NEOANTIGENS GENERATED BY INDIVIDUAL MUTATIONS AND THEIR ROLE IN IMMUNITY AND IMMUNOTHERAPY

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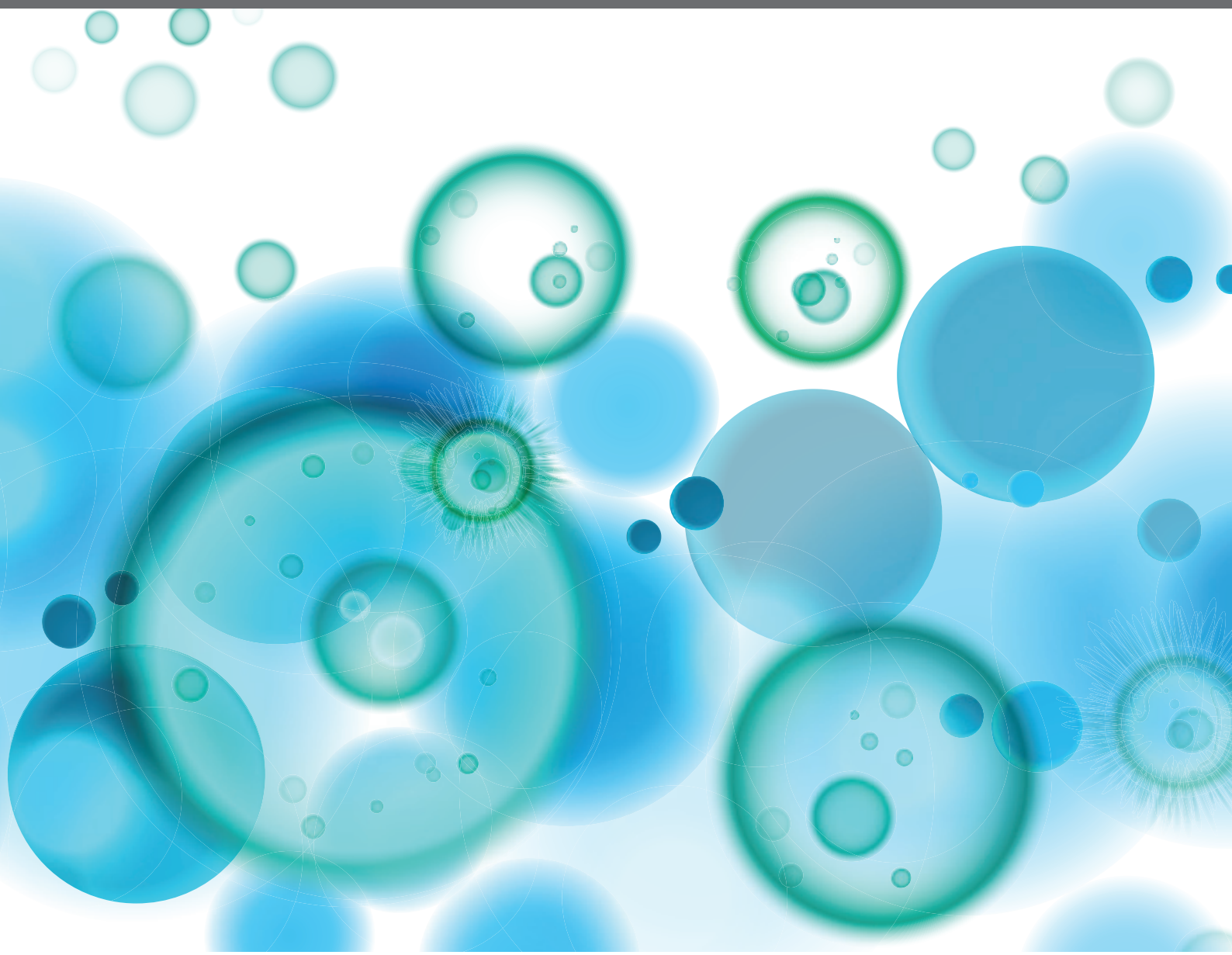

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NEOANTIGENS GENERATED BY INDIVIDUAL MUTATIONS AND THEIR ROLE IN IMMUNITY AND IMMUNOTHERAPY

Topic Editors:
Sebastian Kreiter, Translationale Onkologie an der Universitätsmedizin der Johannes Gutenberg-Universität Mainz, Germany
Mustafa Diken, Translationale Onkologie an der Universitätsmedizin der Johannes Gutenberg-Universität Mainz, Germany

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Cervical Cancer Neoantigen Landscape and Immune Activity is Associated with Human Papillomavirus Master Regulators

*Yong Qin[1], Suhendan Ekmekcioglu[1], Marie-Andrée Forget[1], Lorant Szekvolgyi[2], Patrick Hwu[1], Elizabeth A. Grimm[1], Amir A. Jazaeri[3] and Jason Roszik[1,4]**

[1] Department of Melanoma Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, [2] MTA-DE Momentum, Genome Architecture and Recombination Research Group, Department of Biochemistry and Molecular Biology, University of Debrecen, Debrecen, Hungary, [3] Department of Gynecologic Oncology and Reproductive Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, [4] Department of Genomic Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX, United States

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Edited by:
Mustafa Diken,
TRON – Translational Oncology,
Germany

Reviewed by:
Daniel Olive,
Institut national de la santé et de la recherche médicale (INSERM),
France
Thorbald Van Hall,
Leiden University, Netherlands

***Correspondence:**
Jason Roszik
jroszik@mdanderson.org

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Human papillomaviruses (HPVs) play a major role in development of cervical cancer, and HPV oncoproteins are being targeted by immunotherapies. Although these treatments show promising results in the clinic, many patients do not benefit or the durability is limited. In addition to HPV antigens, neoantigens derived from somatic mutations may also generate an effective immune response and represent an additional and distinct immunotherapy strategy against this and other HPV-associated cancers. To explore the landscape of neoantigens in cervix cancer, we predicted all possible mutated neopeptides in two large sequencing data sets and analyzed whether mutation and neoantigen load correlate with antigen presentation, infiltrating immune cell types, and a HPV-induced master regulator gene expression signature. We found that targetable neoantigens are detected in most tumors, and there are recurrent mutated peptides from known oncogenic driver genes (KRAS, MAPK1, PIK3CA, ERBB2, and ERBB3) that are predicted to be potentially immunogenic. Our studies show that HPV-induced master regulators are not only associated with HPV load but may also play crucial roles in relation to mutation and neoantigen load, and also the immune microenvironment of the tumor. A subset of these HPV-induced master regulators positively correlated with expression of immune-suppressor molecules such as PD-L1, TGFB1, and IL-10 suggesting that they may be involved in abrogating antitumor response induced by the presence of mutations and neoantigens. Based on these results, we predict that HPV master regulators identified in our study might be potentially effective targets in cervical cancer.

Keywords: cervical cancer, neoantigens, human papillomavirus, master regulators, immunotherapy

INTRODUCTION

It is anticipated that most cervical cancer cases will be prevented in the future, but this disease is currently incurable and it causes around 4,000 deaths per year in the US (1). Low vaccination rates (2) forecast that cervical cancer prevalence and mortality will not decrease rapidly and novel treatments are also needed in addition to increasing vaccination uptake. Clinical trials show that

immunotherapy of this malignancy is possible, and multiple new agents are currently being tested, including anti-CTLA-4, anti-PD-1, and anti-PD-L1 antibodies, and therapeutic vaccines as well (3). It is well known that human papillomaviruses (HPVs) cause the overwhelming majority of cervical tumors (4), and HPV proteins are attractive targets (5). Tumor-infiltrating lymphocytes specific to HPV E6 and E7 oncoproteins were successfully expanded and capable of tumor reactivity in an adoptive cell transfer trial in an autologous setting, however, only third of the patients responded in that study (6). *Listeria monocytogenes*-based immunotherapy is another promising approach showing specific activity against high-risk HPV strains (7). The importance of targeting non-viral antigens in HPV-driven cancers has also recently been demonstrated (8).

To increase efficacy of immunotherapies for the treatment of cervical cancer, we need a better understanding of the immune microenvironment of this malignancy. Employing an RNA sequencing-based metric of immune effector function, it was recently shown that immune cytolytic activity is high in cervical cancer compared to other tumors, and it is also higher in cervix tumor samples with a high mutation load (9). These observations suggest the presence of neoantigens derived from somatic mutations that are presented by human leukocyte antigen (HLA) class I molecules and attract cytotoxic T cells. However, as the immune system is unable to eliminate the cancer cells, suppressive mechanisms [such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages, or inhibitory cytokines (for example, IL-10, TGF-beta)] are probably also present in these high mutation burden tumors. To identify the key determinants of immune response or suppression, in the current study, we asked whether increased mutation burden [the number of non-silent exonic mutations, similarly how it was defined in earlier publications (10)] and neoantigen load (the number of mutated peptides derived from genes having non-zero expression that are predicted to bind the patient's HLA) are associated with known antigen presentation and immune cell markers and subtypes.

Although it is known that HPV E6 and E7 oncoproteins play a major role in development of cervix cancer, other "driver pathways" that may also contribute to tumor progression also need to be identified and targeted to be able to successfully fight this disease (11). HPV16 E6 and E7 oncoprotein-related "master regulators" [EGR3, FOSB, NR4A2, PRDM1, SOX9, OVOL1, MNT, PA2G4, Enolase 1 (ENO1), TEAD4, FOXO4, and ZNF365], which have been shown to regulate multiple downstream effects of HPV16 and possibly other HPV types (12), are attractive candidate genes that could potentially regulate the immune microenvironment as well. Therefore, our goal was to also determine the association between expression of these regulators, antigen presentation, antitumor effector function, and immune suppression.

To achieve these goals, we used two large, publicly available cervical cancer data sets, namely the Cancer Genome Atlas (TCGA) cervical cancer data (13) and the exome and RNA sequencing data available in the publication of Ojesina et al. (14) to analyze the neoantigen landscape and the associated immune activity in cervical cancer. Our studies show that mutation load and neoantigen availability is associated with expression of HPV oncoprotein-associated "master regulators," and also with specific genes involved in antigen presentation, immune cytotoxic T-cell function, and immunosuppressive mechanisms. The relationships we identified help to refine our knowledge on immune activity in cervical cancer and are intended to provide attractive targets to increase effectiveness of immunotherapies.

RESULTS

Cervical Cancer Neoantigens Are Detectable and Potentially Immunogenic

First, using the cervical cancer samples available in TCGA CESC (13) ($n = 194$) and in the study published by Ojesina et al. (14) ($n = 79$), we proceeded to predict all potential neoantigens for this cancer type. Only genes with non-zero mRNA expression were included. Our results show that potential neoantigens are detectable in almost all samples in both studies. Only one and two patients did not have any predicted neoantigens in TCGA and Ojesina et al., respectively. The number of predicted neoantigens was from 1 to 4,049 and from 3 to 3,042 in the TCGA (**Figure 1A**) and Ojesina et al. (**Figure 1B**), respectively, and the difference in neoantigen load between the two data sets was not statistically significant. We have identified multiple recurrent mutated antigens in the TCGA (**Figure 1C**, MAPK1 E322K, PIK3CA E545K, PIK3CA E542K, EP300 D1399N, ERBB2 S310F, ERBB3 V104M, KRAS G12D) and also in the Ojesina et al. data set (**Figure 1D**, MAPK1 E322K, PIK3CA E545K, PIK3CA E542K, FBXW7 R465C). The MAPK1 E322K, PIK3CA E545K, and PIK3CA E542K mutations were found in at least three samples in both data sets. Importantly, most of the recurrent neoantigen-generating mutations are found in known oncogenic driver genes.

We then proceeded to look at the immunological aspect in the cervix tumor samples from both studies. Interestingly, we found that HLA class I and class II-related gene expressions that are involved in antigen presentation (HLA-A, HLA-B, HLA-C, HLA-DMA, HLA-DMB, HLA-DOA, HLA-DOB, HLA-DPA1, HLA-DRA, HLA-DRB1, HLA-DRB5, HLA-DRB6, B2M, TAP1, TAP2, PSMB8, PSMB9, and NLRC5) are generally high in cervix tumors ($n = 306$) compared to normal cervix ($n = 11$, ecto- and endocervix) and other cancers (**Figure 2A**). Based on the available immunohistochemistry (IHC) data on cervical tumor samples ($n = 12$) (15), most of tested tissues were positive for protein expression of HLA-B, HLA-DMA, HLA-DOA, HLA-DRA, HLA-DRB5, B2M, PSMB8, PSMB9, TAP1, and TAP2 (**Figure 2B**).

Importantly, the immunosuppressive enzyme IDO1 showed a significantly higher mRNA expression level in cervical cancer than in normal cervix, and also in comparison to other cancers (**Figure 2A**). Moreover, the protein expression of IDO1 was also found to be higher in cervical tumor samples compared to other cancers (**Figure 2B**). These data suggest that IDO1 may be a potential target to abrogate immune suppression.

HPV Master Regulators Are Differentially Expressed in Cervical Cancer Compared to Normal Cervix

We found that HPV E6/E7-related master regulators (ENO1, FOSB, PA2G4, SOX9, TEAD4, FOXO4, and MNT) were

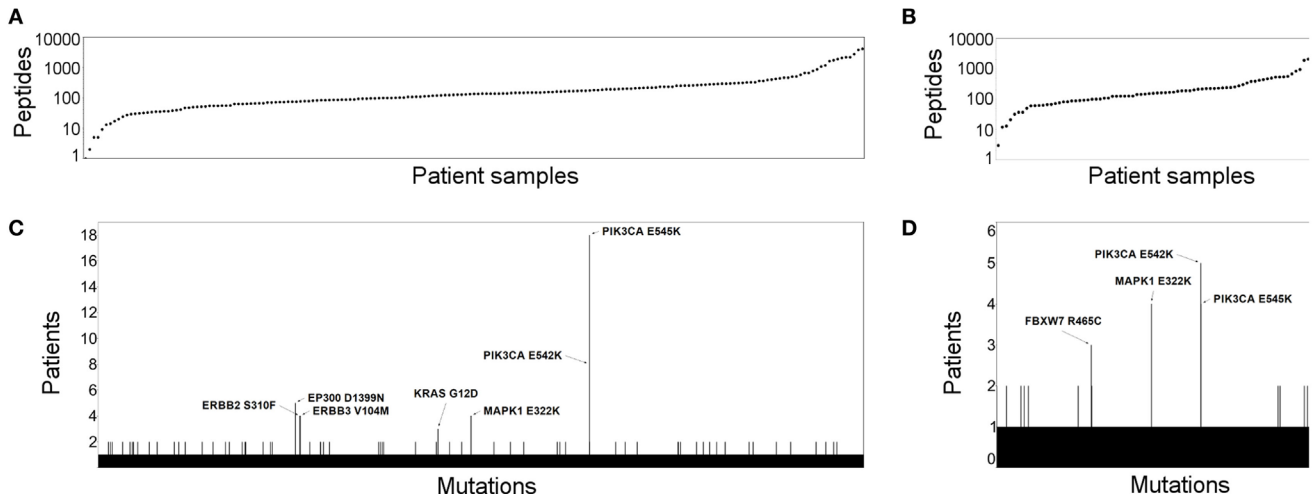

FIGURE 1 | The neoantigen landscape and recurrent targets in cervical cancer. The number of predicted cervical cancer neoantigens is depicted for the Cancer Genome Atlas (TCGA) **(A)** and Ojesina et al. **(B)** data sets. Patient samples are shown in columns, where each dot represents the number of neopeptides predicted to bind human leukocyte antigen class I. Recurrent neoepitopes are also shown for the TCGA **(C)** and Ojesina et al. **(D)** cohorts. Those that were predicted for at least three patients are annotated by gene name and amino acid change.

significantly differently expressed ($p < 0.001$) in the cervical cancer TCGA samples ($n = 306$) than in normal cervix ($n = 11$) tissue (**Figure 2A**). The following genes ENO1, FOSB, PA2G4, SOX9, and TEAD4 were highly expressed in cervical cancer in comparison to normal cervix ($p < 0.001$), while FOXO4 and MNT were significantly lower in cervical cancer ($p < 0.001$) (**Figure 2A**). According to the available data in the Human Protein Atlas (15), most of tested cervical tumor samples were positive for protein expression of EGR3, NR4A2, SOX9, PA2G4, ENO1, and TEAD4 (**Figure 2B**). Protein expression of FOXO4 and ZNF365 was undetectable by IHC in these samples. HPV load, which was calculated as number of HPV RNA-sequencing reads divided by library size (14), also positively correlated with expression of ENO1, PA2G4, and FOXO4 (**Figure 3**).

Mutation and Neoantigen Load Show Association with Potential Immunotherapy Targets

Only a few genes (TAP1, TAP2, HLA-DRA, HLA-DPA1, and NLRC5) related to antigen presentation displayed a positive correlation with mutation or neoantigen load, and only NLRC5, a negative regulator of NF-kappaB and type I interferon signaling pathways (16), which we recently also identified as a target for immune evasion in cancer (17), showed an association with neoantigen load in both cervical cancer data sets (**Figure 3**). Furthermore, MDSC-associated FUT4, which encodes the CD15 protein found on neutrophils and implicated in phagocytosis, was the only immune marker, which showed a negative correlation with mutation and neoantigen load in both data sets.

The immunosuppressive molecule IDO1 (18), which was found to be overexpressed in cervical tumors (19) (**Figure 2**), demonstrated a positive correlation with mutation/neoantigen load in the much larger TCGA data set only (**Figure 3**). Other notable checkpoint-related genes that positively correlated with mutation load in the TCGA are CTLA4, PD-1, and LAG3. Interestingly, PD-L1 correlated positively with neoantigen load, but not with mutation load.

We have also identified that the OVOL1 HPV master regulator positively correlates with mutation and neoantigen load in both data sets, pointing to a potential role in cervical cancer by controlling mesenchymal–epithelial transition (20). Another master regulator, the Hippo pathway target transcription factor TEAD4, negatively correlated with mutation load in both data sets, but showed no correlation with neoantigen load. This association may be important as it has been shown that the Hippo pathway regulates cervical cancer progression (21).

Master Regulators Show Diverse Immune Relationships and May Be Potential Targets

Our correlation analyses revealed that HPV master regulators, which are attractive targets to reverse the effects of HPV oncoproteins, show a close relationship with antigen presentation and immune markers of suppression (**Figure 4**). The ENO1 regulator gene, which we found to be overexpressed in cervical cancer, positively correlated with PD-L1 (CD274) and TGFB1 in both data sets, providing further indication for immunotherapy targeting in this malignancy. PD-L1 also showed a positive correlation with PRDM1, OVOL1, and MNT master regulators. Furthermore, TGFB1 expression also positively correlated with PRDM1, OVOL1, and ZNF365 expressions. ZNF365 was found to negatively correlate with expression of the PSMB8 immunoproteasome gene, highlighting another potential immunosuppressive mechanism by HPV master regulators.

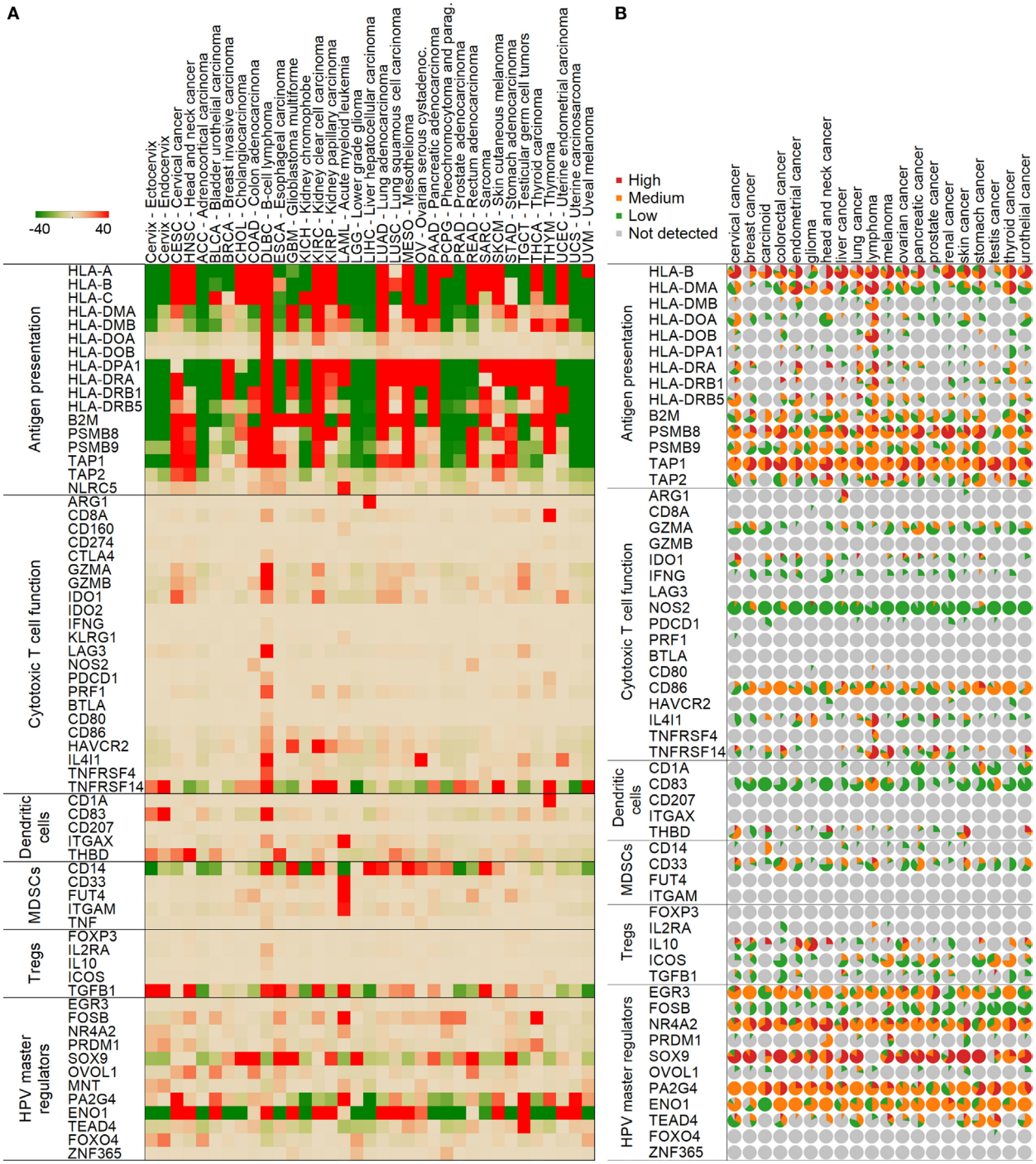

FIGURE 2 | Expression of genes in normal and tumor samples mediating various immune functions. Median mRNA expressions (median centered for tissues) are represented by red (high expression) and green (low) colors **(A)**. A color scale from −40 to 40 transcripts per million was chosen to highlight differences between normal cervix and cervix cancer optimally. Normal cervix is shown in the first two columns followed by cervical cancer and other tumor types in the Cancer Genome Atlas project. Protein expressions from immunohistochemistry in the Human Protein Atlas are shown similarly **(B)**. In the pie charts, the size of the slice represents the ratio of samples with high (red), medium (orange), low (green), or no (gray) expression.

NF-κB inhibitor FOXO4 (22) was the only master regulator that showed an association with the KLRG1 T cell activation maker often associated with senescence, exposing again a potential immunosuppressive mechanism. Expression of FOXO4 also positively correlated with HPV load (in **Figure 3**). Another notable positive correlation we identified was between the immunosuppressive cytokine IL10 and master regulators EGR3 and FOSB. Since it was reported that IL10 plays a role in maintenance

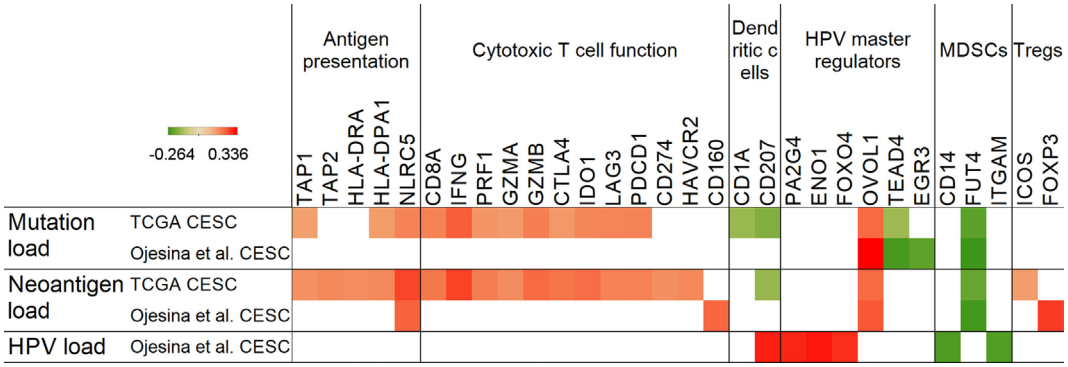

FIGURE 3 | Correlation of mutation, neoantigen, and human papillomavirus (HPV) load with antigen presentation, immune markers, and master regulators. Positive Spearman's rank correlation coefficients (red color) represent positive, while negative coefficients (green) denote negative associations with mutation, neoantigen, and HPV load in the Cancer Genome Atlas (TCGA) and Ojesina et al. data sets. Only genes with $p < 0.05$ correlations are shown.

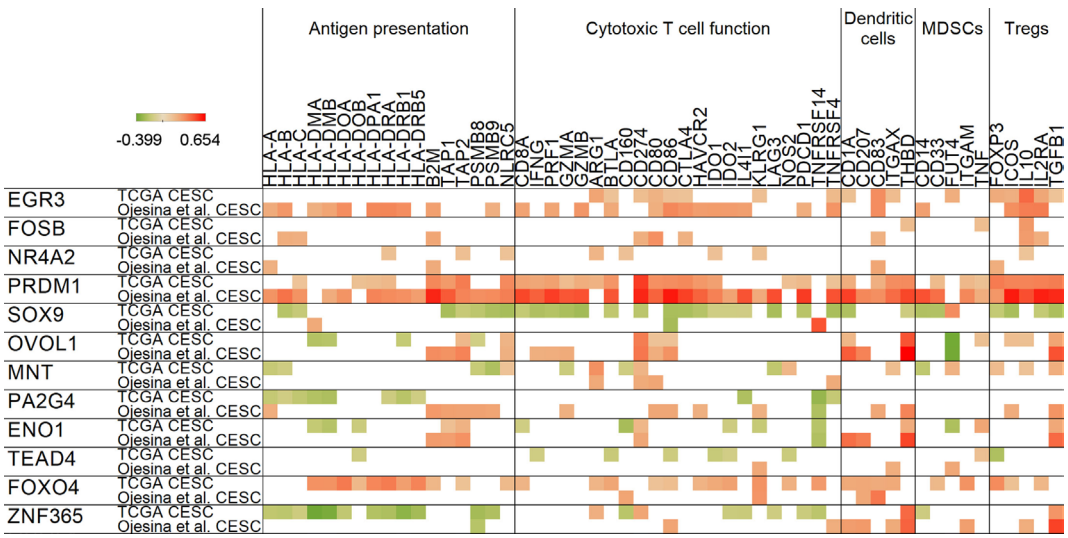

FIGURE 4 | Correlation of human papillomavirus master regulator expressions with antigen presentation and immune-related genes. For all master regulators, Spearman's rank correlation coefficients are shown in rows for both cervical cancer data sets. Red color represents positive, green denotes negative correlations. Only significant relationships ($p < 0.05$) are shown.

of Tregs and immunosuppression in cervical cancer, this correlation might be important in understanding the mechanism (23).

DISCUSSION

Antigen presentation plays a crucial role in human host defense, including immune response to cancer. Immunotherapies are often based on targeting antigens presented *via* major histocompatibility complex/HLA molecules (24). Tumors display tumor-associated antigens on class I and class II HLA molecules at the cell surface, and these antigens can be recognized by CD8 and CD4 T cells. Adoptive T cell therapy uses *ex vivo* expanded tumor-specific T cells that are infused to the patient to elicit tumor regression. This strategy has shown very promising results in the clinic using HPV-specific tumor-infiltrating lymphocytes (6). However, it is clear that the currently available approach alone will not be sufficient to cure cervical cancer. Exome sequencing of a large set of cervical carcinomas revealed an average of 99 missense mutations per sample (14). To determine if these mutations could be targets for individualized T cell immunotherapy, we have evaluated the availability and potential immunogenicity of mutated antigens (i.e., neoantigens) that are predicted to bind HLA in two large cohorts of cervical cancer patients.

We found that most tumors have predicted neoantigens that are capable of binding the HLA molecules of the patient. However, immunogenicity of mutated antigens will need to be validated extensively before clinical application. It was shown by multiple studies that only a minority of predicted neoantigens are immunogenic (25–27). In the case of a set of predicted Cytomegalo-, Epstein–Barr-, and Influenza virus peptides, about

half of all peptides generated a T cell response detectable by IFN-γ production (28). However, it was recently shown that healthy donors may provide a source of neoantigen-specific T cells even when the autologous tumor-infiltrating lymphocytes do not react to the predicted neoantigens (29). Using this strategy greatly increases the chance for developing successful immunotherapies using neoantigens that we identified. Therefore, our data suggest that among the thousands of predicted neoantigens per patient, there may be multiple immunogenic neopeptides that could be effective targets, including those that are derived from recurrent mutations. A few of these recurrent neoantigens are from known oncogenic drivers, for example, PIK3CA, MAPK1, ERBB2, ERBB3, and also from the KRAS G12D mutation, which we recently identified as a promising recurrent neoantigen-based immunotherapy target in pancreatic ductal adenocarcinoma (30). As it was shown in metastatic colorectal cancer, the KRAS G12D mutation can be successfully targeted by specific tumor-infiltrating lymphocytes (31).

Our study also determined a relationship between HPV master regulator genes and antigen presentation and cytotoxic and suppressive immune activity (12). We highlight the duality of the presence of an immune response and the establishment of multiple suppression mechanisms occurring in direct connection to the immune response. The OVOL1 master regulator positively correlated with mutation and neoantigen load, and it was also associated with higher TGFB1 expression. TGF-beta is a well-studied cytokine and immunosuppressive molecule, which has been shown to affect MHC expression (32, 33) and inhibits the ability of dendritic cells to present antigen to stimulate T lymphocytes (34). In addition, OVOL1 also represses c-Myc transcription (35), and as c-Myc level is a known poor prognostic factor in cervical cancer (36). Therefore, OVOL1 may play a complex role in regulating the growth and progression of cervical cancer. Other master regulators, namely PRDM1, ENO1, and ZNF365 showed positive correlations with TGFB1. In the Human Protein Atlas, OVOL1 and PRDM1 showed low, ENO1 medium, while ZNF365 showed no protein expression in cervical cancer (15).

The FOSB gene, which has been shown to be involved in CD95L-initiated apoptosis of T cells (37), was associated with expression of the IL10 immunosuppressive cytokine. FOSB is part of the activator protein-1 (AP-1) complex, and it was previously reported that repression of AP-1 activity and HPV transcription may be effective in controlling cervical tumors (38). FOSB expression is also significantly higher in cervical cancer compared to normal cervix; therefore, it may be a potential target to help overcome immune suppression. EGR3 also showed a positive correlation with IL10 in both data sets. This may be important because EGR3 is a key negative regulator of T cell activation (39). Protein expression data from the Human Protein Atlas indicates low to medium FOSB, and medium to high EGR3 expression.

Another master regulator that potentially plays a role in immune evasion is ENO1. This gene is overexpressed in cervical cancer, and its expression positively correlates with HPV load, and also PD-L1 and TGFB1 expression. Overexpression of the encoded protein ENOA has been detected in several cancers, and ENOA is also able to induce immune response and shows clinical correlations in cancer patients; however, no HLA class I-restricted ENOA peptide has been identified (40). The Human Protein Atlas also shows medium ENO1 expression in cervical cancer. Furthermore, ENO1 is a prognostic marker in the HPV-associated head and neck cancer (41), and it was recently shown that ENO1 silencing impairs cancer cell line growth (42). Based on our results and these previous studies on the role of ENO1 in tumor progression and immune response, we propose that targeting ENO1/ENOA in cervical cancer will provide an additional therapeutic benefit and increase the patient survival.

In conclusion, this study highlights the relationships between the expression of HPV oncoprotein-associated master regulators, neoantigen landscape, the mutation load, and the immune activity in cervical cancer. Analysis of this complicated network and probing of potential interactions will provide attractive targets to increase effectiveness of immunotherapies. In addition to further validation of these targets at the protein level, further *in vitro* and *in vivo* studies will be needed to confirm the regulatory effect of these HPV mater regulators on the expression of major immunosuppression markers in cervical tumors. Moreover, mass spectrometry-based peptide/antigen identification will also be necessary for their use as immunotherapy targets.

MATERIALS AND METHODS

Exome and RNA-Sequencing Data

Mutation, gene expression, and clinical data from the cervical cancer TCGA were obtained from public TCGA repositories and from the publication of Ojesina et al. (14). Raw exome-sequencing data (.bam files) that we used to perform HLA typing were obtained though dbGaP (https://www.ncbi.nlm.nih.gov/gap). HPV load (number of HPV RNA-sequencing reads divided by library size) was available in a supplementary file of the Ojesina et al.'s (14) publication.

Neoantigen Prediction

All 8–12-mer wild type and mutated neopeptides and their HLA-binding affinities were predicted for HLA-A, -B, -C alleles as it was described earlier (43). We used all missense mutations downloaded from the cervical cancer (CESC) TCGA (13) and Ojesina et al. (14) projects. To determine peptide-binding affinities to HLA-A, -B, and -C alleles, we used the NetMHCpan (version 2.8) (44) program, which applies artificial neural networks to predict peptide-MHC class I binding. HLA types of patients were predicted from the raw exome-sequencing data of normal and tumor samples using the Athlates (version 2014_04_26) (45) HLA type prediction software. A peptide was considered a strong binder if the predicted HLA binding affinity was <50 nM, and it was regarded as a weak binder if the HLA-binding affinity was between 50 and 500 nM.

Correlation and Other Statistical Analyses

We calculated all Spearman's rank correlation coefficients using the R software. For comparisons of two groups, we used two-tailed Student's *t*-tests. Differences were considered significant when $p < 0.05$.

AUTHOR CONTRIBUTIONS

Study concept and design: JR. Acquisition, analysis, or interpretation of data: YQ, SE, M-AF, LS, PH, EG, AJ, and JR. Preparation, review, or approval of the manuscript: YQ, SE, M-AF, LS, PH, EG, AJ, and JR.

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The Potential and Challenges of Exploiting the Vast But Dynamic Neoepitope Landscape for Immunotherapy

Els M. E. Verdegaal and Sjoerd H. van der Burg*

Experimental Cancer Immunology and Therapy Group, Leiden University Medical Center, Department of Medical Oncology, Leiden, Netherlands

Somatic non-synonymous mutations in the DNA of tumor cells may result in the presentation of tumor-specific peptides to T cells. The recognition of these so-called neoepitopes now has been firmly linked to the clinical success of checkpoint blockade and adoptive T cell therapy. Following proof-of-principle studies in preclinical models there was a surge of strategies to identify and exploit genetically defined clonally expressed neoepitopes. These approaches assume that neoepitope availability remains stable during tumor progression but tumor genetics has taught us otherwise. Under the pressure of the immune system, neoepitope expression dynamically evolves rendering neoepitope specific T cells ineffective. This implies that the immunotherapeutic strategy applied should be flexible in order to cope with these changes and/or aiming at a broad range of epitopes to prevent the development of escape variants. Here, we will address the heterogeneous and dynamic expression of neoepitopes and describe our perspective and demonstrate possibilities how to further exploit the clinical potential of the neoepitope repertoire.

Keywords: somatic mutations, neoepitopes, immunotherapy, tumor heterogeneity, vaccination, adoptive cell therapy

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****Correspondence:***
Els M. E. Verdegaal
e.m.e.verdegaal@lumc.nl

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INTRODUCTION

Spectacular progress has been made in the treatment of cancer by the introduction of checkpoint blocking antibodies against the inhibitory molecules CTLA-4, and PD-1 or its ligand PD-L1 (1, 2). The efficacy of these antibodies depends on the presence of antigen-specific T cells that can recognize tumor cells but are functionally inhibited in cancer patients (3). In melanoma (4) and lung cancer (5), clinical benefit of checkpoint blocking therapy strongly correlates with the presence of a high mutational load. This led to the hypothesis that a high number of somatic non-synonymous mutations may result in the formation of so-called neoepitopes that are recognized as truly foreign by $CD4^+$ and $CD8^+$ T cells, the response of which is unleashed by checkpoint blocking.

It was suggested that the clinical efficacy of adoptive cell therapy (ACT) also relies on the presence of mutation-specific T cells in the infusion product. Indeed, tumor infiltrating T cell (TIL) used for successful ACT treatment of melanoma patients (6–11), head and neck cancer (12), cholangiocarcinoma (13, 14), and colorectal cancer (15) were shown to contain considerable frequencies of neoepitope-specific T cells. Furthermore, durable clinical responses were obtained when PBMC-derived tumor-reactive T cells, comprising almost exclusively clonally expressed neoepitope-specific

$CD8^+$ and $CD4^+$ T cells, were infused (10, 11, 16). Furthermore, we observed that ACT products administered to responder patients contained T cells that recognized private rather than shared antigens as demonstrated by their almost exclusive recognition of autologous tumor cells and not a series of HLA-matched melanoma cells (**Figure 1**). In contrast, T cells administered to non-responders showed a broad recognition pattern. Moreover, infusion of highly enriched neoepitope-specific T cells resulted in clear tumor regression in a patient who relapsed after bulk TIL therapy (13). Altogether, these data suggest that approaches to select, expand and activate neoepitope specific T cells by (combinations of) checkpoint blocking, ACT and/or vaccination can improve the clinical outcome of this treatment. This, however, does not mean that we should neglect the therapeutic potential of shared tumor-antigens. This is illustrated by the complete tumor eradication of melanoma after transfer of NY-ESO-1-specific $CD4^+$ T cells (17) and genetically engineered NY-ESO-1 specific T cells (18, 19). Although shared tumor antigens are important targets for development of immunotherapy this review focuses exclusively on the exploitation of neoepitopes.

HETEROGENEITY AND DYNAMICS OF NEOEPITOPE LANDSCAPE

Optimal exploitation of neoepitope immunity for cancer therapy requires a thorough understanding of the neoantigen landscape. Several studies have shown that the mutational landscape of a tumor is not cut into stone but dynamically evolves (20–27) with as potential outcome that tumor recognition by the immune system is lost due to reduced or lost expression of neoepitopes in recurrent tumor cell clones (11). Hence, it not only is essential to gain knowledge of the frequency and extent of intratumoral heterogeneity but also of mutational landscape changes during tumor progression and regression after treatment, including immunotherapy.

Heterogeneity of mutations occurs at spatial and temporal levels. First, different areas within a single tumor lesion may harbor different mutations. In individual tumors of eight melanoma patients, the proportion of heterogeneity of somatic mutations was reported to range from 3 to 38%, although it should be mentioned that heterogeneity was particularly abundant in non-expressed genes. Nonetheless, a high degree of heterogeneity was associated with a more aggressive course of the disease (25). Second, mutations may differ between primary and metastatic lesions as well as between various metastases. Analysis of primary breast cancer lesions and matched metastases revealed that the number of genetic alterations was reduced in metastatic lesions. Although this might seem counterintuitive at first glance, it can be explained by a high grade of heterogeneous variants in the primary tumor, from which specific subclones with a less heterogeneous mutation expression but increased proliferative and metastatic potential evolve (20). Indeed, some of the mutations shared between metastatic lesions of various patients are linked to poor survival. The changes in the landscape of expressed (non-silenced) mutations vary depending on the cancer type illustrating that proportionally intratumoral heterogeneity is very high in glioma and low in NSCLC and melanoma [reviewed in Ref. (28)]. However, given the relatively high mutation rate in the latter two tumor types, the absolute number of alterations in expressed mutations is still high (28). In one exceptional case of a NSCLC patient, 99% of the total genetic alterations (point mutations, insertions, and deletions) differed between sequential lesions (24). Finally, mutations may vary between early lesions that are sensitive to treatment and treatment-resistant recurrences. The extent of genetic alterations in these recurrent lesions varies across cancer types and is very low in ovarian cancer (21, 22). At the other end of the spectrum are low-grade gliomas that acquire thousands of somatic mutations that differ from the initial lesions after temozolomide therapy (23) and concomitantly evolve into a high-grade glioma phenotype. Anagnostou et al. elegantly showed that tumor lesions recurring after checkpoint blocking therapy displayed both loss and gain of putative (mutation associated) neoepitopes in four NSCLC and one HNSC patients (27). We analyzed the expression stability of six clonally expressed T cell targeted neoepitopes in serially obtained tumors from two stage IV melanoma patients treated by ACT (11). The data from these paired tumor samples demonstrated that under the attack of T cells neoepitope availability was lost in four out of six cases in tumor subclones that evolved upon disease progression. These two studies show that immune pressure sculpts the mutational landscape of tumors and imply that flexibility toward the neoepitopes targeted is a prerequisite for immunotherapeutic approaches aiming to exploit the neoantigen repertoire. Recently it was reported that the number of recognized neoepitopes in TIL used for ACT of melanoma patients do not directly correlate to treatment outcome (29). There are several reasons to explain this, including the copresence of clinically active T cells reactive to tumor-associated antigens (17), which may have had a major contribution in the clinical responses obtained in patients who received TIL with low neoepitope-reactivity. In those patients who do not show clinical response after transfer of TIL with a high frequency of neoepitope-specific cells, a multitude of factors defined as the immunophenoscore (30, 31), including TME phenotype and tumor escape status, may have hampered clinical effectiveness.

Dynamics in neoantigen expression predict that strategies applying neoepitopes for reinforcement of antitumor immunity should aim at a broad panel of antigens in order to prevent escape variants. Hence, when an immunotherapeutic strategy requires epitope selection the highest priority should be given to neoepitopes derived from driver mutations. These mutations are expected to be expressed in the majority of—if not all—tumor cells and will not be lost by immunoediting because they are essential for the malignant phenotype. However, T cells reactive against these epitopes are infrequently detected even though several driver mutations are frequently present in various tumor types, including colorectal cancer and melanoma (15, 32, 33). Emphasis should also be given to neoepitopes derived from clonally expressed mutated genes other than acquired early during tumor evolution. In contrast to subclonal mutations, these clonal mutations may comprise driver and passenger mutations that are expressed in the "trunk" of the tumor evolutionary tree and therefore expressed in the majority of tumor cells. This notion is sustained by the observation that clinical benefit from checkpoint-blocking therapy is not only correlated with total tumor burden

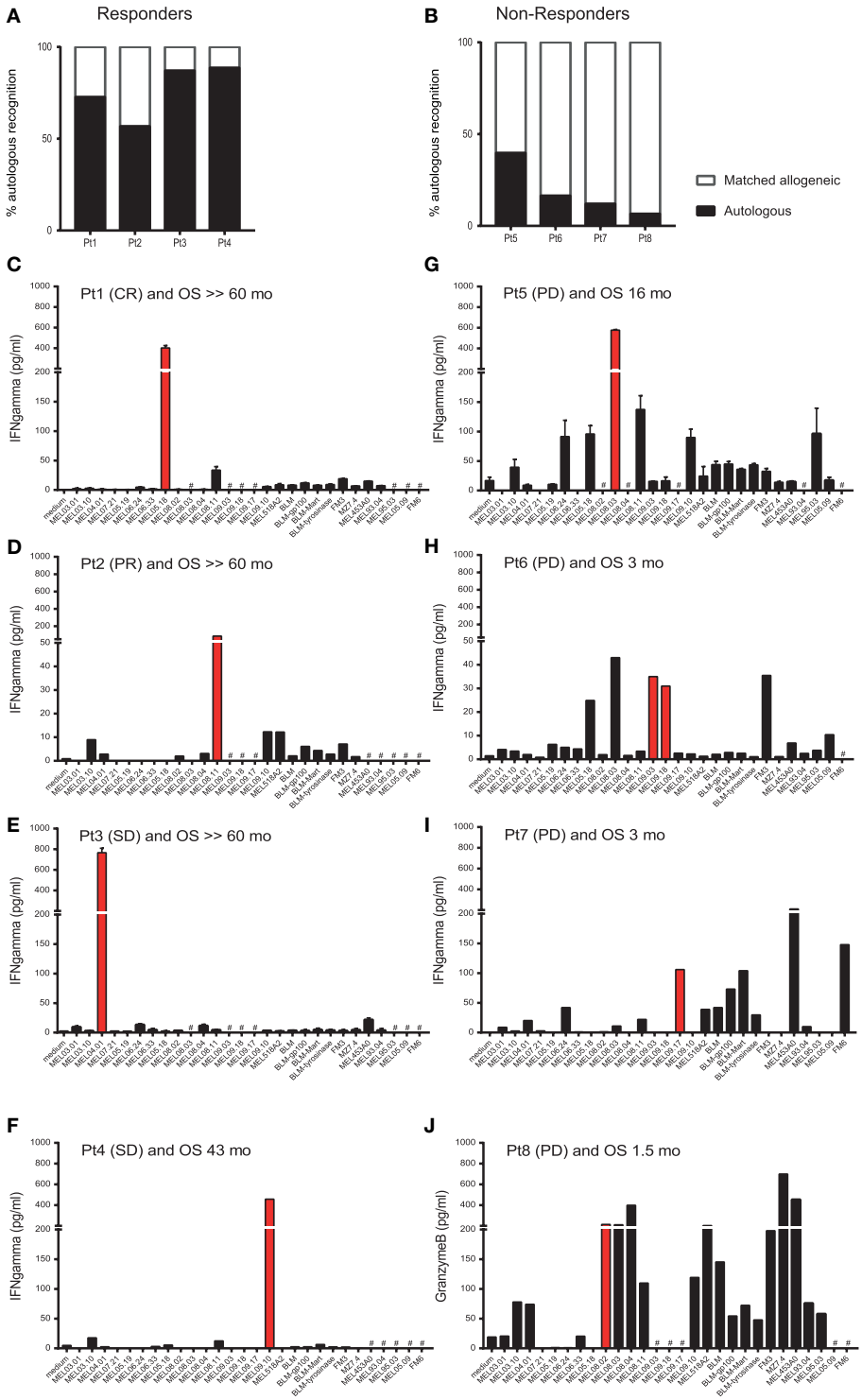

FIGURE 1 | T cell batches administered to responder patients recognize private rather than shared antigens. Tumor-reactive T cell batches were generated by repeated stimulation of PBMC with autologous melanoma cell lines in a mixed lymphocyte tumor cell culture (MLTC). These T cells were administered to melanoma patients by ACT. The patient number, with best overall response [complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD)], and overall survival (OS) in months are given. (# = not done). IFN-gamma production, as an indicator of T cell activation, was measured after incubation of T cells used for ACT with various (partially-)matched HLA class-I melanoma cell lines. The IFN-gamma production of T cells against the autologous tumor cells is depicted as a fraction of the total IFN-gamma production against all tested cells (set at 100%) for responder patients ($n = 4$) and non-responder patients ($n = 4$) in panels **(A,B)**, respectively. The data of each individual patient are given in panels **(C–J)**. Data in panels **(C,D)** were previously reported (11). IFN-gamma production upon recognition of each cell line is represented by separate bars. The red bar in each panel indicates the autologous melanoma cell line that was used to generate the corresponding T cell batch. The patients were treated in a clinical trial approved by the local ethics committee (LUMC study P04.085) and all patients gave written informed consent.

but also correlated with homogeneity of mutations within spatial and temporally different tumor lesions in NSCLC and melanoma patients (26).

EXPLOITING THE POTENTIAL OF THE VAST NUMBER OF PUTATIVE NEOEPITOPES

The correlation between the success of checkpoint blockade and the mutational load in NSCLC, melanoma and mismatch repair deficient tumors (4, 5, 34), demonstrates that metastasized late stage progressive cancers with concomitant high grade of intratumoral heterogeneity can be effectively targeted. It also underscores the adaptive capacity of the immune system to the dynamic mutational and neoepitope landscape. Similarly, we observed in a recurrent subclone after ACT that the expression of a non-targeted neoepitope was increased when compared to the earlier fully regressed lesions and this was paralleled by the emergence of intratumoral T cells specific for this neoepitope (11). However, still roughly halve of the patients do not respond to checkpoint-blocking therapy, part of which can be explained by a weak or absent pre-existing tumor-specific T cell response (3). Therefore, various therapeutic approaches aiming to enhance or induce (neo)antigen-specific T cell responses are pursued.

A logical option to harness the immune system is by identification and targeting of additional neoepitopes. So far, the number of neoepitopes eliciting a T cell response that are identified ranges from one to maximally ten per patient (35) and detection of neoepitope-specific T cells in ACT products or TILs has revealed that only a minority of the putative neoepitopes predicted to bind to HLA elicits spontaneous immune responses (7, 8, 10, 11, 14, 36, 37). The underlying reasons are yet unknown. Most likely the selection of mutated antigens for neoepitope identification based on NGS and RNA sequencing, the prediction algorithms and T cell tests are far from optimal and may be improved. For instance, by more efficient capture of coding DNA regions and/or comprehensive transcriptional analysis as well as by optimization of algorithms that predict peptide processing, HLA binding, HLA-peptide stability and peptide foreignness (38, 39), but also by improving T-cell detection methods. A sensitive and rapid identification method to identify functional immunogenic neoepitopes is the use of DNA barcoded MHC-multimers. This allows screening of a large number of peptides in a relatively small sample of PBMC, TIL or tumor-reactive T cells (40). The sensitivity of detection may be even further enhanced when proliferation of neoepitope-specific T cells is assessed by TCR Vbeta-clonality analysis of PBMC/TIL before and after *in vitro* stimulation (27). The frequency of neoepitope-specific T cells may be low and may therefore limit detection of neoepitope immunogenicity. Selection of tumor-specific T cells from PBMC may be applied to improve outcome of functional immunogenicity tests. Actually, PD-1$^+$ CD8$^+$ and not the more abundant PD-1$^-$ CD8$^+$ T cells from peripheral blood [**Figure 2B** and (36)] and also from TIL (41) were shown to harbor tumor-reactive and neoepitope-specific T cells. Rapid identification of multiple neoepitopes per tumor sample could be readily achieved using PD1$^+$ CD8$^+$ selected TIL (42) isolated directly *ex vivo* from tumor samples. It would be of interest to also investigate PD-1$^+$ CD8$^+$ T cells from PBMC of the corresponding patients to see whether reactivity to a similar repertoire of neoepitopes is detected. Other reasons for a failure to detect more neoepitope specific T cells might be that spontaneously triggered neoepitope-specific T cells are not activated due to neoepitope heterogeneity and in particular neoepitope expression between tumor subclones (11) or because they have become exhausted or anergic (43) in TIL.

There is already some evidence that there are more neoepitopes processed and presented in the HLA molecules at the tumor cell surface than those that spontaneously raise neoepitope-specific T cell immunity. Stronen et al. showed that putative neoepitopes, not recognized by TILs, were able to trigger tumor-reactive T-cell reactivity in PBMC from healthy donors, arguing that a "neglected neoepitope repertoire" exists (37). This is also supported by the work of Carreno et al. showing that vaccination with neoepitopes that are not spontaneously recognized, does result in a putative neoepitope-specific T cell response in three patients with melanoma (44). Two out of seven selected immunogenic HLA-A*0201-restricted neoepitopes used for vaccination of one patient, could be detected by mass spectrometry analysis to be endogenously expressed, processed and presented by HLA on tumor cells (44) and the T cells directed against these epitopes specifically lysed tumor cells expressing these two neo-antigens but not other target cells.

To gain more insight in the number of attended and neglected neoepitopes that are actually presented by HLA at the tumor cell surface mass spectrometry can be utilized. Optimal identification of neoepitopes using this approach would ideally require access to (a) a substantial amount of tumor tissue or preferentially a tumor cell line that can be cultured up to the quantities required; (b) somatic mutation data derived from sequenced exomes and transcriptome; and (c) autologous T cells to confirm immunogenicity of the neoepitope and functional recognition (something for which also HLA-matched naïve T cells from healthy donors can be used) as well as to show the presence of a functional T-cell repertoire in the patient, which is crucial for ultimate immune responsiveness. Identification of tumor-specific T cell epitopes from a fraction of tumor tissue using mass spectrometry may be limited because of the amount of available starting material for representative detection of neoepitopes among the entire HLA-ligandome (45). Nevertheless, immunogenic neoepitopes have been identified directly from melanoma biopsies (46). Mass spectrometry/ligandome data were matched with NGS/transcriptome data for a total of five patients and led to identification of four immunogenic epitopes. In addition to the identified neoepitopes, many known and novel peptide ligands derived from tumor-associated antigens were identified, demonstrating the applicability of mass spectrometry/proteomics for broad MHC peptide ligand identification.

HARNESSING THE IMMUNE SYSTEM WITH NEOEPITOPE SPECIFIC T CELLS

From the above mentioned data, it is expected that also clinical efficacy of ACT or vaccination can be enhanced by focusing on

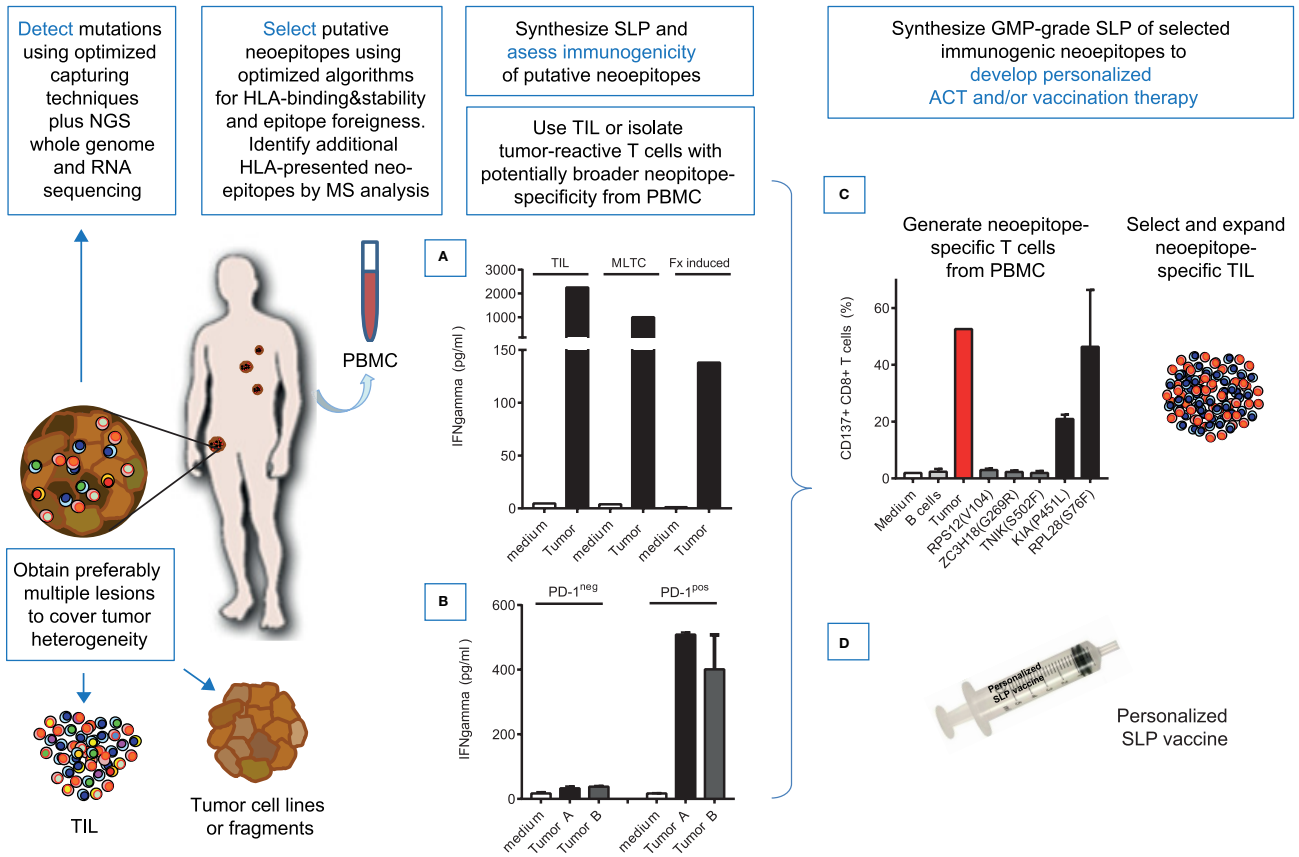

FIGURE 2 | Proposed pipeline for individualized immunotherapy exploiting neoepitope-specific T cells. Tumor tissue is excised and used for: (1) whole-exome and RNA sequencing of tumor cells and matched normal cells using optimized capturing of DNA coding regions to identify somatic mutations in expressed genes. Preferably multiple lesions are used to minimize selection of mutations with heterogeneous or lost expression, (2) to establish a tumor cell line or prepare tumor fragments for generation of neoepitope-specific, tumor-reactive T cells, and (3) to culture tumor infiltrating T cells (TIL). Putative neoepitopes can be selected based on expression of the mutated gene. Further prioritization using optimized processing, MHC-binding and stability algorithms is optional but not essential. Next, synthetic long peptides (SLPs) harboring the selected putative neoepitopes are used to assess immunogenicity using TIL. As an alternative, tumor-reactive T cells obtained by repeated stimulation of PBMC with an autologous tumor cell line (MLTC) or small tumor fragments (Fx induced), exemplified in **(A)** or PD-1 positive cells selected from PBMC, exemplified in **(B)** that are shown to contain a potentially broader repertoire of neoepitope-specific, tumor-reactive T cells can be used. Identification of immunogenic neoepitopes is assessed by IFN-gamma production of tumor-reactive T cells upon coincubation of SLP-loaded autologous B cells as APC. This approach allows identification of $CD4^+$ as well as $CD8^+$ epitopes (10). Subsequently, selected immunogenic neoepitopes that are shown to elicit a T cell response, can be used to select specific T cells from PBMC or TIL for ACT **(C)** or for personalized vaccination **(D)**. **(C)** shows that neoepitope-specific T cells can be obtained from patient's own PBMC by repeated peptide stimulation. PBMC were stimulated at day 0 with SLP harboring selected neoepitopes and stimulated at week 2 with PHA-blasts loaded with the corresponding short minimal $CD8^+$ epitopes. After 4 weeks, the majority of the obtained cells were $CD8^+$ T cells that recognized autologous B cells loaded with the specific SLP, as well as autologous tumor cells. Thus obtained enriched neoepitope- and tumor-reactive T cells can be expanded and used for ACT. Alternatively or in combination with ACT, prevalent neoepitope-specific T cells can be boosted by vaccination with SLP harboring the selected neoepitopes plus an immunostimulatory adjuvant to induce a robust immune response and/or to further support transferred neoepitope-specific T cells *in vivo*.

(clonally) expressed mutations-derived neoepitopes. Once a set of immunogenic neoepitopes has been identified it can be used to induce or increase the frequency of tumor-reactive T cells by vaccination using RNA (47), synthetic long peptides (SLPs) (48), or antigen-loaded DC (49–53). Clinical trials applying vaccination with neoepitope RNA or SLPs recently demonstrated feasibility and clinical effectiveness of neoepitope-based personalized immunotherapy (47, 48).

As an alternative to vaccination, selected neoepitopes can be used to expand neoepitope-specific T cells *in vitro* for use in ACT, for instance by stimulation of patients PBMC with SLPs covering the selected neoepitopes. We showed that SLP-stimulated T cells not only respond to neoepitope peptide-pulsed APC but also recognized autologous tumor cells, indicating that they recognize endogenously naturally presented neoepitopes (**Figure 2C**) and as such have clinical potential. In order to speed-up this process, one may also preselect PD-1 positive cells from PBMC [**Figure 2B** and (36)] either with or without prior stimulation with autologous tumor cells or stimulation with small tumor fragments in case no autologous tumor cell line is available.

CONCLUSION AND PERSPECTIVE HOW TO EXPLOIT THE CLINICAL POTENTIAL OF THE NEOEPITOPE REPERTOIRE

Based on the correlations between successful checkpoint therapy and mutational load as well as successful ACT and the presence of neoepitope-specific T cells, it is fair to assume that these neoepitope-specific T cells strongly contribute to the clinical effect. Clearly, the immunotherapy-mediated increased immunological pressure on the tumor in the end results in the outgrowth of tumor cell clones with downregulated or lost expression of the targeted epitopes. In most cases without direct consequences for the tumor cell itself as most of the targeted mutations are not directly involved in tumorigenesis. Importantly, the number of identified spontaneously recognized neoepitopes probably is only a fraction of the total repertoire of tumor-presented tumor-specific as well as tumor-associated antigens. To prevent neoepitope escape this broader repertoire of neoepitopes should be targeted. This, however, requires crucial improvements both with respect to the identification and the speed of the process itself. These approaches all rely on the successful identification of targetable neoepitopes, which will not be possible for all patients. In cases where no immunogenic epitopes can be identified using TIL, stimulation of PBMC with autologous tumor cells or tumor cell fragments in mixed lymphocyte tumor cell cultures (MLTCs) may result in generation of a T cell product enriched for tumor-reactive T cells probably comprising considerable frequencies of undefined but effective neoepitope-specific T cells (**Figure 1**) with a broader neoepitope specificity when compared with TIL [**Figure 2** and (54)].

Assuming that a selection of immunogenic neoepitopes is available, the question remains how to optimally implicate them in effective treatment. In our opinion, the complexity of tumor biology will eventually require a combined approach to effectively combat the patient's tumor. First of all, the patient must be harnessed with tumor-reactive T cells, which can be accomplished by vaccination targeting neoepitopes (**Figure 2D**) or adoptive transfer of neoepitope specific, tumor-reactive T cells (**Figure 2C**). In addition, radiation and chemotherapy could be applied to induce tumor cell apoptosis, which can be considered as *in vivo* whole tumor cell vaccination, boosting the endogenous T cell response and stimulating antigen spreading and on top of that may promote DC trafficking and T cell priming and trafficking to non-infiltrated "cold" tumors (55–59). Moreover, chemotherapy may normalize the generally suppressive myeloid cell subsets and/or enhance the influx of potent APCs and thereby improve response to therapy (60–63). Finally, checkpoint-blocking therapy should be provided to allow optimal effector cell function of the neoepitope-specific effector T cells at the tumor site.

AUTHOR CONTRIBUTIONS

EV and SB designed and wrote the article.

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'Hotspots' of Antigen Presentation Revealed by Human Leukocyte Antigen Ligandomics for Neoantigen Prioritization

*Markus Müller[1,2], David Gfeller[2,3], George Coukos[3,4] and Michal Bassani-Sternberg[3,4]**

[1] Vital-IT, Swiss Institute of Bioinformatics, Lausanne, Switzerland, [2] Swiss Institute of Bioinformatics, Lausanne, Switzerland, [3] Ludwig Cancer Research Center, University of Lausanne, Epalinges, Switzerland, [4] Department of Oncology, Lausanne University Hospital, Lausanne, Switzerland

The remarkable clinical efficacy of the immune checkpoint blockade therapies has motivated researchers to discover immunogenic epitopes and exploit them for personalized vaccines. Human leukocyte antigen (HLA)-binding peptides derived from processing and presentation of mutated proteins are one of the leading targets for T-cell recognition of cancer cells. Currently, most studies attempt to identify neoantigens based on predicted affinity to HLA molecules, but the performance of such prediction algorithms is rather poor for rare HLA class I alleles and for HLA class II. Direct identification of neoantigens by mass spectrometry (MS) is becoming feasible; however, it is not yet applicable to most patients and lacks sensitivity. In an attempt to capitalize on existing immunopeptidomics data and extract information that could complement HLA-binding prediction, we first compiled a large HLA class I and class II immunopeptidomics database across dozens of cell types and HLA allotypes and detected hotspots that are subsequences of proteins frequently presented. About 3% of the peptidome was detected in both class I and class II. Based on the gene ontology of their source proteins and the peptide's length, we propose that their processing may partake by the cellular class II presentation machinery. Our database captures the global nature of the *in vivo* peptidome averaged over many HLA alleles, and therefore, reflects the propensity of peptides to be presented on HLA complexes, which is complementary to the existing neoantigen prediction features such as binding affinity and stability or RNA abundance. We further introduce two immunopeptidomics MS-based features to guide prioritization of neoantigens: the number of peptides matching a protein in our database and the overlap of the predicted wild-type peptide with other peptides in our database. We show as a proof of concept that our immunopeptidomics MS-based features improved neoantigen prioritization by up to 50%. Overall, our work shows that, in addition to providing huge training data to improve the HLA binding prediction, immunopeptidomics also captures other aspects of the natural *in vivo* presentation that significantly improve prediction of clinically relevant neoantigens.

Keywords: mass spectrometry, immunopeptidomics, antigen processing and presentation, human leukocyte antigen-binding prediction, neoantigens, cancer immunotherapy, personalized cancer vaccines

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***Correspondence:**
Michal Bassani-Sternberg
michal.bassani@chuv.ch

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INTRODUCTION

The adaptive immune system has the capacity to elicit anti-cancer $CD4^+$ and $CD8^+$ T-cell responses, which are triggered by the presentation of cancer-derived antigens as human leukocyte antigen-binding peptides (HLAp) and their recognition by cognate T-cell receptors. HLA class I (HLA-I) and HLA class II (HLA-II) complexes are distinct based on the type of cells that express them, their intracellular processing and loading, and by the type of T-cells that recognize them (1). A dedicated cellular machinery is responsible for the processing of mainly intracellular proteins and their loading on HLA-I complexes, which present these peptides to $CD8^+$ T-cells. Similarly, a parallel machinery processes and loads mainly endocytosed extracellular proteins on HLA-II complexes for their presentation to $CD4^+$ T-cells. The repertoire of HLA presented peptides (HLAp) is remarkably rich and is collectively called the immunopeptidome (2).

In cancer, HLAp derived from processing and presentation of cancer-specific proteins serve as the leading targets for T-cell recognition. Most antigens identified earlier as cancer-specific have been derived from self proteins. Investigated in hundreds of therapeutic clinical trials, these have been mostly clinically disappointing, partially due to central tolerance mechanisms and the elimination of high-avidity T-cells recognizing such normal proteins (3–6). In recent years, the remarkable clinical efficacy of the immune checkpoint blocking therapies has again motivated researchers to discover the immunogenic T-cell epitopes that mediate disease control or long-term cure (7). The observed correlation between mutational load and clinical efficacy highlights the involvement of mutated neoantigens in tumor rejection, and there is now a growing interest in exploiting such targets in the development of personalized vaccines (8–11).

In recent years, significant technological improvements in genomics along with supportive bio-informatics and *in silico* HLA-binding prediction tools have facilitated major breakthroughs in the discovery of neoantigens encoded by non-synonymous mutations that arise during the process of tumorigenesis and are not expressed by normal cells. Mass spectrometry (MS) technology has confirmed the *in vivo* presentation of neoantigens in murine cell line models (12, 13), human cell lines (14, 15), B-cell lymphomas (16), and melanoma tissues (17). In conjunction, the development of immunological screening techniques has facilitated the detection and isolation of T-cells reactive against such mutated epitopes (18–21). Several studies further showed substantial clinical benefit mediated by the administration of highly enriched populations of neoepitopes-reactive $CD4^+$ and $CD8^+$ T-cells (22, 23) and neoantigen-based vaccines formulated as RNA (10) or peptides (9). These patients experienced no major toxicity, suggesting that T-cell responses against neoantigens are likely safe.

Currently, the performance of HLA-I ligand interaction prediction algorithms used for identifying potential neoantigens is still rather poor for infrequent HLA-I molecules, for which binding data are limited, and in general for HLA-II molecules (24, 25). Furthermore, predictors of immunogenicity are still immature (26). Inevitably, false positives are included among the predicted neoantigens, which are then included in a vaccine. MS analysis of HLA-I-binding peptides eluted from tissue samples is a promising approach to discover the actual *in vivo* presented immunopeptidome, including the neoantigens (17). The more specific targeted MS analysis may be used to further validate presentation of *in silico* predicted neoantigens (12). With the current MS instrumentation, MS-based immunopeptidomics approaches have limited sensitivity and are only applicable to a small fraction of patients due to the large amount of biological sample that is required (typically 1 cm^3 of tissue or 1×10^8 cells in culture). Furthermore, they are currently performed in only a few professional labs due to the complexity of these experiments (27).

In addition, interrogating the properties of the thousands of different source-proteins of HLA ligands has identified additional biological determinants, such as their level of translation and expression, turnover rate, proteasomal cleavage specificities, length, and biological functions. Integrating such variables into a single predictor further improves the accuracy of prediction (28, 29). Specifically, recent MS immunopeptidomics studies suggested that HLA-I ligands are not randomly distributed along the proteins' sequences but are located within "hotspots" (17, 28), which fit proteasomal cleavage, peptide processing and HLA-binding rules.

In recent years, it has become common practice in proteomics research to submit MS/MS data to repositories in order to make them available for further research (30). More recently, this practice is also being followed in the field of immunopeptidomics (17, 28, 31). So far, the large body of publically available MS/MS data has been used for training of HLA-I binding prediction (29, 32–34) or to build spectral libraries (35). Although MS-based immunopeptidomics analysis can be directly applied today only to a small number of patients, its emerging use can reveal crucial information on the rules underlying the biogenesis of the immunopeptidome. Indeed, while hunting for neoantigens, such immunopeptidomics MS studies produce massive amount of highly valuable ligandomic data that can be used to refine known HLA-I-binding motifs and to reveal HLA-I-binding specificities of yet unexplored alleles (32, 33). Here, we propose another way to valorize available immunopeptidomics MS/MS data.

We first computationally overlaid HLA-II peptidomics data on top of HLA-I data to highlight the subpopulations of cellular proteins that are naturally accessible and presented by each of the HLA-I and HLA-II presentation machineries and those presented by both (HLA-I/II). Based on the functional annotation of the source proteins and the peptide's length we propose that the HLA-I/II peptides may be processed by the cellular class II presentation machinery within the endosome-lysosome compartments, in a proteasome-independent cross-presentation pathway. Since priming both $CD8^+$ and $CD4^+$ T cell responses would lead to optimal and long lasting immune response required for elimination of tumors *in vivo*, these cross-presented peptides are of particular importance. Next, we provide evidence that data-driven prioritization of predicted neoantigens based on observed "hotspots," which are subsequences of proteins frequently detected in MS/MS immunopeptidomic datasets, will enrich the list of proposed targets with the most likely presented neoantigens. These hotspots reflect the propensity of protein

subsequences to produce HLA peptides averaged over many allotypes and provide complementary information to classical HLA-binding prediction. We show as a proof of concept that by including MS-based hotspot scores into the prioritization scheme we are able to improve the prediction by up to 50%. We envision that as MS-based HLA-I and HLA-II immunopeptidomics datasets become more exhaustive, "hotspot" driven prioritization will have a substantial impact on the selection of neoantigens for vaccination.

MATERIAL AND METHODS

Cell Lines, Tissues, and Antibodies

Detailed information about the biological samples that were included in this database is provided in Table S1 in Supplementary Material. Informed consent of the participants was obtained following requirements of the institutional review board [Ethics Commission, University Hospital of Lausanne (CHUV)]. W6/32 (anti-pan-HLA-I) and IVA12 (anti-pan-HLA-II) monoclonal antibodies were purified from the supernatant of HB95 and HB145 cells, respectively, as previously described (17). We cross-linked the antibodies to Protein-A Sepharose beads (Invitrogen, CA, USA) with 20 mM dimethyl pimelimidate in 0.2 M sodium borate buffer pH9.

Purification of HLA-I Complexes

We included here also unpublished immunopeptidomics data of HLA-I and HLA-II peptides extracted from several biological replicates per cell line or patient material. The cell counts ranged from 1×10^8 to 5×10^8 cells or up to 2 g of tissue per replicate. Purification from these additional samples was performed as previously described (17, 33). Shortly, snap-frozen tissue samples were homogenized for 10 s on ice using ULTRA-TURRAX (IKA, Staufen, Germany) in a tube containing 5–10 ml of lysis buffer and incubated at 4°C for 1 h. Cell pellets were resuspended in 5 ml lysis buffer and incubated similarly. Lysis buffer contained 0.25% sodium deoxycholate (Sigma-Aldrich), 0.2 mM iodoacetamide (Sigma-Aldrich), 1 mM EDTA, 1:200 Protease Inhibitors Cocktail (Sigma, MO, USA), 1 mM Phenylmethylsulfonylfluoride (Roche, Mannheim, Germany), 1% octyl-beta-D glucopyranoside (Sigma). The lysates were cleared by centrifugation with a table-top centrifuge (Eppendorf Centrifuge 5430R, Schönenbuch, Switzerland) at 4°C at 14,200 rpm for 20 min. Immuno-affinity purification from tissues was performed by passing the cleared lysates through Protein-A Sepharose beads, then through Protein-A Sepharose beads covalently bound to W6-32 antibodies, and finally through beads covalently bound to IVA12 antibodies. Purification from cell line lysates required only the two last affinity columns. Affinity columns were then washed with at least 6 column volumes of 150 mM NaCl and 20 mM Tris–HCl (buffer A), 6 column volumes of 400 mM NaCl and 20 mM Tris–HCl and lastly with another 6 column washes of buffer A. Finally, affinity columns were washed with at least 2 column volumes of 20 mM Tris HCl, pH8. HLA complexes were eluted by addition of 1% trifluoroacetic acid (TFA, Merck, Darmstadt, Switzerland) for each sample. To further purify the peptides, the elution samples were loaded separately on Sep-Pak tC18 (Waters, MA, USA) cartridges, which were pre-washed with 80% acetonitrile (ACN, Merck) in 0.1% TFA and 0.1% TFA only. After loading, cartridges were washed twice with 0.1% TFA before separation peptides were eluted with 30% ACN in 0.1% TFA. The peptide samples were dried using vacuum centrifugation (Eppendorf Concentrator Plus, Schönenbuch, Switzerland) and re-suspended in a final volume of 12 µL 0.1% TFA. For MS analysis, we injected 5 µL of these peptides per run.

LC–MS/MS Analysis of HLA-I Peptides

Measurements of HLA-I and HLA-II peptidomics samples were acquired using the nanoflow UHPLC Easy nLC 1200 (Thermo Fisher Scientific, Germering, Germany) coupled online to a Q Exactive or Q Exactive HF Orbitrap mass spectrometers (Thermo Fischer Scientific, Bremen, Germany) with a nanoelectrospray ion source as previously described (33). We packed an uncoated PicoTip with diameter of 50 cm × 75 µm and 8 µm tip opening with a ReproSil-Pur C18 1.9 µm particles and 120 Å pore size resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) resuspended in Methanol. The analytical column was heated to 50°C using a column oven. Peptides were eluted with a linear gradient of 2–30% buffer B (80% ACN and 0.1% formic acid) at a flow rate of 250 nL/min over 90 min.

Data were acquired with data-dependent "top10" method, which isolates the ten most intense ions and fragments them by higher-energy collisional dissociation with a normalized collision energy of 27%. The MS scan range was set to 300–1,650 m/z with a 60,000 (200 m/z) resolution and a target value of 3e6 ions. The ten most intense ions were sequentially isolated and accumulated to an AGC target value of 1e5 with a maximum injection time of 120 ms and MS/MS resolution was 15,000 (200 m/z). The peptide match option was disabled. Dynamic exclusion was set for 20 s.

Data Analysis of HLA Peptides

We employed the MaxQuant computational proteomics platform (36) version 1.5.3.2 to search the peak lists against the UniProt database (Human 85,919 entries, May 2014) and a file containing 247 frequently observed contaminants. All MS/MS datasets were processed in one batch using a global spectrum level false discovery rate (37) cutoff of 1%. Protein N-terminal acetylation (42.010565 Da) and methionine oxidation (15.994915 Da) were set as variable modifications. The second peptide identification option in Andromeda was enabled. The enzyme specificity was set as unspecific. The initial allowed mass deviation of the precursor ion was set to 6 ppm and the maximum fragment mass deviation was set to 20 ppm.

Compiling the Immunopeptidomics Database

An in-house Java program[1] based on the MzJava class library (38) was used to parse the MaxQuant results and organize them in a

[1] www.java.com.

database (*ipMSDB*). The matching and scoring between *ipMSDB* and query peptides was done by another in-house Java program and all further data analysis and visualization was performed in R[2] if not otherwise indicated.

Gene Ontology (GO) Enrichment Analysis and Tree-Map Visualization

The GO enrichment analysis of the source proteins of the presented HLA peptides was performed on the Panther webpage[3] (39). All human proteins were taken as a background and compared to the different protein lists based on the biological process classification. Proteins were quantified by their type I, II, or I/II peptide counts, respectively. A statistical overrepresentation test was performed and the resulting *p*-values were corrected for multiple testing and a *p*-value threshold of 0.05 was applied.

For visualizing of the protein lists, we used the Proteomaps tool[4] (40), which is based on the KEGG protein annotation. We used the same protein lists and the same protein peptide counts as for the GO analysis. The resulting Veronoi-tree-map images were slightly edited for better visibility of the text.

HLA-I, HLA-II, and HLA-I/II Density Profiles and Correlation between Them

HLA-I, HLA-II, and HLA-I/II density profiles were calculated from *ipMSDB* by summing up for each amino acid in a protein sequence the number of HLA-I, HLA-II, and HLA-I/II peptides covering this amino acid. In order to display profiles of HLA-I peptides of typical length, HLA-I peptides shorter than 15 amino acids were also considered separately. The correlation between profiles was calculated in a way that reflects the overlap of the main peaks in a profile ignoring smaller peaks.

Profiles for NetMHCpan version 3.0 (25) HLA-I-binding affinity prediction for the GILT, SEM4D, and MITF proteins were obtained from the web page.[5] All HLA supertype representatives and peptides of length 9–11 were selected. All predicted strongly or weakly binding peptides were retained. The profiles are the NetMHCpan scores of the representative binders summed up over all strong or weak binding peptides for each amino acids of the protein.

Training Predictor and Cross Validation

In order to test whether our *ipMSDB* based features are able to improve the prioritization of predicted immunogenic peptides, we used data from a recent publication by Stronen et al. (41): 1,034 HLA-I peptides of length 9–11 carrying non-synonymous somatic mutations were obtained by genome sequencing from 3 melanoma patients and were screened with T-cell assays for immune recognition. 16 out of 1,034 neoantigens turned out to be immunogenic. Stronen et al. calculated several features for both mutated and wild-type (wt) peptides to enable prioritization of neoantigens: best predicted binding affinity to one of the patients HLA-I alleles (*mutAffinity*, *wtAffinity*), predicted HLA-I-peptide complex stability (*mutPeptideStability, wtPeptideStability*), proteasomal cleavage probability (*mutCleavProb*, *wtCleavProb*), number of mutated and wt reads (*mutReads*, *wtReads*), and RNA abundance (*rnaExpr*). Here, we added three MS-based prediction scores, which evaluate how well the wt counterpart of the predicted mutated peptide is represented in *ipMSDB*. The first score (*nrMatchingPeptides_I*) operates on the protein level and counts the number of all wt HLA-I peptides per protein in *ipMSDB*. The other two scores (*matchScore_I* and *exactMatchScore_I*) operate on the peptide level. In order to calculate *matchScore_I* for a peptide, we sum up the HLA-I density profile height over the position of the peptide. If the peptide is found on multiple proteins and/or several times on the same protein in *ipMSDB*, we take the highest of all the *matchScore_I* values. *exactMatchScore_I* is equal to *matchScore_I* if there is an exact wt peptide match in *ipMSDB* and 0 otherwise.

In order to compare our *ipMSDB* predictors to the features described in Stronen et al. individually, all the 1,018 control and 16 immunogenic peptides were used. We applied a support vector machine (SVM) regression (42) with Gaussian kernel to compare the predictive power of feature sets. The R package e1071, which is an interface to the LIBSVM SVM implementation (43), was used for this purpose. Other than kernel selection no optimization was performed and all SVM parameters were kept at their default values. Combining several features means that each peptide is represented by a feature vector in a *N*-dimensional space, where *N* is the number of features. The task of the SVM regressor is to grade this feature space with values between −1 and 1, where values close to one represent "immunogenic" regions of the feature space. To calculate this peptide immunogenicity grading, the SVM needs to learn from training data, and in order to evaluate the quality of this learning, the grading is compared to independent test data. If the immunogenic test peptides lie in regions in the feature space with grade close to 1 and the control test peptides in regions with grade close to −1, the learning has worked well. To perform the learning and independent testing, the control and immunogenic peptide lists were both randomly split into equally sized training and test parts. One part of the control and one part of the immunogenic peptide list go to the training set (half of all peptides), the other parts to the test set (the other half of all peptides). The SVM was trained on the training set and the trained SVM regression was used to rank the peptides in the test set. The number of immunogenic peptides in the 20 top ranked test peptides was calculated as the prediction performance (value between 0 and 8). The process was repeated 2,000 times in order to calculate average performance values and their standard deviations.

RESULTS AND DISCUSSION

Assembling Large-scale Human Immunopeptidomics Database

The experimental extraction procedure of HLA peptides highly enriches for the true HLA ligands. More than 95% of the HLA-I

[2] www.r-project.org.
[3] http://www.pantherdb.org/.
[4] www.proteomaps.net.
[5] http://www.cbs.dtu.dk/services/NetMHCpan/.

peptides we identified by MS matched the typical properties of sequence length and binding motifs that are necessary for binding to the different HLA-I allotypes (31). In order to build the *ipMSDB* database of HLA peptides, we compiled data from our recent published immunopeptidomics experiments (17, 31, 33, 44) and we added unpublished data (Table S1 in Supplementary Material). Altogether, *ipMSDB* represents an in-depth repertoire of HLA-I and HLA-II peptides purified separately from dozens of different human cell lines and tissues covering many HLA allotypes. Currently, our *ipMSDB* includes 15,422 protein groups with at least one valid peptide match (only MaxQuant leading razor proteins were considered) identified from 67 different biological samples, mainly B-cells (13 samples), T-cells (4 samples), and melanoma tissues (35 samples). At the peptide level, this corresponds to 131,402 unique peptides detected in HLA-I peptide samples and 66,420 unique peptides detected in HLA-II peptide samples. The length distribution (mostly 9 to 11 -mer peptides) of the identified HLA-I peptides highlights the purity of the peptidome (**Figure 1A**). Unlike HLA-I complexes, HLA-II complexes presented families of longer peptides (mainly 13–17 amino acids) (**Figure 1A**), sharing the core binding region of typically 9 amino acids. The binding restrictions of HLA-II peptides are still rather poorly understood and technically it is more challenging to retrieve them directly from immunopeptidomics data as a way to estimate the purity of HLA-II peptidome samples. We expect that the HLA-II peptidomes have similar high purity level because we purified them similarly to the HLA-I. Also, when cells lack HLA-II expression no peptides are detected (17). Interestingly, 6,819 unique HLA-I/II peptide sequences (3.4%) in *ipMSDB* were detected in both HLA-I and HLA-II samples. **Figure 1B** reveals that their length distribution is a mixture of the class I and class II modes.

Remarkably, the broad distribution of the number of peptides presented as HLA-I and HLA-II peptides per source protein implies that the proteins are not randomly selected for presentation (**Figure 1C**; Figure S1A in Supplementary Material). As we have shown previously (31), the number of HLA-I peptides per protein depends on the protein length (Figure S1B in Supplementary Material), but the HLA presentation of proteins also depends on many other factors. The assembly of the above database has allowed preliminary observations that provide important hints on the biogenesis of the immunopeptidome. These could be exploited in the development of algorithms for optimizing the prediction of neoantigens. Our main working hypotheses are presented below.

Hint 1: The Proteome Is Selectively Sampled for Antigen Presentation

We compared the characteristics of the source proteins presented as HLA-I and HLA-II peptides in terms of the biological process GO annotations (see Materials and Methods for details). We further visualized the data with the Proteomaps tool (see Materials and Methods), which is based on the KEGG protein annotation. The Proteomaps tree-map visualization tool shows quantitative composition of proteomes arranged in multiple levels. As a pseudo quantitative score we used the number of assigned HLA peptides per protein in *ipMSDB*. On the lowest level, each protein is represented by a polygon, whose area reflects the number of HLA-I, HLA-II or HLA-I/II peptides, times the protein length, respectively. Functionally related proteins according to a KEGG hierarchy tree were arranged in adjacent and similarly colored regions. On higher levels, similar proteins were grouped into regions. We investigated cellular proteins separately for the following groups:

1. Cellular proteins presented as HLA-I peptides, collectively named "type I." A large fraction comprising 93.2% of the source proteins in *ipMSDB* were presented as HLA-I peptides. HLA-I molecules present most of the cellular proteome. GO annotation enrichment analysis (Table S2 in Supplementary Material) revealed that compared to the reference human proteome, "type I" were enriched in the following biological processes: nuclear chromosomes (p-value = 2.93E−02), nucleus (7.02E−10), and nuclear envelope (2.10E−03) and also lysosome (1.01E−02), endosome (4.67E−04) the Golgi apparatus (4.42E−02), vacuole (2.69E−04) and more general annotation like the ribosome (3.54E−23) and the cytoskeleton (6.81E−07). The MHC protein complex was

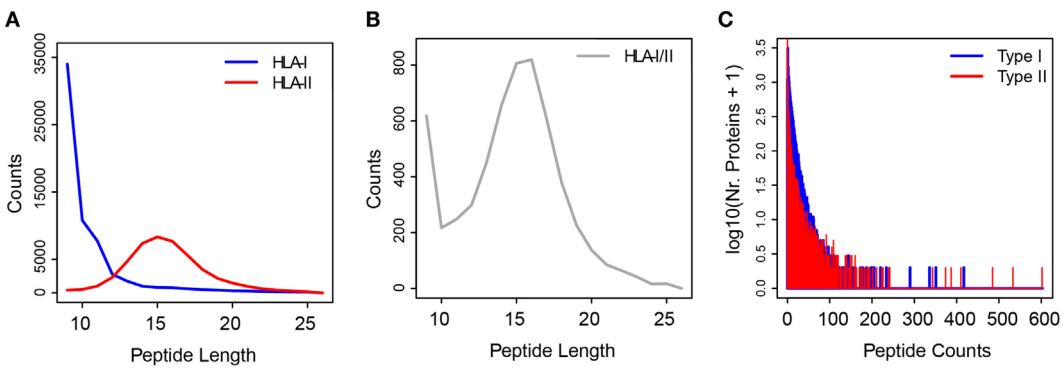

FIGURE 1 | **(A)** Length distributions of HLA-I (in blue) and HLA-II (in red) peptides. **(B)** Length distribution of HLA-I/II peptides samples. **(C)** Histogram of the number of peptides per "type I" (blue) and "type II" (red) source proteins.

enriched (6.88E−03), while membrane proteins in general were depleted (3.87E−02). The proteomaps were in agreement with the GO annotations enrichment analysis (**Figure 2A** and in more details in Figure S2 in Supplementary Material). This pattern was independently observed in B-cells, T-cells and in melanoma tissues. Differences were partially related to differences in protein expression between cell lineages and/or between *in vivo* tissues and cells growing in culture. For example, ribosomal and cytoskeleton proteins were more prominent in melanoma tissues than in B- and T-cells, while proteins related to DNA replication were presented more in rapidly dividing cells growing in culture (Figure S3A in Supplementary Material).

2. Cellular proteins presented as HLA-II peptides were collectively named "type II." "Type II" proteins were enriched in the lysosome (p-value = 7.05E−04), endosome (1.22E−06) Golgi apparatus (1.41E−05), vacuole (2.72E−06), ribosome (6.08E−32), and the cytoskeleton (2.55E−03) biological processes (Table S2 in Supplementary Material). The MHC protein complex was also similarly enriched (3.31E−04) as it was in "type I," while the SNARE complex (1.37E−02) and the vesicle coat (3.37E−02), the proton-transporting ATP synthase complex located to the mitochondria (2.3E−02) were uniquely enriched in "type II" (**Figure 2B**; Figure S4 in Supplementary Material). Some proteins differed in their presentation as HLA-II peptides in melanoma tissues compared to cells growing in culture. For example, complement and coagulation cascade proteins and hemoglobin were detected only in the tissues (Figure S3A in Supplementary Material).
3. Cellular proteins presented by both machineries, in which at least one HLA-I/II peptide sequence was detected are called "type I/II" proteins. This group of proteins was similar to the "type II," as they were enriched in the lysosome (p-value = 9.86E−05), Golgi apparatus (6.79E−03), vacuole (7.59E−05), vesicle coat (1.61E−02), ribosome (4.38E−45), and MHC protein complex (3.76E−06) biological processes, while uniquely to the "type I/II" the extracellular space was enriched (5.57E−05) (Table S2 in Supplementary Material). The typical chromosome or nucleus related proteins that are characteristic for "type I" were not significantly enriched here. Compared to "type I" proteins, "type I/II" proteins comprised of less DNA association, and similarly to the "type II," they included more proteins related to vesicular transport (**Figure 2C**; Figures S3A and S5 in Supplementary Material).

Collectively, these results indicate that the sampling of the self proteome for presentation on HLA-I and on HLA-II complexes is not random and the cellular localization of proteins, possibly also related to the mechanism of their degradation, has an impact. More than that, a subset of the proteome is presented by both machineries and resembles "type II" source proteins.

Hint 2: HLA-I/II Peptides Suggest a Cross-Talk between HLA-I and HLA-II Presentation Pathways

3.4% of peptide sequences in *ipMSDB* were detected as HLA-I/II peptides. Such long peptides detected in HLA-I peptidome could also be a technical artifact of contaminating HLA-II peptides that occurs during the purification. However, several main observations argue against this option: first, a significant part of the long HLA-I peptides fit the P2/PΩ-anchor mode of binding to the expressed HLA-I allotypes. We showed this for the UWB289 ovarian cancer cells that do not express HLA-II, and melanoma tissues from Mel15 and Mel16 patients form which both HLA-I and HLA-II peptidomes were obtained (Figure S6 in Supplementary Material). The remaining peptides could still bind with alternative internal anchors leaving the ends of the peptides to protrude beyond the binding groove (45, 46). Second, we calculated the proportion of long peptides (equal or longer than 14-mers) detected in cell lines

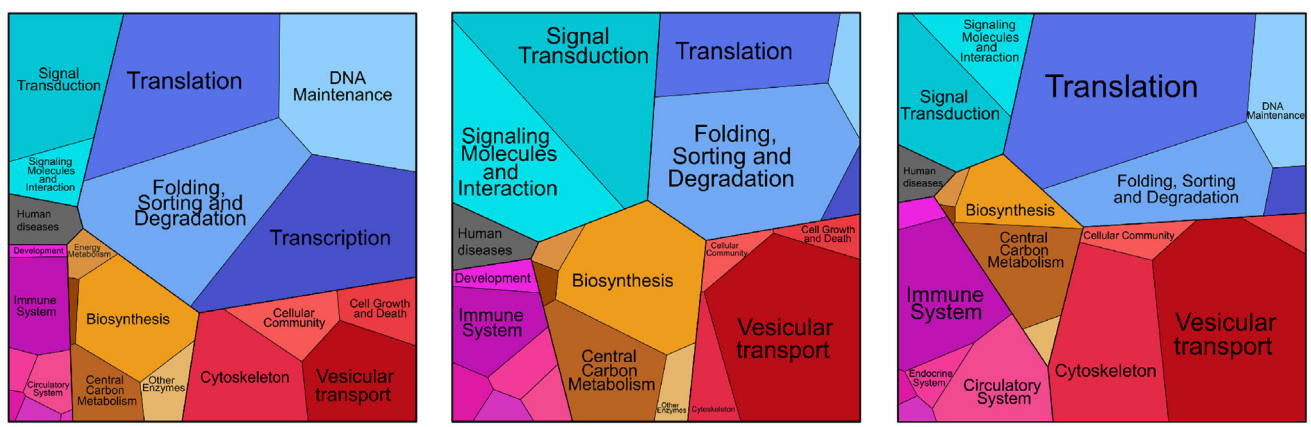

FIGURE 2 | Proteomaps visualization of the level of presentation (number of unique peptide sequences) of the source proteins of "type I" **(A)**, "type II" **(B)**, and "type I/II" **(C)** groups classified according to their annotated cellular functions.

or tissue samples that express HLA-II, and those that lack HLA-II expression, i.e., in which no or only sparse amounts of less than 100 HLA-II peptides could be detected by MS. The average proportion of longer HLA-I peptides in the group expressing HLA-II peptides was 5.6%, whereas the average proportion in the group lacking HLA-II peptides was 4.8% which was not significantly different (standard deviation is 3%). Therefore, it is very unlikely that the long HLA-I peptides were contaminations from HLA-II peptides. Furthermore, HLA-II peptides were purified from the lysates after the HLA-I had been purified, which minimized the chance that HLA-II peptides would contaminate class I peptidome samples. No HLA-II peptides could be detected in samples from which thousands of HLA-I were identified, which supports the claim that there is no significant cross contamination related to sample handling. We further elaborate on the possible biogenesis of the HLA-I/II peptides below.

Cross-presentation has been investigated for many years and it has been shown to be central for the priming of naïve T-cells against exogenous antigens. These antigens are taken up by professional antigen-presenting cells that process and consequently present them on HLA-I molecules (47). Cross-presentation happens *via* two orthogonal routes: a proteasome- and TAP-independent route where proteins digested in endosomes are loaded on HLA-I molecules imported into the endosomes, and a proteasome- and TAP-dependent route where endosomal proteins are exported to the cytosol and processed by the HLA class I presentation machinery (**Figure 3**).

Several lines of evidence, which we discuss below, led us to propose that "type I/II" source proteins are processed partially by the machinery involved in cross-presentation in the endosome-lysosome compartments. We hypothesize that cross-presentation of peptides cleaved in the endosomes consequently leads to the generation and loading of longer HLA-I peptides that are likely to be in common with the peptides generated by the class II processing machinery, and stem from the same source proteins (**Figure 3**). It is important to note that cross-presented peptides may also be generated after the polypeptides have been transferred from the lysosome–endosome compartments into the cytosol. Following the conventional class I presentation pathway that is proteasome- and TAP-dependent, these peptides will then become indistinguishable from the normal pool of HLA-I peptides characterized with a typical length (9–11 aa) (47).

We observed that HLA-I/II peptides stemmed mainly from self proteins localized within the endosome-lysosome

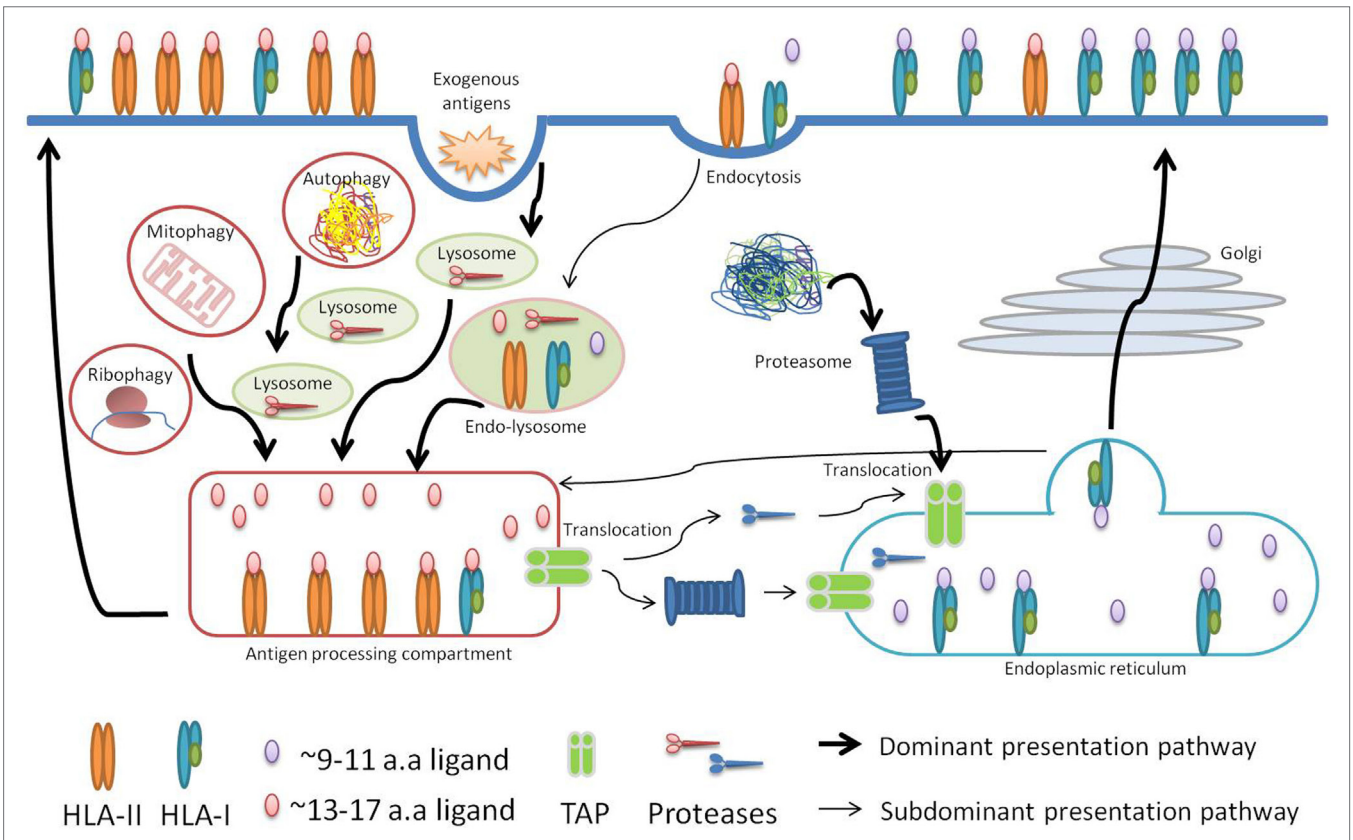

FIGURE 3 | Schematic visualization of our hypothesis. In cancer or upon infection, professional antigen-presenting cells take up antigens released by dying cells, degrade them in the endosome-lysosome compartments, and present their longer peptides as either HLA-II or HLA-I peptides in a proteasome-independent manner. In addition, shorter HLA-I peptides are presented *via* the conventional proteasome-dependent class I pathway. In case cells are directly infected with intracellular pathogens or at steady state conditions, autophagy may similarly lead to the presentation of longer peptides, from the pathogens or from the self proteome, including as HLA-I.

compartments and were enriched with phagosomal structural proteins and phagosomal cargo proteins that are degraded by autophagy (**Figure 2**). Among them are ribosomal proteins and mitochondrial proteins that may be selectively degraded and eliminated in the processes called ribophagy (48) and mitophagy (49), respectively. Previously, we studied HLA-I presentation in several cell lines and we have shown that ribosomes and mitochondrial proteins are presented to a higher extent than what would be expected from their abundance (31). One example of a protein that belongs to the "type I/II" group is the "probable serine carboxypeptidase" (CPVL) localized in phagosomes. It may be involved in the digestion of phagocytosed particles in the lysosome and in trimming of peptides for antigen presentation (50). Another example is the PMEL protein from which several peptides were detected to be presented on HLA-I and HLA-II complexes. PMEL is involved in melanosome formation and disintegration of melanosomes is assumed to take place in the lysosomes (51). Furthermore, the autophagic pathway has a substantial role in the degradation of melanosomes in keratinocytes (52). The confined space within the endosome-lysosome compartments may indeed favor cross-presentation of this set of proteins and could also explain how low abundant proteins may still be presented with multiple ligands and out-compete very abundant proteins. Furthermore, HLA-I/II peptides seem to be more prominent in B-cells and T-cells compared to melanoma tissues where class II presentation machinery might not be fully functional (Figure S3B in Supplementary Material).

Based on these observations, we hypothesize that in cancer or upon infection, professional antigen presenting cells that take up antigens released by dying cells and degrade them in the endosome-lysosome compartments, would present their longer peptides as either HLA-II or HLA-I peptides generated through the proteasome-independent pathway (**Figure 3**). Furthermore, in case cells are directly infected with intracellular pathogens or at a steady state condition, autophagy may lead to the presentation of longer HLA-I peptides from the pathogens or from the self-proteome. For example, a recent study investigated the HLA-I peptidome of cells upon infection with the intracellular pathogen *Toxoplasma gondii* (45), and reported that the *T. gondii* ligands were significantly longer than host ligands. The average length of *T. gondii* ligands was 14.6 amino acids compared to 11.4 amino acids of host ligands for infected and 9.8 amino acids for uninfected cells. Furthermore, they observed that the long ligands did not follow the P2/PΩ-anchor binding mode of HLA-I but instead were predicted to bind *via* a canonical N-terminal binding core preceding the C-terminal extension. Both the length preference and the mode of binding of these peptides may be explained by the alternative processing we describe here. Notably, the 9–11-mers could potentially be mainly driven by ER-resident chaperones and peptidases that are known to play a role in the ER conventional class I presentation pathway.

Hint 3: Protein Hotspots Are Selectively Sampled for Antigen Presentation

Interestingly, we have noticed that there are "hotspots" of antigen presentation within proteins, and that domains within proteins are presented at a higher extent. We separately aligned HLA-I, HLA-II, and HLA-I/II peptides to the protein sequences. As an example, we show the hotspots we detected for the gamma-interferon-inducible lysosomal thiol reductase (GILT) protein (UniProt P13284), the semaphorin-4D (SEM4D) protein (Uniprot Q92854), and the microphthalmia-associated transcription factor (MITF, Uniprot O75030) (**Figures 4A–C**, respectively). More examples are provided in Figures S7A–F in Supplementary Material. GILT is the only enzyme known to catalyze disulfide bond reduction in the endocytic pathway. It facilitates presentation of a subset of HLA peptides from disulfide bond-containing antigens (53). GILT is expressed constitutively in antigen-presenting cells and is induced by gamma-interferon in other cell types. It has an important role in HLA-II-restricted antigen processing and was reported to be expressed in most of primary and metastatic melanomas (54). Indeed, we also detected GILT in B- and T-cells and in melanoma tissues (**Figure 4A**, right inset). SEM4D belongs to the semaphorin family and it regulates the sensitivity of the B-cell antigen receptor that is required for proper B-cell homeostasis (55). We observed that SEM4D was mainly presented in B-cells and also in T-cells (**Figure 4B**). The length distribution of peptides derived from GILT and SEM4D (left insets in **Figures 4A,B**) reveals that the longer HLA-I peptides were also detected as HLA-II peptides. Therefore, these peptides were mainly HLA-I/II (gray line). Globally, such HLA-I/II hotspots overlapped significantly more often with HLA-II hotspots than with HLA-I hotspots (Figure S8 in Supplementary Material).

Naturally, tissue specificity will further restrict the presentation of the antigens. For example, MITF is a transcription factor that regulates the expression of genes with essential roles in cell differentiation, proliferation and survival (56). MITF plays an important role in melanocyte development by regulating the expression of tyrosinase (TYR) and tyrosinase-related protein 1 (TYRP1) (56). Indeed, we also identified MITF, TYR, and melanocyte protein PMEL ligands almost exclusively in the melanoma tissues (**Figure 4C**; Figures S7D,E in Supplementary Material).

It is important to note that HLA-binding affinities cannot accurately predict the hotspots we detected for these proteins (**Figures 4D–F**). Therefore, the immunopeptidomics data provide critical additional information to capture the true *in vivo* presented ligandome. The height of the hotspots and the distribution of hotspots along the protein sequence reflect the level of its presentation. Hotspots may be related to sequence and structure dependent proteasomal or endosomal cleavage preferences. Alternatively, hotspots may be merely the outcome of *de facto* presentation of the rather more stable polypeptides surviving the highly proteolytic cytosolic environment as the expected half life of peptides in the cytosol of living cells is 6–10 s (57). Furthermore, some posttranslational modifications may interfere with protein cleavage and with binding of the modified peptides to the HLA, eliminating them from the presented repertoire. Currently, no prediction algorithms incorporate these factors. Since our database comprises of peptidomes of dozens of different HLA allotypes and binding specificities, these hotspots reflect an average propensity of a

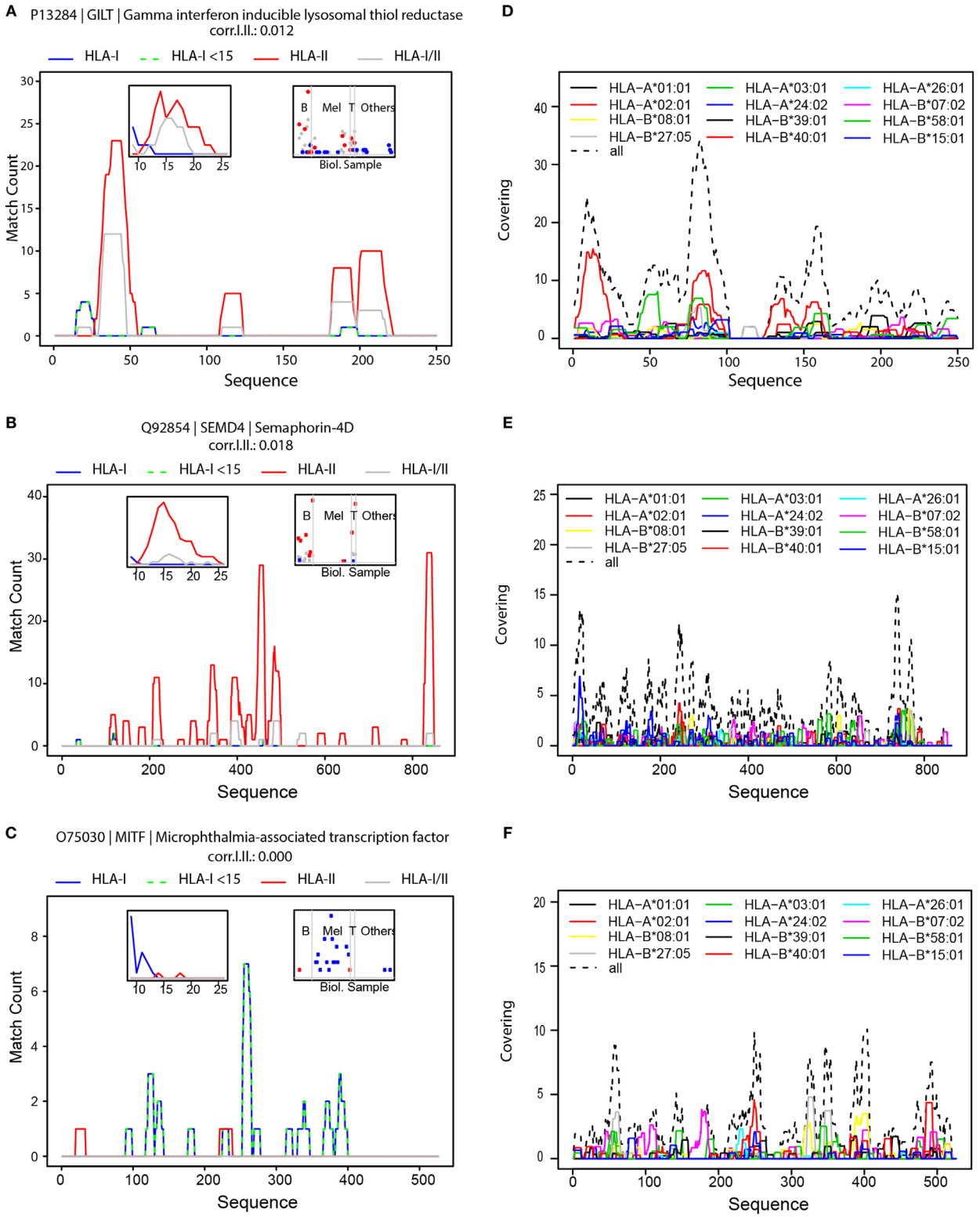

FIGURE 4 | Hotspots of mass spectrometry-detected HLA-I and HLA-II binding peptides and the landscape of predicted HLA-I peptides. Visualization of hotspot of HLA peptides from GILT **(A)**, SEMD4 **(B)** and MITF **(C)**; density profiles of HLA-I peptides (in blue), HLA-I peptides shorter than 15-mers (in green dashed line), HLA-II peptides (in red), and HLA-I/II peptides (in gray). The left inserts are histograms of the length of HLA-I, HLA-II and HLA-I/II peptides, and the right inserts visualize the number of peptides detected in the different samples, grouped as B-cells, Melanoma, T-cells and other samples. Visualization of the landscape of possible presentation of GILT **(D)** SEMD4 **(E)** and MITF **(F)** using alignment of peptide sequences predicted by NetMHCpan to bind any of the 12 HLA-I supertypes.

protein sub-sequence to be presented on a HLA molecule. Hotspots may encompass HLA peptides with N′ or C′ terminal extensions of several amino acids to accommodate peptides that fit a variety of HLA-binding specificities. Hotspots of "type I" proteins are highly enriched in 9–11 HLA-I peptides (Figures S7A,B in Supplementary Material). Similarly, hotspots of "type II" proteins are longer, and the length distribution of their peptides is centered on 15-mer peptides (Figure S7C in Supplementary Material).

Hints on Immunopeptidomics Biogenesis Can Be Applied for Prioritization of Neoantigens

MS-based immunopeptidomics is a powerful approach to shed light on the selective sampling of the proteome. Because it captures the actual presented peptidome, it may bypass the need to computationally predict ligands. Therefore, it is also a promising method to directly identify presented neoantigens. However, enough tumor tissue is available only for the minority of patients and only rarely such neoantigens can be detected due to current limitation in sensitivity. Therefore, the widespread approach for identification of neoantigens for personalized cancer vaccines is still based on *in silico* predictions of the binding affinity to the respective HLA class I allotypes of 9–11-mer peptide sequences harboring the non-synonymous mutations. RNA expression data are further interrogated to exclude non-expressed genes. This *in silico* approach has high false positive rate and, consequently, the list of proposed targets must be further filtered to enrich for true positives. In addition, as we have just discussed above, some HLA ligands are longer and do not conform to the classical binding mode, hence are expected to be false negatives. All the hints presented above could be incorporated into predictors for neoantigen immunogenicity. Here, we focus on Hints 1 and 3.

We propose that large-scale and in-depth peptidomics MS/MS data of naturally presented ligands can be used to complement existing predictors and to improve HLA-I neoantigen prioritization. We test this concept using the Stronen et al. data (see Materials and Methods for more details). To develop this approach, we first defined three immunopeptidomics MS-based features. The first feature, at the protein level, is the number of HLA-I peptides per protein in our database (*nrMatchingPeptides_I*). We anticipate that proteins that are highly presented in their wild-type form will have a better chance to present neoantigens once they are mutated. On the peptide level, a match between a predicted peptide and our database can be *exact*, *included* or *partial* (**Figure 5A**). We expect that if the mutation is in a position in the source protein that is naturally presented, then we have evidence that the neoantigens could also be presented. Obviously, this will not hold if the mutation falls on a HLA-binding site or otherwise strongly weakens HLA binding, but for this proof of concept we will not consider these events. We added two additional features that quantify the overlap of the predicted wild-type (wt) -peptide with other HLA-I peptides in our database, *exactMatchScore_I* and *matchScore_I* (Table S3 in Supplementary Material). Please refer to Section Materials and Methods for a more detailed description of these scores. These scores reflect the propensity of the wt-peptide to be presented as a HLA-I peptide. As a simplification, we assume that a mutation does not completely obfuscate this propensity, just as the wt-affinity is still a good predictor for the mutated peptide affinity and immunogenicity.

Applying all predictors to the data published by Stronen et al. (see Materials and Methods), we show that the MS-based features provide valuable information for the prioritization of the neoantigens (**Figure 5B**). The performance of a single MS-based feature was slightly better than the RNA abundance feature, but lower than the affinity and stability features. Overall, 16 out of 1,034 peptides were immunogenic (1.5%), whereas 3 out of 66 peptides with a positive *matchScore I* (4.5%) and 1 out of 7 (14.2%) with a positive *exactMatchScore I* were immunogenic, i.e., the MS-based scores are able to enrich immunogenic peptides. One striking observation was that all 16 immunogenic peptides belonged to proteins that were present in *ipMSDB*, which is unlikely to happen by chance (872 out of all 1,034 peptides did match *ipMSDB*, which corresponds to a probability of $0.065 = (872/1{,}034)$[16] that the 16 immunogenic peptides are present in *ipMSDB* by chance). Therefore, we improve prediction based on binding affinity of mutated peptides if we remove all predicted sequences which do not belong to any protein in our database. **Figure 5C** shows how many immunogenic peptides are correctly predicted as a function of the rank of the *mutAffinity* score before (gray line) and after such removal (red line).

Furthermore, we used the Stronen et al. data and the cross-validation scheme outlined in Section Materials and Methods to evaluate how much predictive power the *ipMSDB* features add in combination with non-MS features. We trained a SVM regression on the set of training peptides in order to rank the test peptides according to their SVM-predicted immunogenicity. We then calculated the number of correctly predicted peptides within the 20 top-ranked peptides (see Materials and Methods for more details). We examined how the incorporation of the MS-based features into the SVM prediction improves the number of correctly predicted peptides. When the non-MS scores were *mutAffinity*, *mutPeptideStability*, and *rnaExpr*, we improved the prediction by 15.9% (group 1). 38.3% prediction improvement was obtained when the non-MS scores were the scores from group 1 plus *wtAffinity* and *wtPeptideStability* (group 2). *The best improvement of* 50.9% was obtained when the non-MS scores were the scores from group 1 plus *diffAffinity*, *diffPeptideStability*, which incorporate the differences in the binding affinity and in the binding stability, respectively, of the wt and the mutant peptides (Group 3; where *diffAffinity* is *mutAffinity-wtAffinity* and *diffPeptideStability is mutPeptideStability-wtPeptideStability*) in **Figure 5D**. These results indicate that our *ipMSDB* based features are complementary to the affinity based features.

Interesting interactions between the MS-based features and affinity-based features emerged, but given the small size of the dataset the following interpretations are speculative. Figure S9 in Supplementary Material shows that almost all immunogenic peptides (blue dots) had a low *mutAffinity* score (low values are good), and that MS-based features were

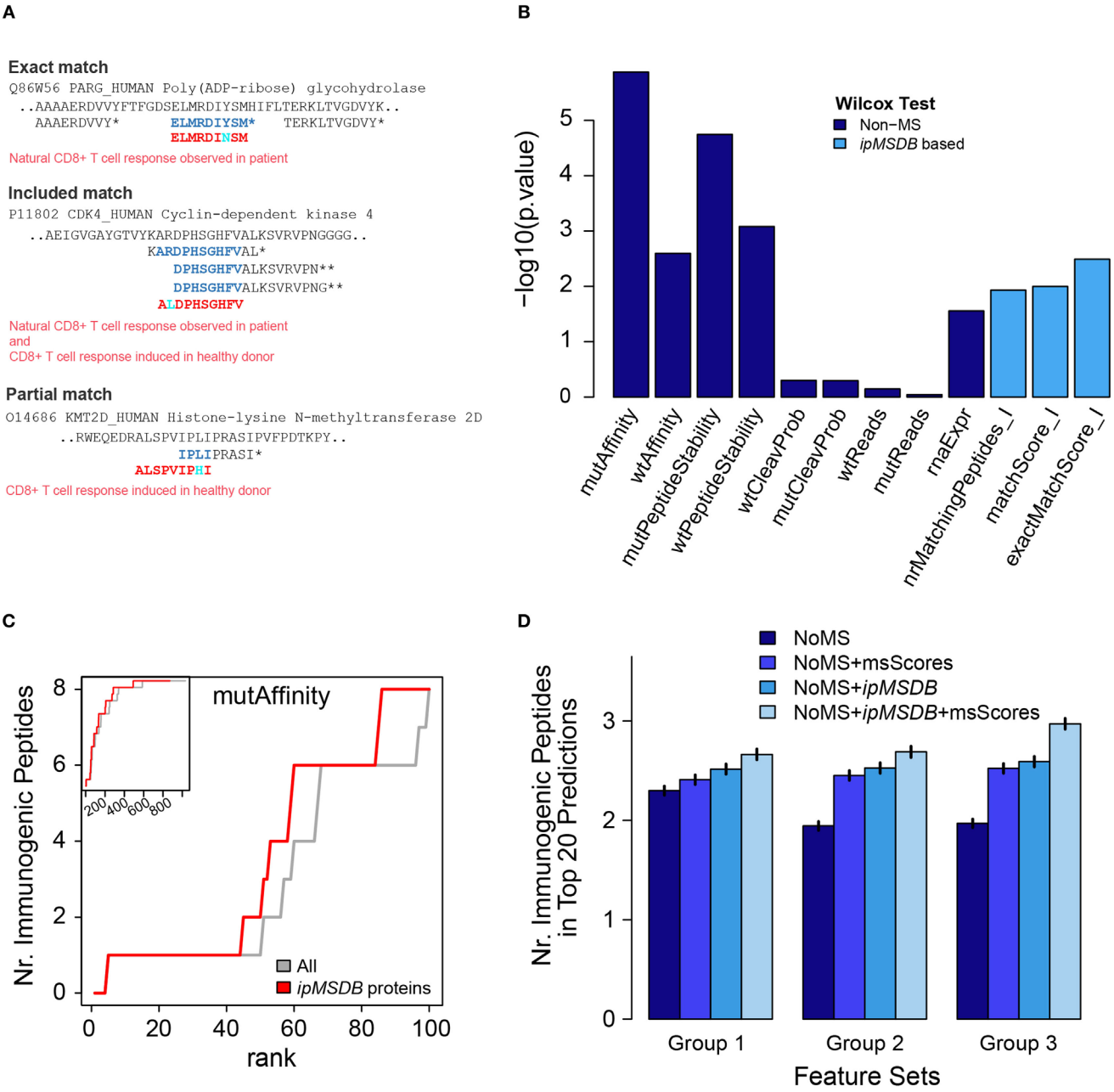

FIGURE 5 | **(A)** Examples of matches of confirmed immunogenic neoantigens from Stronen et al., with peptides in *ipMSDB*. Predicted neoantigen sequences are in red, overlapping amino acids within peptides detected by mass spectrometry (MS) are in blue and mutation are in cyan. Matches can be "exact," signifying that the exact wild-type (wt) counterpart of the neoantigen sequence was detected by MS, "included," i.e., the neoantigen is included within the sequence of a longer wt peptide detected by MS, or "partial," i.e., the neoantigen is partially overlapping at the position of the mutation with the wt counterpart detected by MS. *HLA-I peptides, **HLA-II peptides. **(B)** The higher the $-\log_{10}$ of Wilcox-test *p*-values, (i.e., the smaller the pValue), the more different the distribution of immunogenic peptide values is to the distribution of non-immunogenic peptide values. See Ref. (41) for details about the non-MD scores. **(C)** Predicted peptides were ranked by *mutAffinity* score (HLA-binding affinity of mutated peptide) and the number of correctly predicted immunogenic peptides (maximally 16) is plotted against the rank. Gray line represents before and red line after removal of proteins not present in *ipMSDB*. The main figure shows the first 100 ranks, whereas the inset shows all 1,034 ranks with the *y*-axis ranging from 0 to 16. **(D)** Average number of correctly predicted immunogenic peptides in the top 20 peptides ranked by support vector machine regression. Vertical small bars represent ± 2 times the standard deviation of the average values. msScores are *exactMatchScore_I* and *matchScore_I*. In group 1, non-MS scores were *mutAffinity*, *mutPeptideStability*, and *rnaExpr.* In group 2, the non-MS scores were the scores from group 1 plus *wtAffinity* and *wtPeptideStability* that take into consideration also aspects related to the wt peptide counterparts. In group 3, non-MS scores were the scores from group 1 plus *diffAffinity*, *diffPeptideStability*, which incorporate the differences in the binding affinity and in the binding stability, respectively, of the wt and the mutant peptides. *diffAffinity = mutAffinity−wtAffinity* and *diffPeptideStability = mutPeptideStability−wtPeptideStability.*

able to rescue the two peptides with the higher *mutAffinity* value (larger orange diamond and red square). Peptides with an *exact* match to the immunopeptidomics MS database (red points) lied close to the diagonal, i.e., had similar *mutAffinity and wtAffinity* scores in which the mutation does not seem to change HLA binding. On the other hand, *included* or *partial* matches can also identify peptides which had different affinities in their mutational and wild type state. In order to confirm these interactions, refine them or find new ones, many more datasets will be required. Also, more work is needed to extend the prediction to peptides, which are often missed by MS/MS analysis such as very hydrophobic peptides or peptides that fragment very poorly.

One of the advantages of our approach is that it may mitigate the limitation of predicting binding affinity for very rare HLA-I alleles and for HLA-II molecules. We deduced information from hundreds of thousands of ligands detected in tens of different individual donors, representing the overall distribution of HLA allotypes in the human population. As the binding specificities of different HLA allotypes may be redundant, in the future, an exhaustive database will provide a good approximation to the definite immunopeptidome. As we know from many studies, immunogenicity of peptides is not highly correlated with binding affinity (26, 58, 59), and peptides with (measured) low affinity may still induce an immune response, especially upon vaccination. However, the peptides should still be naturally presented on the target cells to induce an effective T-cell response. We envision that the pan-HLA-peptide interaction predictors will provide estimation of the binding affinity, and together with an exhaustive immunopeptidomics database, the prioritization of neoantigens will include hotspot features, which capture other aspects of the natural *in vivo* presentation.

In this preliminary, proof of concept study we showed how immunopeptidomics database comprising many melanoma tissue samples contained information that enabled prioritization of neoantigens predicted in similar melanoma samples. We anticipate that the same tumor type will have to be adequately represented in the database to overcome and reflect tissue specific expression signatures. The scoring scheme we introduced here may already be implemented providing large in-depth immunopeptidomics data matching the investigated tumor is present. Yet, much more data from T cell based assays of both immunogenic and confirmed non immunogenic neoantigens from multiple patients across different tumor types will be critical to sorely benchmark and optimize this algorithm.

CONCLUSION

Given the rise in the number of research labs performing large-scale immunopeptidomics and the growing interest in detecting neoantigens by MS, it is very likely that within the coming years, comprehensive databases of naturally presented immunopeptidomes from thousands of donors and HLA allotypes will be characterized. This will inevitably lead to a deeper understanding not only of the binding specificities of each of the HLA molecules (28, 29, 32–34) but also of the rules governing sampling of proteins and of the cellular machineries that are involved, in a cell type specific manner (28). The immunopeptidomics data do not contribute to the understanding of immunogenicity seen from a tolerance perspective, since they do not provide information about which neoepitopes are sufficiently "foreign" to induce a T cell response. Therefore, high-throughput functional T-cell screening assays will be fundamental in resolving the propensity of these presented peptides to induce the $CD8^+$ and $CD4^+$ immune response (20). We envision that combining improved HLA-binding predictions together with information about *in vivo* presentation and their recognition by effector T-cells will significantly improve the accuracy of neoantigen prediction algorithms. Consequently, more patients could benefit from the promising personalized neoantigen-based treatments.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the institutional review board [Ethics Commission, University Hospital of Lausanne (CHUV)] with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Commission, University Hospital of Lausanne (CHUV).

AUTHOR CONTRIBUTIONS

Conception and design of the work, analysis and interpretation of data, manuscript writing: MM and MB-S; critical revision for important intellectual content: DG and GC. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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An Analysis of Natural T Cell Responses to Predicted Tumor Neoepitopes

Anne-Mette Bjerregaard[1], Morten Nielsen[1,2], Vanessa Jurtz[1], Carolina M. Barra[2], Sine Reker Hadrup[3], Zoltan Szallasi[1,4] and Aron Charles Eklund[1]**

[1] Department of Bio and Health Informatics, Technical University of Denmark, Kongens Lyngby, Denmark, [2] Instituto de Investigaciones Biotecnológicas, Universidad Nacional de San Martín, Buenos Aires, Argentina, [3] Section for Immunology and Vaccinology, National Veterinary Institute, Technical University of Denmark, Kongens Lyngby, Denmark, [4] Computational Health Informatics Program (CHIP), Boston Children's Hospital, Harvard Medical School, Boston, MA, United States

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Angelika B. Riemer, Deutsches Krebsforschungszentrum (DKFZ), Germany

***Correspondence:**
Anne-Mette Bjerregaard ambj@bioinformatics.dtu.dk; Aron Charles Eklund eklund@bioinformatics.dtu.dk

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Personalization of cancer immunotherapies such as therapeutic vaccines and adoptive T-cell therapy may benefit from efficient identification and targeting of patient-specific neoepitopes. However, current neoepitope prediction methods based on sequencing and predictions of epitope processing and presentation result in a low rate of validation, suggesting that the determinants of peptide immunogenicity are not well understood. We gathered published data on human neopeptides originating from single amino acid substitutions for which T cell reactivity had been experimentally tested, including both immunogenic and non-immunogenic neopeptides. Out of 1,948 neopeptide-HLA (human leukocyte antigen) combinations from 13 publications, 53 were reported to elicit a T cell response. From these data, we found an enrichment for responses among peptides of length 9. Even though the peptides had been pre-selected based on presumed likelihood of being immunogenic, we found using NetMHCpan-4.0 that immunogenic neopeptides were predicted to bind significantly more strongly to HLA compared to non-immunogenic peptides. Investigation of the HLA binding strength of the immunogenic peptides revealed that the vast majority (96%) shared very strong predicted binding to HLA and that the binding strength was comparable to that observed for pathogen-derived epitopes. Finally, we found that neopeptide dissimilarity to self is a predictor of immunogenicity in situations where neo- and normal peptides share comparable predicted binding strength. In conclusion, these results suggest new strategies for prioritization of mutated peptides, but new data will be needed to confirm their value.

Keywords: neoepitopes, neoantigens, prediction, immunogenicity, mutations, MHC binding

INTRODUCTION

Tumor cells can be naturally recognized by the adaptive immune system based on the sequence and abundance of the immunogenic peptides presented on the tumor cell surface. The majority of known tumor antigens are either normal peptides expressed at an unusually high level, or peptides derived from translation of somatic mutations (neoepitopes).

Neoepitopes are important in several successful approaches to enhance tumor killing by T cells. Inhibitors of immune checkpoints such as programmed cell death protein 1 and cytotoxic T lymphocyte-associated protein 4 counter the inhibition of T cell responses often observed in cancer

patients. With both of these approaches, a higher mutation load predicts greater clinical benefit, suggesting that neoepitopes are important for immune response, at least in selected cancer types (1, 2). Adoptive transfer of expanded tumor-infiltrating lymphocytes (TILs) increases the proportion of tumor-responsive T cells, and selective expansion of neoepitope-specific T cells has been successfully demonstrated (3). Recent studies using peptide vaccines of patient-specific neoepitopes have shown that targeting neoepitopes may be an effective method to treat cancer (4, 5). Thus, rapid and accurate identification of patient-specific neoepitopes is an important goal.

Neoepitopes can be identified in various ways. Early studies used T cell reactivity screening of cDNA expression libraries (6). More recently, a common approach is to first identify somatic mutations by sequencing DNA and/or RNA from a tumor and matched normal specimen. These somatic mutations can be used to infer changes in protein sequences, resulting in "neopeptides" that are potentially present in tumor cells but not in normal cells. Such candidate neopeptides can next be synthesized and tested for reactivity by autologous T cells using various assays such as ELISPOT, fluorescently labeled HLA tetramers, or barcode-labeled HLA multimers (7). However, most neopeptides do not serve as neoepitopes. For a neopeptide to become a neoepitope, at least two properties must be fulfilled: the peptide must be processed and presented by HLA, and the presented peptide must be recognized by a suitable T cell. The problem of predicting neoepitopes can, therefore, be split into two individual problems: (1) predict neopeptide antigen processing and presentation (presentation) and (2) predict which peptides, if presented by HLA, can trigger a T cell response (immunogenicity).

Predicting HLA presentation is typically done based on prediction of HLA binding between an individual peptide sequence and the relevant HLA alleles, using tools such as NetMHC (8) or NetMHCpan (9). If available, mRNA expression data may be used to eliminate neopeptides from genes that are not expressed. Abelin et al. (10) trained a prediction algorithm considering mass spectrometry (MS) data from eluted peptides, peptide expression and cleavage, outperforming NetMHC 4.0 and NetMHCpan 2.8, although this predictor is not yet publicly available. It should be mentioned that the newest version (4.0) of NetMHCpan is also trained on MS eluted ligand data as well as binding affinity data (11).

To predict immunogenicity, one proposal has been to use the "differential agretopic index" (DAI), defined as the difference in binding strength between the mutated neopeptide and its unmutated normal peptide counterpart (12). The reasoning behind this is based on the mechanism of immune tolerance ensuring that no T cell will recognize HLA presented self peptides. Given this, one way for a neopeptide to become a neoepitope would be to have significantly improved HLA-binding capacity compared to the normal peptide. In this situation, only the neopeptide will be presented by surface HLA, and hence, no tolerization is present against the normal peptide. Consequently, tolerization against the neopeptide is expected to play a minor role for the immunogenicity in this situation. In contrast, when the neopeptide and the normal peptide are both HLA binders, tolerization against the normal peptide has taken place, and this tolerization is expected to impact the immunogenicity of the neopeptide. Consequently, in this situation, the immunogenicity will depend on the lack of similarity between the mutated neopeptide and the normal counterpart.

Typically, only a minority of the tested neopeptides evoke a T cell response, suggesting that current methods to select candidate neopeptides are insufficient. In order to characterize the properties of neoepitopes, at least two groups have analyzed the characteristics of combined lists of published, confirmed neoepitopes. Van Buuren et al. (13) compiled a list of 17 neoepitopes that were identified without predictions and thus tested in an "unbiased" manner and found that their prediction algorithm would have correctly predicted 12 of the 17, for a sensitivity of 70%. However, this analysis does not provide a specificity estimate, and the data set is too small to analyze the relative importance of their individual selection criteria. Fritsch et al. (14) did a similar study on a larger group of 40 published neoepitopes, and found that in the majority of cases both the mutated and unmutated peptide were predicted to bind to HLA. Importantly, neither of these studies analyzed their data considering the set of neopeptides that did *not* elicit a T cell response. Recently, several studies have assessed immunogenicity of larger sets of neopeptides and have published lists of neopeptides, which both did and did not elicit a T cell response. We set out to analyze these data to investigate if any broad patterns emerge that might enable better predictions of neoepitopes.

MATERIALS AND METHODS

Data Collection and Correction

Data was gathered from the 13 published papers (**Table 1**). For 10 of 13 studies, both neopeptides and the corresponding unmutated peptide were provided. For the other three studies, the corresponding normal peptides were missing or partially missing. For the missing normal peptides, we used "pepmatch," a program available as part of MuPeXI (15), to identify the most similar peptide from the normal human peptidome. The normal human peptidome was defined as all unique peptides of lengths 8–11 extracted from human proteins available in Ensembl release 85, based on human genome GRCh38. Out of 820 neopeptides analyzed with pepmatch, 20 matched a reference peptide exactly with no mismatches, and 14 matched a reference peptide with more than a single mismatch. Additionally, one study included peptides originating from indels resulting in one peptide originating from a frameshift mutation being tested. An additional 7 peptide-HLA combinations were duplicates and were removed from the dataset together with the 35 non-single nucleotide variant (SNV) peptides; we note that none of these elicited an immune response. Thus, the final dataset included 1,948 peptide-MHC combinations of 27 HLA alleles and 1,874 unique mutated peptides. It should be noted that we included all 11 peptides from the study by Strønen et al., only two of these were found in autologous TILs, whereas the rest of the immunogenic peptides were identified in peptide stimulated PBMCs from healthy donors.

TABLE 1 | Data included in this study.

Reference	Publication date	First and last author	Journal	Tumor type	Patients	Peptides tested	T cell responses	Test method	Peptide lengths
(16)	2013–05	Robbins et al. and Rosenberg	Nat Med	SKCM	3	227	10	ELISPOT	9–10
(17)	2013–11	Van Roij et al. and Schumacher	J Clin Oncol	SKCM	1	–	1	FLT	9
(18)	2014–03	Wick et al. and Nelson	Clin Cancer Res	HGSC	3	109	1	ELISPOT	8–11
(19)	2014–06	Rajasagi et al. and Wu	Blood	CLL	2	48	3	ELISPOT	9–10
(20)	2014–07	Lu et al. and Robbins	Clin Cancer Res	SKCM	2	10	2	ELISA	8–11
(1)	2014–12	Snyder et al. and Chan	N Engl J Med	SKCM	1	–	1	ICS	9
(2)	2015–04	Rizvi et al. and Chan	Science	NSCLC	1	–	1	FLT	9
(21)	2015–10	Cohen et al. Robbins	J Clin Invest	SKCM	8	427	9	FLT	9–10
(22)	2016–01	Kalaora et al. and Samuels	Oncotarget	SKCM	1	2	1	ICS	9, 11
(23)	2016–03	McGranahan et al. and Swanton	Science	NSCLC	2[a]	642[a]	3/8[a]	FLT/BLM	9–11
(24)	2016–05	Strønen et al. and Schumacher	Science	SKCM	4	56	11	FLT	9–11
(25)	2016–05	Bassani-Sternberg et al. and Krackhardt	Nature Commun	SKCM	1	8	2	MS-FLT	8–10, 12
(26)	2016–08	Bentzen et al. and Hadrup	Nat Biotechnol	NSCLC	2[a]	703[a]	9[a]	BLM	9–11
	Total	13		4	30	1,874	53	5	5

1,874 unique tested peptides, 1,948 peptide-HLA combinations, from 27 HLA alleles.

FLT, fluorescently labeled tetramers; BLM, DNA barcode-labeled multimers; ICS, intracellular cytokine staining; MS, mass spectrometry; SKCM, skin cutaneous melanoma; NSCLC, non-small cell lung cancer; CLL, chronic lymphocytic leukemia; HGSC, ovarian high grade serous carcinoma.

[a]*Peptides and patient overlap between studies.*

Binding Prediction

We used a custom Python script to extract additional information, including the amino acid change, peptide mutation position, and number of mismatches, in addition to running NetMHCpan-4.0 (11) for HLA binding prediction and extracting the output predicted affinity and eluted ligand likelihood percentile rank (EL%Rank) score. NetMHCpan 4.0 is trained on both *in vitro* binding affinity and MS eluted ligand data and includes distinct prediction modes for each of the two data types. The default mode for NetMHCpan-4.0 (and the mode recommended for eluted ligand and epitope prediction) is eluted ligand-likelihood predictions. However, the user has the possibility to use the binding affinity mode by selecting the −BA. In this study, the recommended mode was used, evaluating the peptides on EL%Rank score. This score indirectly accounts for peptide cleavage and translocation when predicting peptide binding, as part of the dataset used for training consisted of MS identified HLA eluted ligands.

Anchor Mutation Annotation

Anchor positions for each HLA allele were manually defined from NetMHCpan-3.0 sequence motifs (9). Peptides were annotated according to whether the mutation occurred in the given HLA allele anchor position (Table S1 in Supplementary Material, column: "BindingPosition," "Anchor").

Analysis and Statistics

The resulting data was analyzed in R, and plots were generated using R packages ggplot2 and ggbeeswarm. *P*-values for difference in proportion were calculated using a two-sided Fisher's exact test and/or Student's *t*-test.

Self-Similarity Predictions

The similarity between pairs of neo- and normal peptides was calculated using the kernel similarity measure proposed by Wen-Jun Shen et al. (27). The measure gives a value between 0 and 1 for the similarity of two peptides, where a value of 1 indicates a perfect match. In basic terms, this similarity is calculated from matching, at different length scales, all kmers (a substring of length k) in one peptide to the kmers in the other peptide using a Blosum similarity measure. In Figure S1 in Supplementary Material, we show the average similarity between a set of 9-mer peptide pairs with single mutations at different positions, forming 3,420 single mutant peptide pairs (20 random natural peptides each mutated to 19 single mutant variants at each of the 9 peptide positions). From this plot, it is clear that single mutation variations toward the N and C terminal of the peptide have very limited impact on the similarity between two peptides (the similarity is high) compared to mutations in the central part of the peptides (where the similarity is lower).

Receiver Operator Characteristic (ROC) Curve Generation

Generally, a ROC curve is created by plotting the true positive fraction (or sensitivity) against the false positive fraction (or 1—specificity) at various threshold settings. In the ideal case, where a threshold exists that can perfectly separate the positive and negative data point, the area under the ROC curve (AUC) is 1, and in the situation where the predictive model has no predictive power, the ROC curve forms a straight line $x = y$ and the AUC is 0.5. The plots where generated in R using the packages ggplot2 and plotROC.

The full dataset including all predictions, deselected peptides, HLA alleles, and additional peptide-specific information can be found in Table S1 in Supplementary Material.

RESULTS

We searched for published studies in which putative neoepitopes were first identified by tumor DNA sequencing and then

experimentally tested for T cell reactivity. We chose to focus on studies in which native T cell reactivity against the minimal peptide of a neoepitope was assessed and did not include vaccine studies in which an induced T cell response was assessed. We considered only neopeptides derived from a SNV/missense mutation. We identified 13 relevant studies from which we created a dataset consisting of 1,948 unique peptide-HLA complexes, of which, 53 were reported to elicit a T cell response (**Table 1**; Table S1 in Supplementary Material). This represents 1,874 unique peptides; some of which were evaluated in combination with more than one HLA allele.

First, we searched for broad trends in factors that might influence neopeptide immunogenicity (**Table 2**). We analyzed the proportion of neopeptides eliciting a response according to neopeptide length and found that 9-mers had the highest relative frequency of response (4.3%), substantially higher than 10-mers (2.4%, $P = 0.063$) or 11-mers (0.2%, $P = 0.00001$). Note that this analysis accounts for the larger number of 9 and 10mer peptides experimentally evaluated compared to 8 or 11mer (**Table 2**). We next compared the three HLA genes and did not find a statistically significant difference between HLA-A and HLA-B ($P = 0.50$). The number of HLA-C restricted responses was too low to make any meaningful analyses related to the relative importance of this locus.

Even though the neopeptides included in this study were selected by the original study authors based on predicted binding affinity, we asked whether predicted HLA binding could be used to further prioritize the neopeptides. We did this by examining the predicted HLA binding strength of neopeptides and normal peptides with NetMHCpan-4.0 using the EL%Rank score (results for binding affinities are included in Table S1 in Supplementary Material). We found a broad range of predicted binding values of the neopeptides from each study (**Figure 1A**), and immunogenic neopeptides (neoepitopes) were overall predicted to bind significantly more strongly ($P < 0.0001$, Student's *t*-test, AUC = 0.72) than non-immunogenic peptides (**Figure 1B**). Similar but less significant differences were observed when comparing the HLA-binding strength of the immunogenic and non-immunogenic peptides in terms of predicted binding affinity and predicted EL%Rank scores (data not shown). We also analyzed the DAI described by Duan et al. (12), but this was only moderately predictive (AUC = 0.57). The vast majority (75%) of the neoepitopes were predicted to be very strong HLA binders with EL%Rank scores less than 0.5. Only seven neoepitopes had a predicted EL%Rank score greater than 2, and 5 of these were 10- or 11-mer peptides, which all contained nested submer peptides with improved binding to the HLA allele, suggesting that these peptides were not mapped to the minimal epitope (27). The remaining two were 9-mer peptides both containing segments of double or triple cysteines (KVCCCQILL, NLNCCSVPV). Such cysteine-rich peptides are handled poorly by the MHC binding prediction tools due to the bias against cysteines in the peptide data used to train these methods. In fact, replacing the cysteines in the two peptides with X (making NetMHCpan ignore these residues) confirms the strong binding strength (EL%Rank score less than or equal to 1, data not shown). Overall, we hence find that neoepitopes, in accordance with earlier studies analyzing HLA ligands and T cell epitopes in general (9, 11), are characterized by strong predicted binding to the restricting HLA molecules. We find that 96% of the neoepitopes (given the handling of outliers described above) are identified at a EL%Rank threshold of 2.

We plotted the predicted binding strength of neopeptide and normal peptide and observed the previously described pattern (14) that the data can be split into two broad groups (**Figure 1C**). One group (CB for conserved binding) is defined by peptides where the neo- and normal peptides have comparable binding strength (peptides located close to the diagonal), and one group (IB for improved binding) where the neopeptides have improved binding compared to the normal peptides (peptides located in the upper left corner). As a reflection of the processes applied to selected neo-peptides, very few examples are found where the neopeptide has decreased binding compared to normal.

Next, we split the peptides into two equal sized groups of IB and CB. The split was determined based on the ratio between the EL%Rank scores for the mutated and corresponding normal peptide ($EL\%Rank_n/EL\%Rank_m$). The IB group included neopeptides, which had at least a 20% improved binding (ratio $\geq$1.2) whereas the CB group included the remaining peptides (ratio <1.2). Note that this ratio-based measure shares a high overlap with the DAI. As expected, a very large proportion (45%) of the peptides in the IB group are characterized by mutations in the HLA anchor positions, whereas the proportion of peptides with such mutations is low in the CB group (14%) (**Figure 1C**).

Given the split of peptides into IB and CB, we now investigated how the similarity of the neopeptide to "self" (here taken as the normal counterpart peptide) would impact the peptide immunogenicity. This we did by calculating the similarity between each neo- and normal peptide using the kernel similarity measure proposed by Wen-Jun Shen et al. (27). In short, the similarity in this measure is estimated from the combined set of overlapping kmer (substring peptides of length *k*) peptides. An inherent bias of this approach is that it focuses on the central part of the peptide (for details see Materials and Methods). This bias makes it an

TABLE 2 | Associations between peptide characteristics and T cell responsiveness.

	T cell response		Total	Proportion responding	*P*
	No	Yes			
Peptide length					
8mer	24	1	25	0.040	1.00
9mer	742	33	775	0.043	N/A
10mer	720	18	738	0.024	0.063
11mer	408	1	409	0.002	0.00001
12mer	1	0	1	0.000	1.00
HLA gene					
HLA-A	1,440	42	1,482	0.028	N/A
HLA-B	414	9	423	0.021	0.50
HLA-C	41	2	43	0.047	0.35

P-values represent a test for difference in proportion responding, between the given row and the corresponding most frequent row (9mers or HLA-A).

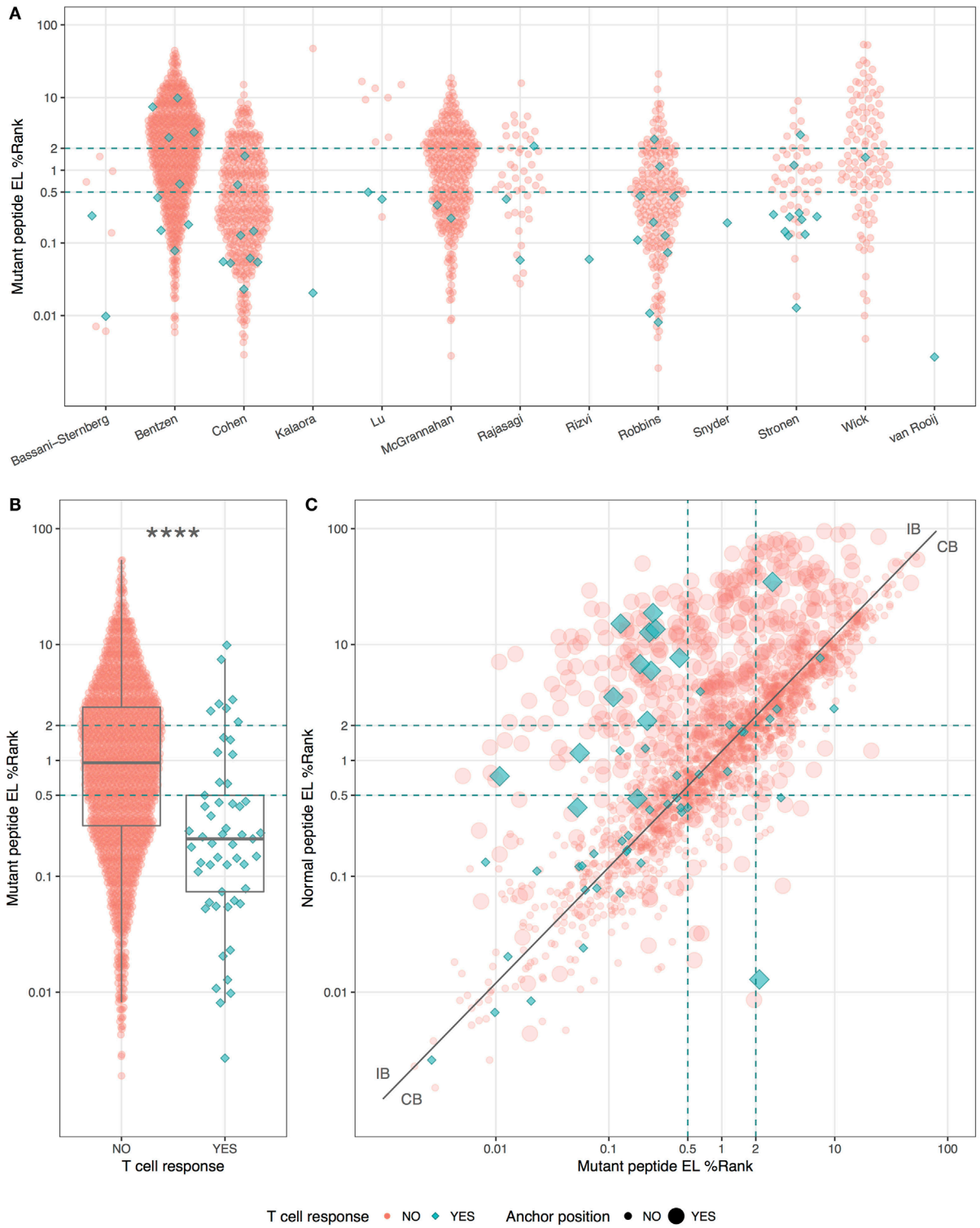

FIGURE 1 | HLA-binding properties of neopeptides. Predicted eluted ligand likelihood percentile rank (EL%Rank) score of neopeptides, corresponding to individual studies **(A)** or summarized according to mutant peptide T cell response **(B)**. As there is an overlap in patients between the Bentzen and the McGrannahan study, only unique observations are plotted, the first refereeing to the peptides tested with barcode labeled multimers and the second with fluorescently labeled tetramers **(A)**. **(C)** Predicted EL%Rank score for neopeptides and their corresponding normal peptides, with mutant peptide T cell response and anchor position mutations indicated. The curve corresponding to the median $EL\%Rank_n/EL\%Rank_m$ value equal to 1.2, used to define the groups of peptides with improved binding strength (IB) and conserved binding strength (CB), is shown as a solid line. Thresholds for weak (2 EL%Rank) and strong binders (0.5 EL%Rank) are indicated with dashed lines. ****$P < 0.0001$.

ideal first approximation to the HLA fingerprint on T cell interactions with peptide–HLA complexes; here, the C and N terminal positions of the peptide are generally found to play a minor role due to their important contribution to the HLA binding (28). The measure gives a value between 0 and 1 for the similarity of two peptides, where a value of 1 indicates a perfect match. Using this measure, the self-similarity scores of the immunogenic and the non-immunogenic neopeptides were compared for either the complete data set, or the two groups (IB and CB) defined above in terms of the difference in HLA binding strength between mutant and normal peptide (**Figure 2**).

These results demonstrate that self-similarity in general is a relatively poor predictor for peptide immunogenicity. However, this situation is changed when focusing on the CB subset of peptides where the neo- and normal peptide share comparable HLA binding strength. Here, we found the immunogenic peptides to be significantly less similar to self, compared to the non-immunogenic peptides ($P = 0.02499$, Student's t test). For the IB subset of peptides with improved HLA binding strength of the neopeptide compared to self, immunogenic and non-immunogenic peptides were found to have the same level of self-similarity. The difference in self-similarity score is even more evident when directly comparing IB versus CB peptides among the immunogenic neoepitopes only. Here, we find that CB neoepitopes are indeed characterized by a lower self-similarity score compared to IB neoepitopes ($P = 0.00395$, Student's t-test).

We summarize these findings in **Figure 3** where receiver operator characteristic (ROC) curves for the predictive performance of HLA binding of the neopeptides, the DAI score, and the self-similarity score are shown for the complete data set and for the two peptide groups IB and CB. In short, a ROC curve is a graphical illustration of the power of a predictive model, in this case, how good predicted HLA binding, DAI and the self-similarity score are at sorting the immunogenic peptides before the non-immunogenic peptides (for details see Materials and Methods). The plots in **Figure 3** confirm the above findings, namely that binding strength to HLA is an overall good predictor for neopeptide immunogenicity (the mutant peptide EL%Rank score achieves the highest predictive performance in all 3 plots), that DAI demonstrates poor predictive performance for the data included in the given study (the AUC is low and close to 0.5 in all cases), and that neopeptide self-similarity can be used as an additional correlate besides binding to peptide immunogenicity for peptides with comparable HLA binding between neo- and normal peptide (AUC = 0.65 in **Figure 3** CB).

Taken together, these results support the notion that immunogenicity of neopeptides should be predicted using different approaches according to the relationship of the neopeptide HLA binding strength to the binding strength of the counterpart normal peptide. In cases where the neo- and normal peptides both are binders and share similar binding strength, self-similarity plays an important part in the prediction of the neopeptide immunogenicity. This is in contrast to the situation where only the neopeptide is predicted to bind HLA. Here, self-similarity plays a limited role, if any, for the prediction of neopeptide immunogenicity.

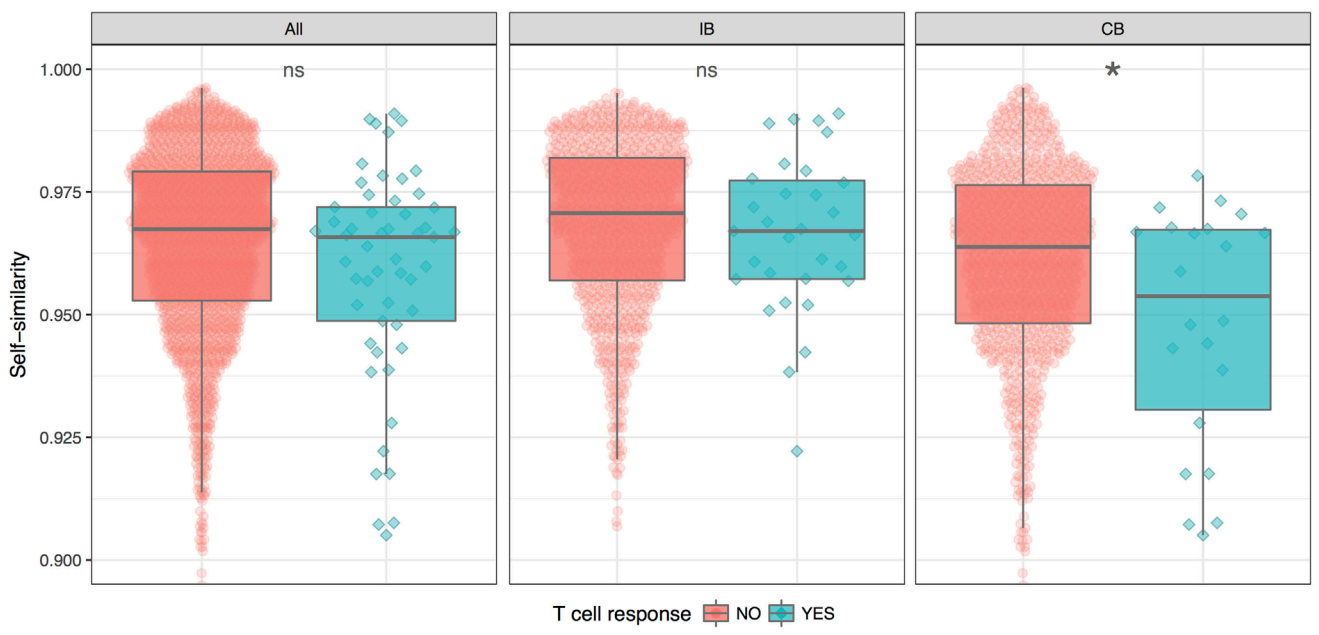

FIGURE 2 | Similarity between neo- and normal peptides. The plot shows the average and SE for the immunogenic (blue) and non-immunogenic (red) peptides for each of the three peptide groups; all (all peptides in the given study), IB (neopeptides with increased binding compared to the normal peptide), and CB (neopeptides with comparable binding compared to the normal peptide). For details, see text. The difference in similarity scores is significant only for CB (*$P = 0.025$, Student's t-test).

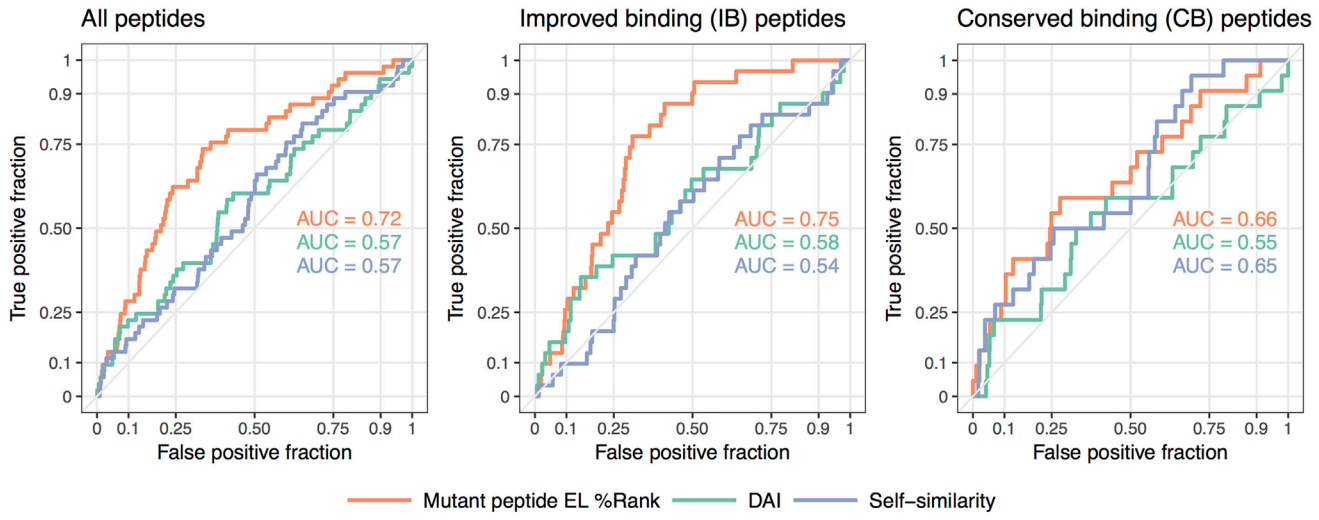

FIGURE 3 | Receiver operator characteristic analyses of the predictive performance of the NetMHCpan-4.0 eluted ligand likelihood percentile rank (EL%Rank) score, the "differential agretopic index" (DAI), and the self-similarity measure. The diagonal line corresponding to AUC = 0.5 is included as a guide to the eye.

DISCUSSION

Previous studies have analyzed published neoepitopes for patterns in peptide binding affinity (13, 14). To the best of our knowledge, this is the first study analyzing published neoepitopes along with neopeptides from the same studies, which failed to elicit a T cell response. By comparing these two sets of peptides, we identified several patterns that may improve prioritization of candidate neoepitopes.

First, we observed that 9-mer neopeptides were substantially more likely to elicit a T cell response than other peptide lengths. However, it is possible that this reflects the relative inaccuracy in earlier versions of HLA binding prediction algorithms in predicting HLA binding affinity for peptides with length different from 9. Newer versions of, e.g., NetMHCpan better account for peptide length preferences. Indeed, NetMHCpan-4.0 predicts overall stronger affinity for the 9-mers in this study compared to other lengths.

Second, we found that predicted HLA binding was a strong correlate to neopeptide immunogenicity, despite the fact that all neopeptides analyzed in this study were selected in the original publications based on such predictions. Analyzing the HLA binding strength in terms of the EL%Rank score of NetMHCpan-4.0, we found that the immunogenic neopeptide bound HLA significantly stronger than the non-immunogenic peptides. In terms of absolute prediction scores, 96% of the neoepitopes were found to bind with a EL%Rank score of 2 or less. This binding threshold and sensitivity value is in agreement with earlier studies of HLA ligands and T cell epitopes outside the cancer epitope field (9, 11), and suggest that neoepitopes bind HLA with similar binding strength as pathogen derived epitopes. In the context of HLA binding, we also found that the DAI alone is, overall, less predictive than the HLA binding strength of the neopeptide.

Finally, we found that different characteristics were associated with immunogenicity when splitting the peptides into two groups based on neopeptides with conserved binding strength (CB) or improved binding strength (IB) compared to the normal peptide. For the IB neopeptides, no difference in self-similarity between immunogenic and non-immunogenic peptides were observed. In contrast, for the IB neopeptides, the immunogenic peptides were found to share significantly lower self-similarity compared to the non-immunogenic. Given the limitations of the current study (in particular related to the very small number of neoepitopes included), we believe this result to reflect the impact of T cell tolerance on neopeptide immunogenicity. Tolerization against self is only relevant for antigen presented peptides. If a normal peptide fails to bind HLA, no tolerization would have happened against this peptide, and we hence expect similarity toward this peptide to play a minor role in the prediction of immunogenicity. In contrast, we would expect tolerization to take place against a HLA binding self-peptide, and hence also that similarity toward such self-peptides plays a prominent role when predicting neopeptide immunogenicity. This hypothesis is reflected directly in our results.

We are aware that this study suffers from several important limitations. First, 3 of the 13 studies did not provide the peptides, which did not elicit T cell recognition or activation. The non-immunogenic peptides are important for discovering patterns that distinguish non-immunogenic neopeptides from immunogenic neoepitopes. However, a more profound limitation of this study is the small amount of data available. We anticipate that newer high-throughput T cell reactivity screening systems will provide much more data, which will enable a more detailed analysis. Also, it is clear that the model used to assess peptide similarity is very simplistic, and most likely could be refined substantially by for instance taking into account the direct impact on the TCR fingerprint imposed by variations in HLA anchor positions, and by incorporating an amino acid similarity measure different from the protein evolution-based Blosum score used here. Finally, it will be important to evaluate the effects of other factors such as

antigen processing, HLA binding stability (29, 30), gene expression, mutant allele frequency, and clonality (23), each of which may be associated with immunogenicity.

AUTHOR CONTRIBUTIONS

Design of the study: A-MB, AE, MN, SH. Data collection: A-MB, VJ. Data analysis and interpretation: A-MB, VJ, CB, MN, AE. Drafting the article: A-MB, AE, MN. Critical revision of the article: SH, VJ, CB, ZS. Final approval of the version to be published: A-MB, AE, ZS, SH, MN, CB, VJ.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01566/full#supplementary-material.

FIGURE S1 | Average similarity between peptide pairs with single mutations at different locations within the peptide. The plot was estimated from 3420 single mutant peptide pairs (20 random natural peptides each mutated to 19 single mutant variants at each of the 9 peptide positions).

TABLE S1 | Characteristics of peptides analyzed in this study.

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CORRECTION
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Corrigendum: An Analysis of Natural T Cell Responses to Predicted Tumor Neoepitopes

Anne-Mette Bjerregaard[1], Morten Nielsen[1,2], Vanessa Jurtz[1], Carolina M. Barra[2], Sine Reker Hadrup[3], Zoltan Szallasi[1,4] and Aron Charles Eklund[1]**

[1] Department of Bio and Health Informatics, Technical University of Denmark, Kongens Lyngby, Denmark, [2] Instituto de Investigaciones Biotecnológicas, Universidad Nacional de San Martín, Buenos Aires, Argentina, [3] Section for Immunology and Vaccinology, National Veterinary Institute, Technical University of Denmark, Kongens Lyngby, Denmark, [4] Computational Health Informatics Program (CHIP), Boston Children's Hospital, Harvard Medical School, Boston, MA, United States

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****Correspondence:***
Anne-Mette Bjerregaard
ambj@bioinformatics.dtu.dk;
Aron Charles Eklund
eklund@bioinformatics.dtu.dk

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An outdated version of Supplementary Table 1 was uploaded to the final version of the paper for publication. This table has not been under peer review and does not include the information described in the paper such as the similarity measurement column. The correct Supplementary Table 1 has now been published in the original article. The authors apologize for this oversight. This error does not change the scientific conclusion of the article in any way.

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REVIEW
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Neoantigens Generated by Individual Mutations and Their Role in Cancer Immunity and Immunotherapy

*Mirjana Efremova, Francesca Finotello, Dietmar Rieder and Zlatko Trajanoski**

Biocenter, Division of Bioinformatics, Medical University of Innsbruck, Innsbruck, Austria

Recent preclinical and clinical studies have proved the long-standing hypothesis that tumors elicit adaptive immune responses and that the antigens driving effective T-cell response are neoantigens, i.e., peptides that are generated from somatically mutated genes. Hence, the characterization of neoantigens and the identification of the immunogenic ones are of utmost importance for improving cancer immunotherapy and broadening its efficacy to a larger fraction of patients. In this review, we first introduce the methods used for the quantification of neoantigens using next-generation sequencing data and then summarize results obtained using these tools to characterize the neoantigen landscape in solid cancers. We then discuss the importance of neoantigens for cancer immunotherapy using checkpoint blockers, vaccination, and adoptive T-cell transfer. Finally, we give an overview over emerging aspects in cancer immunity, including tumor heterogeneity and immunoediting, and give an outlook on future prospects.

Keywords: next-generation sequencing, immunoediting, tumor heterogeneity, somatic mutations, cancer vaccines

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****Correspondence:***
Zlatko Trajanoski
zlatko.trajanoski@i-med.ac.at

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INTRODUCTION

In the past decade, driven by technological advances major progress in cancer research and cancer therapy was made. First, the development of next-generation sequencing (NGS) technologies and large-scale projects such as The Cancer Genome Atlas (TCGA) resulted in a comprehensive characterization of the human cancer genomes. And second, novel drugs targeting immune checkpoint molecules have been approved in several malignancies and are showing remarkable clinical effects. These drugs augment T-cell activity by blocking cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed cell death protein 1 (PD-1), or PD-1 ligand. Long-term data of patients who received anti-CTLA-4 antibodies in unresectable or metastatic melanoma indicate curative potential in a fraction of patients (1). Moreover, efficacy of anti-PD-1 antibodies has been shown not only in melanoma, but in an increasing number of other cancers (2). Not surprisingly, there are now enormous efforts for the development of novel immunotherapeutic strategies with over 1,000 clinical trials with monotherapies or combination therapies (3).

One specific advantage of cancer immunotherapy is the potential to adapt to the evolution of the tumor since specific T cells can develop which are targeting newly developed tumor clones. T cells recognize tumor-specific antigens bound to the major histocompatibility complex (MHC) molecules of tumor cells. Antigens with high tumoral specificity have the potential to elicit tumor-specific immune responses and are, therefore, of great interest for cancer immunotherapeutic strategies, including therapeutic vaccines and engineered T cells. There are three classes of antigens with high tumoral specificity: (1) viral antigens that are derived from genes expressed in virus-infected tumor cells; (2) cancer-germline antigens, also known as cancer-testis antigens. These

are proteins that are expressed only by germline cells and have aberrant expression in tumor cells; and (3) neoantigens, i.e., are peptides that are generated from somatic mutations. During tumor progression, mutations accumulating in the tumor genome can affect protein-coding genes and result in altered protein sequences. Mutated proteins are proteolytically cleaved into short peptides and presented on the tumor cell surface by the MHC—called human leukocyte antigen (HLA) in humans. These mutated neoantigens, which are present in the malignant cells but not in the normal cells can be recognized as foreign by tumor-infiltrating lymphocytes (TILs) and elicit potent tumor-specific immune responses. Neoantigens released after tumor cell death initiate a number of processes that ultimately lead to T cells that recognize cancer cells through the interaction of distinct T-cell receptors (TCR) with specific neoantigen–MHC complexes.

The tumor–immune cell interaction can be conceptualized as a number of processes conceptualized as the cancer-immunity cycle (4). The first step in this cycle is the generation of neoantigens (neoepitopes) and, therefore, the identification and characterization of neoantigens is of utmost importance for deriving novel mechanistic insights on cancer immunity and developing efficient cancer immunotherapies. In this review, we give an overview of the current advances in the computational prediction of neoantigens and discuss the development of cancer immunotherapies targeting neoantigens, including vaccination, checkpoint therapy, and adoptive cell transfer.

QUANTIFYING NEOANTIGENS USING NGS DATA

Neoantigens can be experimentally determined using proteomic analysis of MHC ligands by liquid chromatography and tandem mass spectrometry (5–7). However, this approach is labor intensive and requires large amount of material for the analysis, which is seldom available from human biopsies. Alternatively, when NGS data are available from matched tumor and normal samples, neoepitopes can be predicted by integrating four computational tasks: (i) prediction of somatic DNA mutations; (ii) identification of mutated proteins; (iii) *in silico* HLA typing; and (iv) selection of the mutated peptides with high binding affinity to the predicted MHC/HLA molecules and high expression of the mutation-encoding gene [see recent comprehensive review (8)]. Somatic DNA mutations are usually computed from whole-exome (WES) or whole-genome sequencing (WGS) data from matched tumor-normal samples using computational tools for variant detection, and can be further processed with software for variant annotation to predict the affected proteins (9). Patient-specific NGS data from WES, WGS, or RNA sequencing (RNA-seq) can be also used to predict HLA types with computational tools like Polysolver (10) and Optiptype (11), which are able to extract the reads covering the HLA locus and predict the major alleles at 4-digit resolution or more. Finally, machine learning algorithms such as NetMHCpan (12) trained on experimental data can be used to predict which short peptides spanning protein regions affected by mutations bind with high affinity to the predicted HLA types.

The single tools performing the three computational tasks described above require a number of intermediate steps for data preprocessing and formatting which is usually carried out in specialized bioinformatics labs. In order to broaden the utility of the computational genomics tools, a number of computational pipelines that integrate the individual steps were recently developed. Such pipelines for *in silico* prediction of personalized neoantigens from NGS data with different degrees of functionality include pVAC-seq (13), FRED 2 (14), INTEGRATE-neo (15), and MuPEXI (16). However, although an improvement to the use of individual steps, assembling analytical pipelines and executing workflows with a number of consecutive steps is laborious and depends on many parameter settings. The recently developed pipeline TIminer (17) integrates cutting-edge bioinformatics tools for the analysis of both, RNA-seq data and somatic DNA mutations in order to characterize the tumor–immune interface. This pipeline enables: (1) genotyping of HLAs using exome-sequencing or RNA-seq data, (2) prediction of tumor neoepitopes using specific HLA types and mutations, and (3) characterization of TILs from bulk RNA-seq data.

The available computational pipelines predict neoepitopes that bind to class-I MHC molecules. Peptides binding to class-I MHC molecules, which exist on almost all nucleated cells, are presented for recognition by cytotoxic $CD8^+$ T cells. Class-II MHC molecules are present only on professional antigen-presenting cells, such as dendritic cells, macrophages, and B lymphocytes, and display antigens to $CD4^+$ helper T cells. Although coordinated $CD4^+$ and $CD8^+$ responses are required for tumor control and rejection, the suboptimal performance of the current algorithms for prediction of class-II neoantigens limits their translational potential for personalized cancer medicine. The need for better methods for prediction of class-II neoantigens has increased ever since studies showed that $CD4^+$ T cells recognize a higher number of neoantigens than was previously known and can generate potent antitumor response (17). More recently, a proof-of-concept by Sahin et al. and Ott et al. using a combined strategy for class-I and class-II neoantigen prediction was presented (18, 19).

There are several challenges with MHC–peptide-binding prediction algorithms. First, experimental data from measurements of the biochemical affinity of synthetic peptides, needed for the training of these algorithms, are limited for MHC class-II alleles. Therefore, while effective in predicting many epitopes, these approaches may nevertheless be limited in their accuracy due to the sparsity of both positive and negative training data sets and result in high false-positive rate. For example, in Robbins et al., 229 tumor-specific neoepitopes were predicted across three melanoma patients, but only 11 of these neoepitopes elicited a T-cell response (20). In addition, these methods do not necessarily consider the endogenous processing and transport of peptides prior to HLA binding. In order to improve neoantigen predictions, Abelin et al. developed a new biochemical and computational pipeline for LC–MS/MS analysis of endogenously processed HLA-associated peptides along with a predictor that outperformed current algorithms that are trained on peptide affinity data (21).

NEOANTIGEN LANDSCAPE IN SOLID CANCERS

Given the availability of NGS data from cancer samples from large-scale projects such as the TCGA, as well as the improved performance of the computational tools, a number of studies analyzed neoantigens and association with clinical parameters and molecular entities. A seminal work by Holt and colleagues showed an association between neoantigen load and survival (22). We recently generated high-resolution maps on neoantigens and the immunophenotypes in colorectal cancer (CRC) (23) using genomic data sets from the TCGA cohort ($n = 598$) (24). The neoepitopes were barely shared between patients: only 4% of the predicted neoepitopes were shared between 2 and more patients. The shared neoepitopes are identical peptides that originate from one or more genes. Importantly, we observed that the genetic basis of the tumors determines the tumor escape mechanisms. For example, hypermutated tumors had higher intratumor heterogeneity, indicating that the larger mutational load results not only in a larger neoantigen load but also in a more diverse neoepitope landscape, and therefore likely promotes T-cell activation and infiltration.

We then extended the analysis and characterized more than 8,000 patients from the TCGA comprising 19 solid cancers (25) (results available at https://tcia.at). As expected, our pan-cancer analysis showed that the number of neoepitopes correlated with the mutational load. The results of this analysis are shown in **Figure 1**. Moreover, the number of neoepitopes correlated also with the infiltration of TILs. The fraction of neoepitopes generated from driver genes was 7.6%. Thus, the majority of neoepitopes had its origin in passenger genes. Similar to the CRC study, the results showed that the neoepitopes were seldom shared. From the total of 911,548 unique predicted neoepitopes, only 24 were common in more than 5% of patients. As expected, the most frequent predicted neoepitopes were induced by mutations in driver genes, such as BRAF, RAS, and PIK3CA. Thus, the results show that the neoantigen landscape in solid cancers is not only highly diverse both, between and within cancers, but also extremely sparse. The sparsity of the neoantigen space clearly argues against vaccination strategy based on off-the-shelf vaccines. Rather, cancer vaccination strategies based on neoepitopes has to be personalized. This can be achieved by using whole-exome NGS for the identification of somatic mutations and bioinformatic prediction of neoepitopes, followed by synthesis of peptide- or DNA/RNA-based vaccines. Proof of concept for this type of individualized cancer vaccination was recently shown in several clinical studies (18, 19, 26).

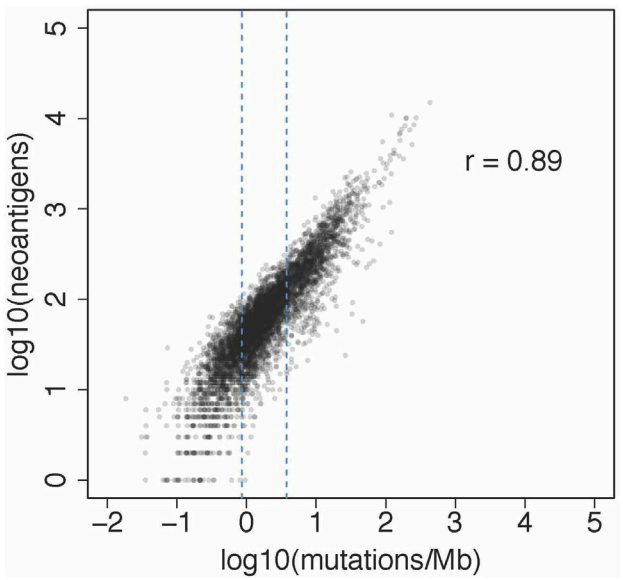

FIGURE 1 | Association between neoantigens and mutations from a pan-cancer analysis reported recently (25). The plot shows the results of the analysis of 6,726 patients from 19 solid cancers. The number of neoantigens per subject ranged from 1 to 15,035 and the number of mutations per MB per patients from 0.019 to 933.085.

NEOANTIGENS AND CANCER IMMUNOTHERAPY

Self-antigens that are aberrantly expressed in cancerous tissues can provoke an immune response and have been used in the past in clinical studies. However, expression of these antigens in normal tissues can initiate central and peripheral tolerance mechanisms. Lately, more efforts have been focused on antigens derived from mutated proteins. Since T cells recognizing neoantigens are not influenced by central immune tolerance because of the lack of expression in healthy tissues, targeting of tumor neoantigens may be more specific and less toxic than other approaches, making neoantigens attractive targets for immunotherapy, including therapy with antibodies directed against immune checkpoint blockers, therapeutic vaccination, or adoptive T-cell transfer with TCR-engineered neoantigen-specific T cells.

Neoantigens and Response to Therapy with Checkpoint Blockers

An increasing number of studies have shown a strong association of the mutation/neoantigen burden with TIL infiltration and activity, as well as better response to therapy and overall survival in non-small cell lung cancer (NSCLC) and melanoma patients (27–29). Both types of cancers accumulate high number of mutations as a result of exposure to mutagens, such as tobacco smoke and ultraviolet light. Tumors with microsatellite instability due to deficiency in the mismatch repair system show high mutational burden, T-cell infiltration, improved survival, and durable clinical benefit when treated with checkpoint blockers. Similarly, tumors with mutations in other DNA repair pathways (30–32) showed an enhanced T-cell response and better response to checkpoint blockers. A recent study by Le and colleagues provided further evidence of the sensitivity of mismatch-repair-deficient cancers to checkpoint blockade, irrespectively of the tissue of origin (33). The authors evaluated the efficacy of anti-PD-1 treatment in patients with mismatch repair deficiency from 12 different cancer entities and reported objective responses in

53% and complete responses in 21% of these patients. Moreover, they demonstrated through functional analysis in a responding patient that tumor-reactive lymphocytes were directed against mutated neoantigens. As a comparison, MSS CRC and cancers with low mutational load such as prostate cancer have shown little or no benefit from immunotherapies, providing additional evidence for the importance of neoantigens in the antitumor immune response. However, there are also cases of cancers with high neoantigen burden showing no response to immune checkpoint therapies [e.g., 50% of the microsatellite instable (MSI) cancers], as well as cancers characterized by low neoantigen load that are susceptible to immunotherapy (34). Thus, it can be argued that the high neoantigen load represents merely a higher likelihood of the presence of immunogenic neoantigen suggesting that neoantigen landscape alone is not sufficient in predicting immunotherapy responses.

Cancer Vaccination

Individualized vaccines, designed to present neoantigens to prime and activate dendritic cells have also been used to selectively target neoantigens. Vaccines have been shown to both expand pre-existing neoantigen-specific T-cell populations as well as broaden the TCR repertoire. In addition to enhancing the strength and persistence of T cells, vaccines can elicit an immune response for neoantigens that were undetectable prior to vaccination (26). Therefore, even if the neoantigens that spontaneously induce T-cell responses are lost due to immunoediting, the neoantigens that do not naturally elicit a response can serve as targets for vaccines (35). Two recent studies (18, 19), used vaccines based on neoantigens recognized by $CD4^+$ and/or $CD8^+$ T cells to demonstrate that personal neoantigen vaccines, alone or in combination with checkpoint blockade, can induce both effective and safe immune response. The authors reported T-cell infiltration induced by vaccination and specific killing of tumor cells expressing neoantigens. Ott et al. used synthetic long 15–30-mers peptides and immunized six melanoma patients, two of which had lung metastases (35). Four out of six patients had no disease recurrence, whereas metastatic patients were further treated with anti-PD-1 therapy and showed complete tumor regression. Sahin et al. used an RNA-based approach using predicted neoantigens recognized by $CD4^+$ and $CD8^+$ T cells in 13 melanoma patients (18). Neoantigen-based vaccination reduced metastatic events and caused objective response in two over five metastatic patients and, even more strikingly, a complete response in a third patient treated with the vaccine in combination with anti-PD-1 immunotherapy. Both reports further indicate that neoantigens are important targets for mediating response to checkpoint therapies and, additionally, that the tumor-reactive T cells target diverse tumor clones, thereby dealing with extensive tumor heterogeneity.

Adoptive T Cell Transfer

Neoantigens can be used to expand neoantigen-specific T cells *in vitro* for use in adoptive T-cell transfer. Several studies reported tumor regression achieved by transfer of autologous TILs resected from patients with metastatic melanoma (20, 36). The Rosenberg group demonstrated dramatic tumor regression in a metastatic cholangiocarcinoma patient treated with a personalized adoptive cell transfer, where over 95% of autologous T cells consisted of $CD4^+$ T cells that recognized a single HLA class-II-restricted neoantigen (37). They also demonstrated the therapeutic efficacy of neoantigen-specific $CD4^+$ T cells. In a study by van Rooij et al., in which exome sequencing and MHC tetramer screening was used, compelling evidence was provided that immunotherapy with checkpoint blockers in a melanoma patient induces expansion of already existing T cells which are targeting neoantigens (38).

Despite the success of immunotherapies targeting neoantigens, many questions remain unanswered. To begin with, only few of the predicted neoantigens elicit potent immune responses. Even though only a small percentage of the *in silico* predicted neoantigens are actually immunologically recognized, Strønen et al. provided evidence of the existence of a neglected neoantigen repertoire that induces T-cell reactivity and may broaden neoantigen-specific T-cell reactivity and enable the targeting of neoantigens that have not been recognized by the patient's own immune system (39). This is further supported by two other studies (18, 35) that showed that responses against neoantigens can be induced *de novo*. One possible explanation for the hidden neoantigen repertoire is the immunodominance of tumor antigens: the immune system targets particular tumor antigens but ignores others (40), a phenomenon that often occurs with viral antigens. In addition, lack of T-cell priming against tumor-associated antigens can result in the exclusion of T cells from the tumor microenvironment. For example, Spranger et al. (39) reported that oncogenic WNT/β-catenin signaling pathway prevents T-cell and $CD103^+$ DC infiltration in melanoma and generates resistance to checkpoint blockade therapy.

In conclusion, genomic approaches can facilitate the development of personalized immunotherapies directed at neoantigens. Therapeutic stimulation of broad neoantigen-specific T-cell responses through vaccines targeting multiple antigens could help overcome the effects of tumor heterogeneity as well as avoid resistance, by providing broader coverage of the whole tumor cell population. As highly homogeneous tumors have been shown to be more immunogenic and since clonal neoantigens seem to drive antitumor responses following therapy with antibodies against immune checkpoints, a potential approach is to target clonal neoantigens, i.e., those that are expressed in all tumor cells within a patient, in order to overcome the significant challenge posed by intratumor heterogeneity. However, different cancers undergo different evolutionary trajectories: some are dominated by Darwinian selection pressures that shape their clonal composition, whereas others follow neutral evolution. In order to successfully target the whole tumor population and prevent escape of resistant clones, comprehensive genomic and immunogenomic analyses of pre- and post-treatment samples are needed for longitudinally evaluating changes in the tumor. Ultimately, a deeper understanding of the evolutionary and immune-related forces that shape the tumor progression will be fundamental to improve the efficacy of immunotherapy and minimize resistance.

TUMOR HETEROGENEITY, IMMUNOEDITING, AND ACQUIRED RESISTANCE TO CANCER IMMUNOTHERAPY

Tumor Heterogeneity

Mutational processes and genomic instability can result in extensive tumor heterogeneity, which has important clinical and immunological implications. While it is well established that intratumoral heterogeneity has an impact on the response of cancer patients to treatments with targeted therapies (41–43), the role of the immune surveillance and sensitivity of the tumors to therapy with checkpoint blockers are only beginning to emerge. Recent studies provided insights into the effect on intratumoral heterogeneity on the immune response and showed that the mutational load in combination with the intratumoral heterogeneity is a better predictor of response to checkpoint blockers than the neoantigen burden alone. More homogeneous tumors and tumors with high clonal neoantigen burden have been associated with higher T-cell infiltration, better prognosis, and better sensitivity to immunotherapeutic approaches (44–46). McGranahan et al. demonstrated that tumors from melanoma and NSCLC patients enriched with clonal neoantigens displayed an inflamed phenotype and were more sensitive to checkpoint blockade therapy (44). These findings raise the question whether immunotherapy will be also effective in heterogeneous tumors as the targeting of subclonal neoantigens by cytotoxic T cells is not sufficient to eradicate the whole tumor.

Although intratumoral heterogeneity presents a challenge for conventional and targeted therapies, increased mutational diversity may provide a beneficial opportunity for immunotherapies by generating potential neoantigens that can be recognized by T cells (47). Very high intratumoral heterogeneity has also been correlated with better prognosis, implying a possible trade-off between acquiring an immunogenic mutation that can elicit an immune response or a driver mutation that can confer a fitness advantage to the tumor cells (45). Highly heterogeneous tumors are possibly driven by neutral evolution, resulting into many subclonal mutations with little or no impact on cancer progression, but potentially generate neoantigens able to elicit an immune response (48).

Cancer Immunoediting and Acquired Resistance to Immunotherapy

The cancer immunoediting hypothesis postulates a dual role of immunity in the complex interaction between the tumor and the host: the immune system, by recognizing tumor-specific antigens, not only can protect the host by eliminating tumor cells but can also sculpt the developing tumor by editing the cancer genome, and thereby modifying the heterogeneity of the tumor. Strong immunoediting would render tumors more homogeneous by eradicating immunogenic clones (49). Elimination of neoantigens by a T cell-dependent selection process has been suggested as a mechanism of cancer immunoediting in mouse models and human studies (50). Experimental evidence from mouse models and human studies now provides strong support for the existence of cancer immunoediting in many cancers.

The definitive work supporting the existence of cancer immunoediting was published in 2001 by the Schreiber lab and showed that immunodeficient $Rag2^{-/-}$ mice develop spontaneous and carcinogen-induced tumors more rapidly and more frequently than genetically matched wild-type controls (51). Moreover, the tumors arising in immunodeficient animals were frequently rejected following transplantation into immunocompetent recipients, however, when implanted into secondary immunodeficient hosts the effects were not observable. Hence, it seems that tumors from those mice were more immunogenic compared to the tumors from wild-type mice. In a more recent study, cancer immunoediting was investigated in the same mouse model of sarcoma using NGS of the tumor exome and algorithms for predicting neoantigens (52). Their results demonstrated that MCA tumors with a mutant form of spectrin β2 (also known as SPTBN1) were rejected, whereas other tumors developed because of a T-cell-dependent selection of tumor cells that lacked expression of the spectrin β2 antigen. Similar observations were obtained using an oncogene-driven model of cancer in genetically engineered, immunodeficient mice (53) in which primary sarcomas were edited through selection of cells that either did not express antigens or were unable to present antigens to T cells. These studies demonstrated that dynamic interactions between tumors cells and T cells lead to immunoediting. In contrast to carcinogen-induced highly mutated tumors, non-immunogenic tumors with low neoantigen burden do not undergo spontaneous immunoediting (54). In addition, longitudinal samples of pre- and post-treatment samples have shown that different therapies also impose strong selective pressure that can affect the tumor clonal architecture and change the evolutionary path of tumor progression. For instance, patients with a high number of subclonal mutations due to treatment with an alkylating agent were reported to have a poor response to anti-CTLA-4 therapy (44).

Even though immunoediting is more difficult to study in humans, there have been several studies exploring the neoantigen dynamics over time and before and after therapy in patients. A pan-cancer study of TCGA patients in which observed and expected numbers of neoepitopes were analyzed provided the first evidence of immunoediting in human cancers (28). The authors showed that neoantigens are depleted in some cancer types relative to their expected numbers, indicating immune-mediated elimination of tumor subclones that contain neoantigens. Using a similar approach, we recently provided additional data that support the existence of immunoediting in MSI CRC (55).

More recent studies explored the evolution of the neoantigen landscape over time and in response to therapy-induced immune editing. Verdegaal et al., using longitudinal samples from two melanoma patients treated by adoptive T-cell transfer, observed loss of the mutant allele in two cases and reduced expression of T-cell-recognized neoantigens in another one, suggesting potential T-cell dependent selection of antigen-negative variants (56) However, they additionally reported an increased expression of one mutated gene over time. Anagnostou et al.

analyzed matched pretreatment and resistant tumors in patients with NSCLC that acquired resistance following a response to therapy targeting PD-1 and/or CTLA-4 (57). The authors identified immunogenic neoepitopes that were not detectable in the resistant tumors due to an elimination of tumor subclones or chromosomal deletions, and proposed therapy-induced immunoediting of neoantigens as a mechanism of acquired resistance to checkpoint blockade therapy.

Apart from antigen loss, other immunoediting mechanisms such as defects in antigen processing and presentation (58) or in pathways involved in interferon receptor signaling (59) may give rise to acquired resistance to therapy. Gao et al. reported that melanoma patients failed to respond to anti-CTLA-4 therapy due to the loss of IFN-γ signaling caused by genomic defects, such as loss-of-function mutations in JAK1/JAK2 or copy-number alterations in IFN-γ pathway genes (60). In another recent study, pre-treatment and relapse samples from melanoma patients subjected to anti-PD-1 blockade therapy were analyzed to identify resistance-associated mutations. The results showed clonal selection of loss-of-function mutations in JAK1 and JAK2 in two patients, which led to lack of response to interferon gamma, and a truncating mutation in the antigen-presenting protein B2M in another case, resulting in decreased immune cell recognition of tumor cells (61). Moreover, vaccines can increase tumor-infiltrating $CD8^+$ T cells that secrete IFNγ, leading to upregulation of the PD1–PDL1 pathway and other inhibitory pathways (62) and creating a negative feedback loop that can suppress tumor immunity.

OUTLOOK

In the past few years, driven by novel mechanistic insights into cancer immunology and data from clinical trials with checkpoint blockers, tumor neoantigens came into focus in cancer immunology. It became obvious that targeting neoantigens can improve antitumor immunity and minimize off-target toxicities. However, several issues need to be addressed in order to fully harness the power of cancer immunotherapy by targeting neoantigens. First, and most important, considerable research efforts are required to identify the rules that govern the immunogenicity of neoantigens. The majority of the experimentally verified neoantigens that induce antitumor responses are from passenger genes, likely due to the large fraction of passenger mutations (roughly about 90%) compared to driver mutations. Major drawback for developing computational tools for predicting immunogenicity of neoantigens is the dearth of available data. As of today, there are probably few hundred doublets (HLA-neoantigens) and about a dozen triplets (HLA-neoantigens-αβTCR sequences) available for training. Thus, novel medium-to-high-throughput methods are required to generate large enough datasets for data-driven modeling. Second, improved computational methods need to be developed to accurately predict class-II MHC binding neoantigens. Again, major limitation is the limited availability of both positive and negative training data sets. And third, one almost completely unexplored area are neoantigens that are post-translationally modified and the impact of these epitopes on the antitumor immunity. Efforts are underway to tackle these challenges and we will very likely witness in near future exciting developments and discoveries, which will ultimately result in benefit for an individual patient.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of the manuscript.

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Identification and Characterization of Neoantigens As Well As Respective Immune Responses in Cancer Patients

*Eva Bräunlein[1] and Angela M. Krackhardt[1,2]**

[1] Medizinische Klinik III, Klinikum rechts der Isar, Technische Universität München, Munich, Germany, [2] German Cancer Consortium of Translational Cancer Research (DKTK), German Cancer Research Center (DKFZ), Heidelberg, Germany

Cancer immunotherapy has recently emerged as a powerful tool for the treatment of diverse advanced malignancies. In particular, therapeutic application of immune checkpoint modulators, such as anti-CTLA4 or anti-PD-1/PD-L1 antibodies, have shown efficacy in a broad range of malignant diseases. Although pharmacodynamics of these immune modulators are complex, recent studies strongly support the notion that altered peptide ligands presented on tumor cells representing neoantigens may play an essential role in tumor rejection by T cells activated by anti-CTLA4 and anti-PD-1 antibodies. Neoantigens may have diverse sources as viral and mutated proteins. Moreover, posttranslational modifications and altered antigen processing may also contribute to the neoantigenic peptide ligand landscape. Different approaches of target identification are currently applied in combination with subsequent characterization of autologous and non-self T-cell responses against such neoantigens. Additional efforts are required to elucidate key characteristics and interdependences of neoantigens, immunodominance, respective T-cell responses, and the tumor microenvironment in order to define decisive determinants involved in effective T-cell-mediated tumor rejection. This review focuses on our current knowledge of identification and characterization of such neoantigens as well as respective T-cell responses. It closes with challenges to be addressed in future relevant for further improvement of immunotherapeutic strategies in malignant diseases.

Keywords: neoantigens, immunopeptidomics, T-cell responses, immune monitoring, adoptive T-cell transfer

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Mustafa Diken, Translational Oncology at the University Medical Center of Johannes Gutenberg University, Germany

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****Correspondence:***
Angela M. Krackhardt
angela.krackhardt@tum.de

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NEOANTIGENS AS HIGHLY RELEVANT AND ATTRACTIVE TARGETS OF TUMOR-SPECIFIC IMMUNE RESPONSES

Tumor immunologists have been fascinated on the possibility of tumor rejection by the immune system and recognition of tumors as "foreign" in comparison to healthy tissues for a long time. Tumor-associated antigens representing a group of antigens with accentuated but not unique prevalence in the tumor have been investigated as target antigens in a broad variety of tumor entities (1). However, therapeutic efficacy of such targeting approaches could be only rarely demonstrated (2) or has been accomplished outside of the self-educated T-cell receptor (TCR) repertoire (3). Central tolerance to self-antigens may represent one of the main reasons for the

limited efficacy of such approaches. In contrast, tumor-specific antigens (TSA) are characterized by their unique presentation in tumor cells and, therefore, lack of negative thymic depletion of respective specific T-cell populations. Virus-associated antigens have traditionally been acknowledged as TSA in tumors with viral etiology as Merkel cell carcinoma, adult T-cell leukemia, and human papilloma virus (HPV)-associated tumors (4–6). In fact, HPV-induced tumors can be prevented by vaccinations and induced adaptive B-cell responses can be followed over years (7, 8). Mutations have been also early acknowledged to be highly interesting and potentially recognized by specific T cells (9–12), although the significance for a broader patient population remained elusive. A potentially more general role of mutations in tumor rejection has been demonstrated for a larger cohort of cancer patients only after introduction of immune checkpoint modulating antibodies, such as anti-CTLA4 and anti-PD-1, and association of the burden of non-synonymous mutations with response (13–16). Since then, neoantigens have become a major focus of interest either as potential biomarkers or as targets for directed immunotherapies. In fact, novel immunotherapeutic approaches targeting neoantigens by defined vaccines or directed T-cell transfer hold great promise to further improve therapeutic efficacy of immunotherapeutic approaches (17–21).

LANDSCAPE OF NON-PATHOGEN-DERIVED NEOANTIGENS

Currently, a diversity of tumor-specific alterations may serve as suitable sources for non-pathogen-derived neoantigens (**Figure 1**). Single nucleotide variants (SNV) resulting in non-synonymous substitutions have been a major focus of interest since a correlation of the non-synonymous mutation burden within the tumor and response to checkpoint modulators has been established (13, 14, 22). SNVs are typically present in malignancies induced by ultraviolet light exposition or tobacco smoke (23–25). Most of the SNV-derived neoantigens gain their immunogenic foreignness throughout altered amino acids involved in direct T-cell contact although also anchor positions may be affected resulting in potential lack of presentation of the wild-type peptide (26). Recurrent mutations may serve as public neoantigens enabling the development of targeted approaches applicable to broader patient cohorts (27–29). Nonetheless, the majority of immunogenic mutations appear to derive from patient-specific alterations. In addition to the potentially singular nature of a mutated peptide ligand, immunogenic neoantigens derived from non-synonymous mutations have been reported to be enriched for a distinct tetrapeptide signature homologous to epitopes derived from pathogens as suggested by data from Snyder and colleagues (13). However, subsequent studies could not confirm a prevalent role of such a defined peptide motif (22, 30).

Frame shifts in antigen-coding regions due to insertions or deletions have been described as additional promising source of TSA (31, 32). A recent report indicated frameshift-derived mutations to be enriched especially in cancer entities known to respond to immune checkpoint modulators and predicted neoantigens derived from these mutations correlated with response to immune checkpoint modulation as well as upregulation of immune signatures (33). Due to the high frequency of nucleotide insertions or deletions in defined genes, resulting mutated peptides may be also used as shared public neoantigens possibly of use for a broader patient population (34). Of note, the fraction of human leukocyte antigen (HLA) class I bound peptides derived from non-canonical reading frames was found to comprise 10% of all ligands identified on the surface of an expanded B-cell line (35) and thereby provides an additional highly interesting source of TSA as recently summarized (36).

Chromosomal translocations may lead to expression of novel epitopes spanning the respective breakpoint mutation, therefore, representing another source of potential neoantigens. Analyses of immune responses against such neoantigens have provided encouraging rational for clinical applications (37, 38). However, in case of the Philadelphia chromosome defined t(9;21) bcr/abl translocation, vaccination studies have shown variable efficacy (39, 40). One reason might rely in limitations of natural processing of the expected mutated ligands (41). Thus, further studies are required to investigate this anticipated group of highly attractive neoantigens.

Besides the above described sources of altered peptides, B-cell derived malignancies inherit an exceptional source of potentially immunogenic tumor-specific peptides spanning the monoclonal hypervariable recombined immunoglobulin-coding region (42). It has been recently shown for lymphoma that such idiotype-derived ligands are actually presented by MHC class II molecules as detected by mass spectrometry (MS)-based immunopeptidomics and that these are immunogenic (43).

Tumor-specific antigenic peptides may additionally derive from cellular processes specifically altered in tumor cells resulting in a modified peptide repertoire presented by MHC complexes on the tumor surface. Examples comprise peptides with posttranslational modifications as phosphorylation and deamidation potentially resulting in TSA (44–46). Moreover, tumor-specific peptides may derive from alternative splicing in the proteasome (44, 47, 48). As it has been recently reported that spliced peptides substantially contribute to the immunopeptidome (49), it might be highly attractive to more comprehensively investigate the cancer-related MHC peptide ligandome for the presence and immunogenicity of such peptides. However, peptide ligands derived from altered cellular processes currently require MS for detection and there are no algorithms for reliable prediction of such antigens. Moreover, it will be important to investigate in larger studies if these peptides represent really unique TSA suitable for therapeutic targeting approaches.

IDENTIFICATION OF TUMOR-SPECIFIC NEOANTIGENS

Neoantigens have been primarily identified on the base of defined T-cell responses resulting in a qualitative view on relevant antigens (10, 11). However, general rules could not be deduced from these early reports. Large-scale analyses of genomes and

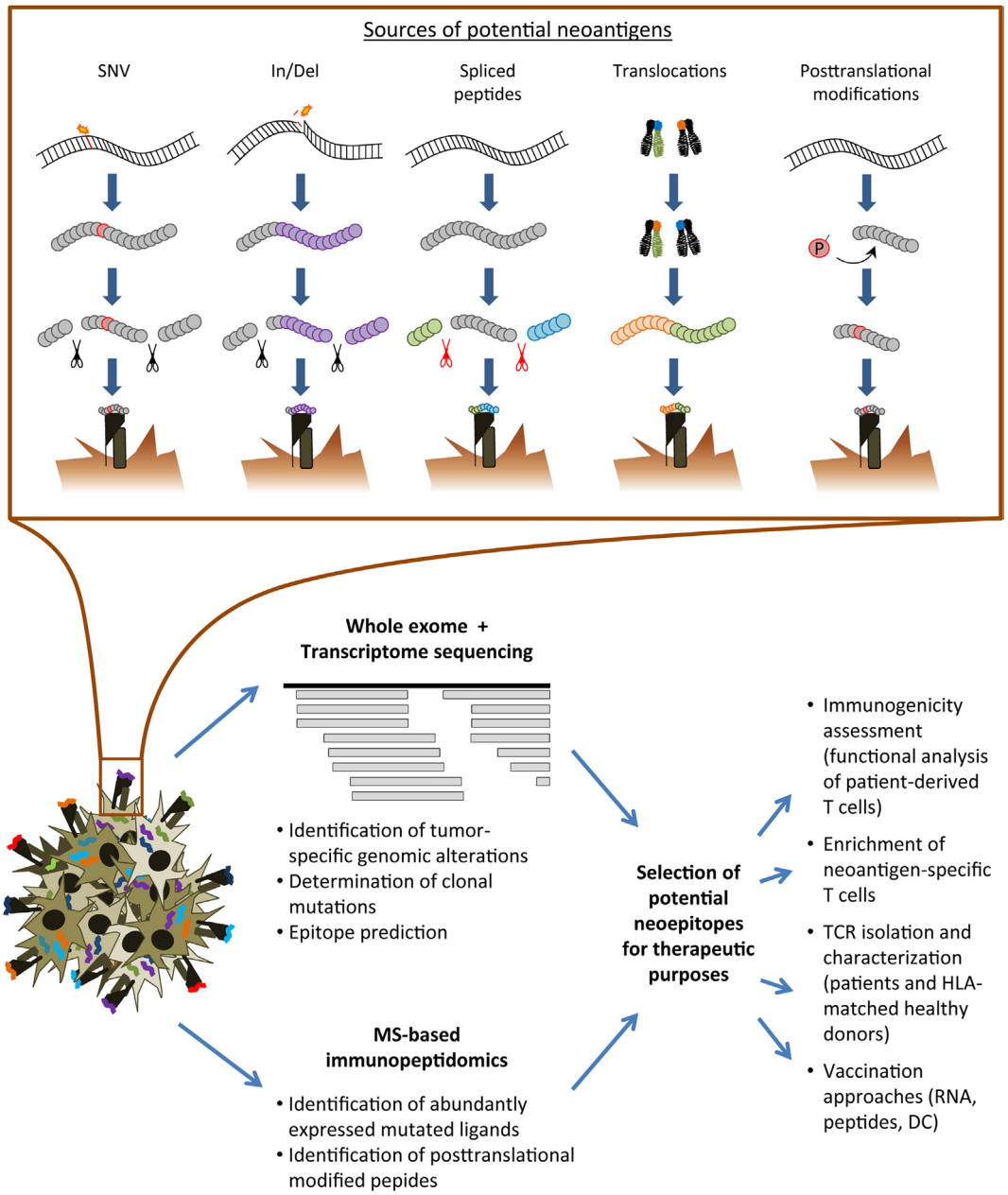

FIGURE 1 | Overview of the neoantigen landscape and identification strategies. Upper row: sources of conceivable neoantigens exemplarily shown for HLA class I ligands. Lower row: schematic overview of analysis pipelines for the immunogenicity assessment of tumor-specific alterations. SNV, single nucleotide variant; In/Del, insertion/deletion; MS, mass spectrometry; TCR, T-cell receptor; HLA, human leukocyte antigen; DC, dendritic cell.

immunopeptidomes, advanced computational analyses, and development of bioinformatics algorithms to predict immunogenicity of tumor-specific peptide ligands greatly enhanced the field (50, 51). This approach resulted in the successful identification of neoantigens in a diversity of malignant diseases although the number of positive hits validated by respective T-cell responses was highly diverse (15, 52–55). Differences of tumor entities as well as inter- and intraindividual heterogeneity of tumors, metastases, and interrogated T-cell repertoires may play an important role for the diversity in the validation rate of predicted epitopes. However, additional aspects govern the quality of such predictions. Technical features as the depth of sequencing and the quality of tumor material, source material for sequencing and algorithms used for SNV calling may have a major impact on the results (56–58). In addition, prediction algorithms for more frequent HLA alleles provide superior results in comparison to less frequent HLA alleles emphasizing the need of larger training datasets (59). Besides, different pipelines for HLA binding prediction have been developed and are currently used in parallel leading to limited comparability of obtained results (60).

Moreover, reliable prediction algorithms are currently missing for many aspects of antigen processing and presentation apart from peptide binding. However, there are approaches to improve and harmonize current epitope predictions. A recent implementation of several steps of analysis into one single tool called MuPeXI was provided aiming at integration of predictions and data processing into one straightforward pipeline (61). Application of newly gained knowledge derived from large-scale analyses of pre-existing datasets, such as the pan-cancer analysis of tumor-specific alterations caused by insertions and deletions (33) will further improve our understanding of tumor-specific changes on the genomic level, thereby steadily broadening the current view of potential immunogenic features. Moreover, the bias of epitope prediction may be circumvented by therapeutic approaches as vaccinations based on long peptides or RNA fragments encompassing several point mutations. Two such approaches used in early clinical trials have recently shown encouraging results (20, 21).

Direct identification of mutated peptide ligands by immunoprecipitation of peptide-HLA-complexes and subsequent peptide ligand analysis by MS provides a promising tool for a more straightforward approach with the perspective to define especially those neoepitopes that are indeed well presented on the tumor cell. Feasibility of the detection of naturally presented mutated HLA ligands by this technology has been primarily shown for murine tumors (52, 62) and human cell lines (63). Improved sensitivity as well as optimized bioinformatics algorithms resulted also in the identification of neoantigens directly eluted from primary human tissues (43, 59). In addition, MS data may help to improve current prediction algorithms (64, 65). Feeding of databases such as IEDB and the human immunopeptidome project of the human proteome organization (66) with experimental data is, therefore, of fundamental importance. However, technical issues as requirement for large amounts of tumor material, low yield in peptides after immunoaffinity purification, limited reproducibility and biases from fragmentation methods currently represent major limitations (66). Improvements in this field will likely have a great impact on neoantigen identification to be used for personalized therapies.

VALIDATION OF T-CELL RESPONSES AGAINST NEOANTIGENS

As described above, the identification of all putative mutations within the entire exome (67, 68) paved the way to systematic screens of T cells for respective responses. Pushing the development of technologies for rapid assessment of neoantigen-specific T-cell responses, groundbreaking studies mainly focused on diseases with high mutational burden, especially melanoma and non-small cell lung cancers (50, 54, 69, 70). However, some malignancies with comparably low amounts of tumor-specific mutations also elicit mutation-specific immune responses, including cervical, gastric, and triple-negative breast cancers (55, 71, 72).

As a fairly straightforward approach, the exact expected epitope or longer peptides to be processed by dendritic cells (DCs) are synthesized and screened for recognition by tumor-specific T cells (73). As another possible strategy, T-cell populations may be identified using MHC multimers containing the expected epitope of respective mutated antigens (70, 74, 75). However, MHC multimer analyses may have limitations for fine characterization of neoantigen-specific T-cell populations and may differ to in-depth functional T-cell analyses (59). As an alternative to long peptides, which have to be processed by professional antigen-presenting cells, minigenes comprising respective mutation can be transduced and used for large-scale screening approaches, again circumventing the need of knowing the exact epitope (50). Still, the exact epitope has yet to be determined in additional screenings in case that further characterization of specific immune responses is desired (72, 73). Patient-derived tumor cell lines or spheroids can be used for screening of neoantigen-specific reactivity, although stable expansion of *in vitro* cultures starting with primary human material is often not successful.

The therapeutic potential of targeting somatic mutations throughout vaccination approaches has been also investigated *in vivo* using different mouse models. Specific immune responses could be elicited and successful tumor shrinkage has been observed after application of neoantigen vaccines (67, 68, 76). However, results obtained with murine models rather serve as a proof of principle for a defined immunotherapeutic approach. Another possibility for screening of personalized neoantigen-specific T-cell responses may be achieved by the establishment of individual patient-derived xenografts (PDX). It has been shown that the clonal architecture of patient tumors transplanted in murine hosts exhibit a clonal architecture comparable to tumors grown in the patient (77–80). Therefore, PDX mirror escape mechanisms, which may be translated into the clinical setting. However, some limitations within this approach including changes in the tumor microenvironment and the long time it takes to grow individual xenografts (81) currently prevent larger applications of PDX models in prompt and patient-resembling immunogenicity assessments.

SOURCES OF NEOANTIGEN-SPECIFIC T CELLS

For the above described validation of altered target structures, different TCR repertoires may be interrogated. The application of checkpoint inhibitors unleashing the patient's own immune system emphasized the inherited potential of autologous immune cells to fight cancer. Numerous studies have confirmed neoantigen-specific reactivity within the TIL repertoire (51, 53, 55, 59, 73, 82). In addition, immune responses against mutated peptide ligands can be also detected in the peripheral blood of cancer patients (59, 83) and responses overlapping between PBMC-derived lymphocytes and TIL have been additionally reported (54). Investigation of the TCR beta repertoire of tumor patients vaccinated with a DC vaccine after treatment with Ipilimumab suggested a promotion of neoantigen-specific diversity in TCR beta usage and clonal composition (18). As another important aspect, analysis of treatment-naïve patients in comparison to patients with previous immunotherapies is expected to help to decipher clinically relevant immunoreactivity (84, 85).

For those patients lacking endogenous tumor-specific immune responses or harboring terminally exhausted T cells, the investigation of alternative TCR repertoires provides a meaningful source to empower the patient's immune system (86). Neoantigen-specific T cells can be also isolated from HLA-matched healthy donors (59, 87). The xenogeneic source of murine TCR (e.g., isolated from HLA-transgenic mice) may provide an alternative source for neoantigen-specific TCR (88). As such, a xenogeneic model is generally rather easily accessible, it may be used to build up a robust workflow for patients lacking specific immune responses. However, it remains questionable, whether this approach confers a significant advantage for neoantigens, as HLA-matched healthy donors should inherit comparable high chances for detectable antigen-specific T-cell frequencies due to circumvention of thymic depletion. Moreover, there might be an enhanced risk for toxicity due to crossreactivity against human peptide ligands, which are not processed or presented by the murine immunopeptidome. However, both repertoires may serve as base for genetic engineering of neoantigen-specific TCR to be used for the adoptive transfer of redirected T cells. Further improvements regarding cost efficacy and time restrictions might enable an automated production of redirected neoantigen-specific T-cells.

Not only the mere detectability of neoantigen-specific T cells, but also the quality of respective T-cell responses is currently under detailed investigation. Various aspects, such as the frequency, phenotype, functional capacities, dynamic changes during clinical course, and the contribution of $CD4^+$ and $CD8^+$ lymphocytes to tumor rejection (21, 75, 89), are taken into consideration. These analyses may help to understand qualitative characteristics of neoantigens representing immunodominant and suitable rejection antigens inducing an effective T-cell mediated tumor reactivity.

FUTURE CHALLENGES AND CLINICAL IMPLICATIONS

With respect to neoantigen-targeted therapies but also biomarker development, one central question relies in the selection of those neoantigens, which are in fact relevant in the clinical setting. In this regard, the presence of clonal versus subclonal neoantigens may be highly relevant and tumor heterogeneity may represent a major hurdle for an effective anti-tumor response (70, 90, 91). Driver mutations clearly represent a highly attractive group of potential neoantigens to be targeted for neoantigen-specific therapies as targeting such antigens may limit or decelerate immune evasion due to their frequent clonal nature (17, 19, 92). However, other alterations as genetic changes of tumor cells affecting antigen processing and presentation may still result in immune evasion (19). In fact, defects in antigen presentation incorporate a major risk for immune escape and represent a frequent form of acquired resistance in a diversity of immunotherapies (93–96). A multivariate analysis support the notion of multiple determinants being responsible for the therapeutic outcome (97). A recent study by Riaz and colleagues investigates changes in the tumor evolution and the tumor microenvironment under immune checkpoint inhibition and thereby emphasizes the interdependence of the tumor mutanome and TIL composition (85). In this regard, the assessment of primary and secondary resistance to immune-mediated therapies may potentially lead to improved identification of those patients who may primarily profit from immunotherapies alone and those who may need additional therapeutic approaches. Strategies to restore antigen presentation to be used in combinatorial treatment approaches may become particularly important including the sequential or consecutive application of innovative and well-established therapies as recently reviewed (96, 98). A systematic approach of TCR repertoire profiling across different tumor regions in lung adenocarcinoma hints toward a complex interaction between intratumoral heterogeneity and distribution patterns of clonal T cells (99). In combination with further functional dissection of tumor-specific TCR, information of spatial distribution of neoantigen-specific T cells will likely provide important insights into the dynamics and interactions of tumors and respective neoantigen-specific T-cell responses.

Future directions may, therefore, aim at the comprehensive analysis of immunogenic potential of respective neoantigens by interrogation of diverse repertoires and building up multi-omics and large screening libraries. Therefore, combinatorial analyses of tumor-derived mutations and other molecular characteristics of the tumor cells, tumor microenvironment, and respective immune responses are required for a better understanding of tumor dynamics and selection of suitable structures capable to induce tumor rejection.

AUTHOR CONTRIBUTIONS

EB and AK wrote and critically revised the manuscript.

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The Potential of Donor T-Cell Repertoires in Neoantigen-Targeted Cancer Immunotherapy

Terhi Karpanen and Johanna Olweus**

Department of Cancer Immunology, Institute for Cancer Research, Oslo University Hospital Radiumhospitalet, and K.G. Jebsen Center for Cancer Immunotherapy, University of Oslo, Oslo, Norway

T cells can recognize peptides encoded by mutated genes, but analysis of tumor-infiltrating lymphocytes suggests that very few neoantigens spontaneously elicit T-cell responses. This may be an important reason why immune checkpoint inhibitors are mainly effective in tumors with a high mutational burden. Reasons for clinically insufficient responses to neoantigens might be inefficient priming, inhibition, or deletion of the cognate T cells. Responses can be dramatically improved by cancer immunotherapy such as checkpoint inhibition, but often with temporary effects. By contrast, T cells from human leukocyte antigen (HLA)-matched donors can cure diseases such as chronic myeloid leukemia. The therapeutic effect is mediated by donor T cells recognizing polymorphic peptides for which the donor and patient are disparate, presented on self-HLA. Donor T-cell repertoires are unbiased by the immunosuppressive environment of the tumor. A recent study demonstrated that T cells from healthy individuals are able to respond to neoantigens that are ignored by tumor-infiltrating T cells of melanoma patients. In this review, we discuss possible reasons why neoantigens escape host T cells and how these limitations may be overcome by utilization of donor-derived T-cell repertoires to facilitate rational design of neoantigen-targeted immunotherapy.

Keywords: neoantigen, immunotherapy, T cell, donor, allogeneic hematopoietic stem cell transplantation, donor lymphocyte infusion, minor histocompatibility antigen, graft versus tumor effect

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Mustafa Diken, Immunotherapy Development Center (IDC) TRON Translational Oncology at the University Medical Center of Johannes Gutenberg University, Germany

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Daniel Olive, Institut National de la Santé et de la Recherche Médicale, France

****Correspondence:***
Terhi Karpanen
terhi.karpanen@medisin.uio.no;
Johanna Olweus
johanna.olweus@medisin.uio.no

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INTRODUCTION

Neoantigens derived from somatic mutations in cancer cells and recognized as foreign by T cells are arising as the most attractive targets of cancer immunotherapy. They are expressed exclusively in malignant cells, making them truly tumor specific, and the T-cell repertoire recognizing them is not affected by central tolerance mechanisms. Recent studies have demonstrated a correlation between the clinical benefit of cancer immunotherapies such as checkpoint inhibition, with mismatch-repair deficiency, burden of somatic nonsynonymous mutations and neoantigen load (1–5), and neoantigen-reactive T cells have been detected in many tumors (**Table 1**).

Many tumors harbor a large number of mutations that potentially can give rise to neoepitopes (26). All mutations leading to single amino acid substitutions, reading-frame alterations, splice variants, inversions, fusions, and aberrant posttranslational modifications, have the potential to generate neoantigens. For a neoantigen to be immunogenic, it has to be expressed at sufficient levels, have the correct subcellular localization to enter proteasomes, be efficiently processed and

Abbreviations: alloHSCT, allogeneic hematopoietic stem cell transplantation; CAR, chimeric antigen receptor; DLI, donor lymphocyte infusion; GvHD, graft versus host disease; GvT, graft versus tumor; HLA, human leukocyte antigen; mHAg, minor histocompatibility antigen; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocyte.

TABLE 1 | Only a small fraction of candidate neoantigens elicits spontaneous responses in the cancer patient's autologous T-cell repertoire.

Tumor type (source of neoantigen-reactive T cells)	Total number of nonsynonymous mutations/number of patients analyzed	Mutations encoding neoepitopes/mutations screened (immunogenic peptides identified/ peptides screened[a])	% Mutations encoding neoepitopes (% immunogenic peptides[a])	Test method (pipeline for preselection)	Reference
Autologous, spontaneous					
Melanoma (TIL)	1,084/1	2/448[a]	0.4[a]	pMHC (RNAseq, NetChopCterm3.0, NetMHC3.2)	(6)
Melanoma (TIL)	1,116/3	7/191	3.7	IFNg (NetMHCpan2.4)	(7)
		11/227[a]	4.8[a]		
Melanoma (TIL)	np/2	2/288	0.7	TM and IFNg (none)	(8)
Ovarian cancer (TAL)	93/3	1/79	1.3	ELISpot (NetMHCpan2.4)	(9)
Gastrointestinal cancers (TIL)	773[b]/10	18/1,452	1.2	TM and ELISpot/CD137 (none)	(10, 11)
Melanoma (FTD and TIL)	19,597/8	10/369[a]	2.7[a]	pMHC (RNAseq, IEDB)	(12)
Melanoma [TIL or PBMC-derived infusion product]	2,386/5	8/1,543	0.5	ELISA (RNAseq)	(13)
Melanoma ($CD8^+PD\text{-}1^+$ PBMC)	1,479/4	7/691	1.0	TM and ELISpot/CD137 (RNAseq)	(14)
Melanoma (TIL)	1,100/3	2/201	1.0	pMHC (RNAseq, NetChopCterm3.0, NetMHC3.2, or NetMHCpan2.0)	(15)
		2/391[a]	0.5[a]		
Melanoma (TIL)	>4,000/1	10/720	1.4	TM and IFNq ELISA (RNAseq, IEDB)	(16)
Melanoma (TIL)	np/4[c]	12/675	1.8	TM and CD137 (IEDB)	(17)
NSCLC (TIL and T cells from adjacent normal tissue)	np/2	3/642[a]	0.5[a]	pMHC (NetMHCpan2.8)	(18)
Melanoma (TIL)	1,019/1	1/2	50.0	ELISA (MS/MS)	(19)
NSCLC (TIL and PBMC)	np/2	9/705[a]	1.3[a]	DNA-barcoded pMHC staining (NetMHCpan2.8)	(20)
Autologous, therapeutically induced					
NSCLC (PBL after anti-PD-1)	324/1	1/99	1.0	pHMC (NetMHC3.4)	(3)
		1/148[a]	0.7[a]		
Colorectal cancer (PBMC after anti-PD-1)	1,477[b]/1	3/15	20.0	ELISpot (ImmunoSelect-R)	(5)
		3/15[a]	20.0[a]		
Melanoma (PBMC after anti-CTLA-4)	2,329/1	2/8	25.0	ELISpot (LC-MS/MS)	(21)
		2/8[a]	25.0[a]		
Melanoma (PBMC after peptide loaded dendritic cell vaccination)	1,099[d]/3	9/21	42.9	pMHC (MS/MS)	(22)
		9/21[a]	42.9[a]		
Lung squamous cell carcinoma (PBMC after peptide vaccination)	93/1	4/5	80.0	ELISpot (NetMHC3.4)	(23)
		6/11[a]	54.5[a]		
Melanoma (PBMC after peptide vaccination)	4,729/6	15/91	16.5 (CD8)	ELISpot (RNAseq, NetMHCpan2.4)	(24)
		58/97	59.8 (CD4)		
Donor derived					
CLL (PBMC after alloHSCT)	51/2	3/25	12.0	ELISpot (NetMHCpan2.4)	(25)
		3/48[a]	6.3[a]		

(*Continued*)

TABLE 1 | Continued

Tumor type (source of neoantigen-reactive T cells)	Total number of nonsynonymous mutations/number of patients analyzed	Mutations encoding neoepitopes/mutations screened (immunogenic peptides identified/ peptides screened[a])	% Mutations encoding neoepitopes (% immunogenic peptides[a])	Test method (pipeline for preselection)	Reference
Melanoma (PBMC from healthy individuals)	6,413/5	4/11	36.4	ELISpot (LC-MS/MS)	(21)
		4/11[a]	36.4[a]		
Melanoma (PBMC from healthy individuals)	1,100/3	10/45	22.2	pMHC (RNAseq, NetMHC4.0)	(15)
		11/57[a]	19.3[a]		

Summary of studies in which mutations encoding candidate neoantigens were identified by whole-exome sequencing and systematically screened for recognition by T-cells. For each study, the table indicates the number of mutations encoding neoepitopes (immunogenic peptides) that were identified among the number of mutations screened, and/or, when indicated by an [a], the number of immunogenic peptides (neoepitopes) identified among the total number of peptides screened (multiple candidate peptides can be screened for a single mutation).

[b]Total number of mutations (when number of nonsynonymous mutations was not reported).

[c]Only the four patients for which tandem minigene constructs were available are included.

[d]Nonsynonymous mutations in lymph node or axilla.

np, information not provided; TIL, tumor-infiltrating lymphocytes; TAL, tumor-associated lymphocytes; FTD, fresh tumor digest; PBMC, peripheral blood mononuclear cells; NSCLC, non-small-cell lung carcinoma; PBL, peripheral blood lymphocytes; CLL, chronic lymphocytic leukemia; TM, tandem minigene; MS/MS, tandem mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; pMHC, peptide-major histocompatibility complex molecule multimers; IEDB, The Immune Epitope Database and Analysis Resource.

transported to the endoplasmic reticulum, be loaded on human leukocyte antigen (HLA)-molecules with high enough affinity, form a complex with HLA with sufficient stability, and be efficiently recognized by the patient T-cell repertoire. Current sequencing techniques and computational sequence analysis tools enable rapid calling of somatic mutations from individual tumors. However, identification of verified neoepitopes from the large pool of candidate peptides remains a major challenge on the way to efficiently exploit each patient's unique set of neoantigens for targeted immunotherapy. The vast majority of neoantigens are personal and few shared neoantigens have been found to be immunogenic, highlighting the need for efficient strategies for fast identification of personal neoepitopes. Prediction methods for HLA-binding affinity, at least for the most frequent HLA class I alleles, can narrow down the number of candidate neoepitopes, but are insufficient in predicting proteasomal processing, transport of the peptides, stability of the peptide–HLA complexes, and recognition by T cells. This makes the identification of clinically relevant neoepitopes with therapeutic potential challenging.

In this review, we discuss possible reasons for the insufficiency of the patient's T cells to respond to neoantigens and experience gained from the use of donor T cells in the treatment of hematological malignancies. We will also focus on recent insights gained from the use of T-cell repertoires from healthy individuals to identify immunogenic neoantigens and present possibilities these insights open for the efficient clinical exploitation of personal neoantigens.

THE AUTOLOGOUS T-CELL REPERTOIRE OF THE PATIENT FREQUENTLY FAILS TO CONTROL CANCER PROGRESSION

The potential of neoantigen-specific T cells to induce cancer regression has unequivocally been demonstrated. Infusion of selected patient-derived, neoantigen-reactive T cells were shown to induce objective clinical responses in a patient with epithelial cancer, treated with enriched CD4 T cells recognizing a mutant erbb2 interacting protein (ERBB2IP)-derived peptide (10), and in a colorectal cancer patient treated with a cytotoxic T-cell pool consisting of four different clonotypes specific for KRAS G12D-derived peptides presented by HLA-C*08:02 (27). Analysis of T cells derived from tumor-infiltrating lymphocytes (TIL) or peripheral blood has, however, shown that the frequency of neoantigens that can elicit such responses is low: Only about 1.2% of mutations with neoantigenic potential are spontaneously recognized in patients with melanoma, gastrointestinal, lung, and ovarian cancers. T cells responsive to 68 of the 5,842 candidate neoantigens screened were found in 36 patients included in 11 studies (**Table 1**; 7–11, 13–17, 19). Similarly, very few mutations were identified that evoke an immune response in patients treated with checkpoint inhibition (3). A multitude of mechanisms might collectively be responsible for this. Tumors can actively suppress existing T-cell responses, extensively reviewed elsewhere (28). Strategies include secretion of immunosuppressive cytokines, attraction of regulatory T cells or myeloid-derived suppressor cells, upregulation of the expression of inhibitory molecules, such as immune checkpoint receptors on T cells (29) and their ligands in tumor cells, loss or mutation of HLA-molecules (30–33), target antigens (34), or activating co-receptors resulting in induction of T-cell anergy. Peripheral tolerance mechanisms can also lead to clonal deletion and permanent loss of T cells recognizing abundantly expressed antigens in murine models (35). Parallel tracking of neoantigen-specific T cells and cognate tumor cells in an ovarian cancer patient with progressive disease showed that expansion of a tumor clone was accompanied by disappearance of the reactive T cells, suggesting deletional tolerance (9, 36). The possibility to therapeutically reverse immunosuppression has been demonstrated by checkpoint inhibition, which has led to impressive clinical responses in multiple cancer types, reviewed in Ref. (37). A large number of patients do, however, not respond, and the great majority of treated patients eventually relapse. Two recent papers have shed light on potential mechanisms for this.

Persistent stimulation by cognate antigen or exposure to inflammatory signals can lead to T cell exhaustion, which impairs

T-cell effector functions. This dysfunctional state is characterized by an altered transcriptional program, including high expression of multiple inhibitory receptors such as programmed cell death 1 (PD-1). Recent studies indicate that exhausted T cells acquire an epigenetic profile that is distinct from that of effector and memory T cells and can only minimally be remodeled by PD-1 blockade therapy (38, 39). This epigenetic programming of T cells from a functional to dysfunctional state is suggested to occur in two phases, initially to a plastic state from which T cells can be rescued, and subsequently to a fixed dysfunctional state where T cells are resistant to reprogramming (40). This could possibly explain frequent clinical relapses following treatment with immune checkpoint inhibitors and has profound implications for the development of future immunotherapies. If the tumor-responsive T-cell repertoire of the patient is incapable of exerting lasting tumor control even if inhibitory signals are discontinued, new and innovative ways to activate the immune system are required.

BROADENING THE ENDOGENOUS T-CELL RESPONSE TO NEOANTIGENS

Several studies have demonstrated that personal neoantigen vaccination protocols can elicit neoantigen-specific T-cell responses that are not detectable before vaccination (22–24), suggesting that insufficient priming partially accounts for limited neoantigen-specific T-cell responses in cancer patients. Two clinical studies pioneering mRNA-based (33) and peptide-based (24) personalized neoantigen vaccines in metastatic melanoma recently demonstrated clinical relevance of vaccination-induced responses. Thus, reduction in the frequencies of metastatic events (33) and direct recognition of tumor by some of the induced T-cell specificities (24, 33) were observed. T-cell responses to the majority of the vaccine antigens were *de novo* responses, supporting the view that the potential of the T-cell repertoire can be optimized by more effective priming. Such *de novo* responses are expected to mobilize naïve T cells that are not exhausted or dysfunctional. In the study by Ott et al., 16% of the peptides used for vaccination induced a CD8 response and 60% a CD4 response (24). Clinical response rates might, however, increase if an even higher number of verified CD8 epitopes could be included and a larger fraction of the induced T-cell specificities would translate into tumor-reactive responses (24). Thus, further studies to improve on antigen selection might be advantageous.

DONOR-DERIVED T CELLS CAN MEDIATE GRAFT-VERSUS-TUMOR EFFECTS FOLLOWING ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION (alloHSCT)

In alloHSCT, donor-derived T cells can overcome the insufficiency of patient immunity. Alone or in combination with donor lymphocyte infusions (DLI), alloHSCT is frequently used to treat hematological cancers and still remains the only potentially curative treatment for many hematological malignancies [reviewed in Ref. (41)]. In an HLA-matched alloHSCT, the desired graft-versus-tumor reactivity (GvT) is thought to be mainly mediated by donor T cells recognizing peptides from polymorphic proteins, so called minor histocompatibility antigens (mHAg). These are generated by genetic differences between the donor and the host and presented by matched HLA on the malignant cells [reviewed in Ref. (42)]. Immunogenic mHAgs in the recipient are recognized by T cells from a donor lacking the immunogenic allele. mHAgs can be encoded by the Y chromosome or be autosomal. Autosomal mHAgs are most commonly derived from nonsynonymous single nucleotide polymorphisms, which result in single amino acid differences in the encoded proteins. Thus, mHAgs are seen as "neoantigens" by the donor T cells. Hematopoietic cells are preferentially recognized as they are more easily accessible than cells in solid tissues and they frequently express high levels of HLA class I and II, costimulatory receptors and adhesion molecules [reviewed in Ref. (42)]. However, donor T-cell reactivity to broadly expressed immunogenic mHAgs on healthy tissues bears the risk of potentially detrimental graft-versus-host disease (GvHD). DLIs can induce complete remissions in patients with relapsing leukemia after alloHSCT (43–46). The GvT effect is considered to be dependent on the presence of host antigen-presenting cells capable of efficiently displaying recipient's hematopoietic lineage-restricted mHAgs to the donor T cells (47).

The powerful immune responses of GvT and GvHD demonstrate the ability of donor T cells to attack and kill defined cell types dependent on recognition of antigens differing between host and donor by a single amino acid. In fact, it is possible that tumor-specific neoantigens also serve as clinically relevant targets mediating GvT following alloHSCT. This could be suggested by the fact that syngeneic alloHSCT from a genetically identical twin can result in similar long-term disease-free survival rates as alloHSCT from an HLA-matched donor, but in the absence of allogeneic GvT (48–50). The relevance of neoantigens as targets for GvT was shown by a study in which two chronic lymphocytic leukemia patients with durable remission after alloHSCT were monitored for cytotoxic T-cell responses against predicted tumor-specific neoantigens and found to mount long-term responses against personal neoantigens derived from three different genes (25). As donor T cells have not been exposed to the peripheral tolerance mechanisms of the tumor, they can strongly recognize defined mHAgs or neoantigens presented by patient cancer cells. The possibility to specifically target donor T cells to patient neoantigens has, however, not been therapeutically explored thus far.

DONOR-DERIVED T-CELL RESPONSES REVEAL A HIGH FREQUENCY OF IMMUNOGENIC NEOANTIGENS

Advancements in sequencing techniques and computational sequence analysis tools have enabled fast identification of somatic mutations in expressed genes in individual tumors. The precision level of computer algorithms predicting potential neoepitopes recognized by T cells is, however, not known. A main challenge, therefore, remains to rapidly select among the large number of candidate neoantigens those that translate into clinically efficient immune responses. The uncompromised T-cell repertoires from HLA-matched donors hold an unrealized potential

when addressing these challenges as they retain their inherent capability to respond to immunogenic neoantigens. Donor T-cell repertoires could thus be applied for identification of neoepitopes, independently of blood sampling from the patient. This was recently demonstrated by Strønen et al. by coculturing the non-adherent fraction of peripheral blood mononuclear cells with autologous monocyte-derived dendritic cells electroporated with *in vitro*-translated tandem minigene library RNA encoding potential neoepitopes from three melanoma patients, and by detecting neoantigen-responsive T cells with fluorescently labeled HLA-multimers. The study revealed that 11 out of 57 predicted HLA-A*02:01-binding neoepitopes were recognized by the nontolerized CD8 T-cell repertoires of healthy blood donors, 10 of which were ignored by the autologous TILs of the patient (15). Importantly, donor T-cell populations also recognized cognate neoepitopes when endogenously presented by the patient's tumor cells, suggesting high-avidity T-cell responses. This study showed that a much higher frequency of neoantigens was immunogenic than was anticipated from analyzing the patient's autologous *in vivo* T-cell responses. T-cell receptors (TCRs) isolated from the neoantigen-reactive donor T cells efficiently retargeted third party T cells to recognize patient-derived melanoma cells harboring the targeted mutations, suggesting that patient T cells redirected with neoantigen-targeted TCR could be effective in gene therapy (15).

DONOR-DERIVED IMMUNITY TO DESIGN PERSONALIZED IMMUNOTHERAPY

When the endogenous T-cell repertoire of the cancer patient is insufficient at controlling the disease, donor-derived immunity might provide rescue. One of the most successful examples is the adoptive transfer of T cells genetically modified by chimeric antigen receptors (CARs). T cells engineered to express CARs, harboring the antigen-recognition domain of an antibody grafted onto signaling domains that confer T-cell activation, can mediate selective killing of defined cell subsets. Treatment with CD19-targeted CAR T-cells consistently leads to complete response rates of 70–90% in acute lymphoblastic B-cell leukemia and has shown promise in non-Hodgkin B-cell lymphoma (51). The success of CAR T-cells to treat B-cell malignancies has, however, yet to be extended to other hematological cancers or solid tumors. A major obstacle is the identification of cell-type specific cell-surface

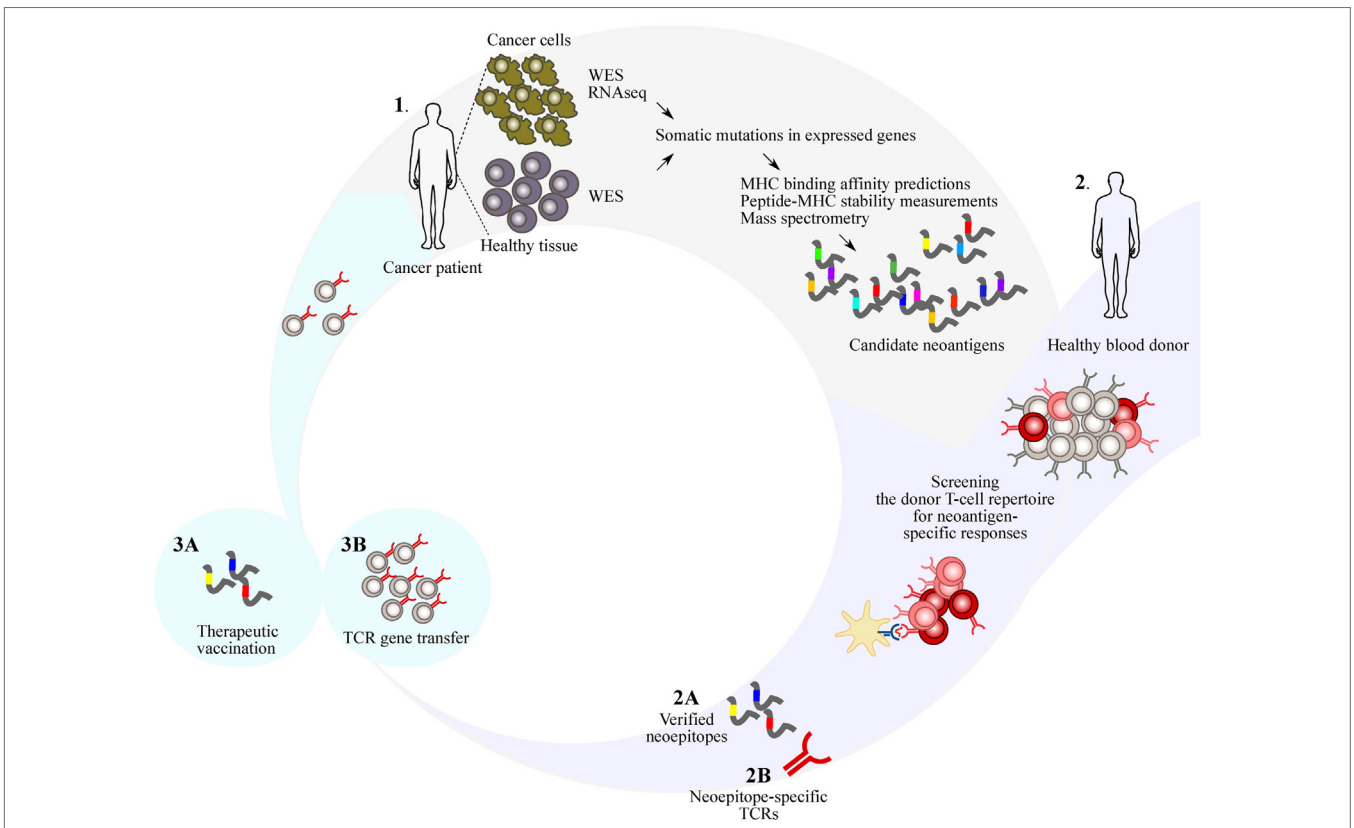

FIGURE 1 | Identification of immunogenic neoantigens is the major technical challenge in genome-based personalized immunotherapy. **1**. Advances in sequencing techniques and computational sequence analysis tools have enabled rapid identification of somatic mutations in expressed genes that are capable of generating potential neoantigens. Human leukocyte antigen (HLA) binding affinity algorithms can narrow down the number of potential neoantigens, but are insufficient in predicting aspects contributing to immunogenicity. **2**. Nontolerized T-cell repertoires of healthy donors HLA-matched with the patient can be used to identify neoepitopes **(A)**, and T-cell receptors (TCRs) from the neoantigen-responsive donor T cells can be isolated **(B)**. **3**. The identified neoantigens can be used for therapeutic vaccination to (prime and) expand neoantigen-specific T cells in the patient repertoire **(A)**. Alternatively, the TCRs identified from the neoantigen-responsive donor T cells can be used to retarget patient T cells to recognize the tumor **(B)**. WES, whole-exome sequencing; RNAseq, RNA sequencing.

molecules that can be safely targeted. In contrast to CARs, TCRs can recognize also intracellular antigens when presented in the context of surface MHC-molecules, opening the possibility to employ neoantigen-responsive TCRs to redirect patient T cells. TCRs from neoantigen-responsive donor T cells can be isolated and introduced into patient's naïve or central memory T cells with high proliferative and functional potential. Adoptive transfer of genetically engineered T cells can create specificities not present or irreversibly inhibited in the patient, thus broadening the spectrum of the naturally occurring antitumor immunity (**Figure 1**). However, to make gene therapy with personalized, neoantigen-specific TCRs a feasible clinical option, the time required to identify clinically effective and safe TCRs has to be reduced. Furthermore, the cost and labor-intensive procedures associated with, and facilities required for, current retro- or lentiviral protocols for gene transfer are prohibiting widespread use of neoantigen-specific TCR gene therapy. Non-viral approaches for gene transfer might represent promising alternatives [discussed in Ref. (52)]. To this end, a recent study demonstrated efficient *in vivo* delivery of CAR genes using T-cell-targeted nanoparticles in mice, possibly representing a practical way to rapidly deliver genes (53).

A systematic screening for immunogenic neoantigens from the big pool of candidate epitopes using donor T-cell repertoires could advance our understanding of the rules determining immunogenicity. This could in turn enable development of more accurate prediction tools to identify neoepitopes. The need for sampling of blood from often heavily pretreated patients, and the use of patient T cells impaired by a variety of immunosuppressive mechanisms, would thus be circumvented. Proof of principle that such a screening with donor T-cell repertoires is possible was shown in Ref. (15), but the development of faster culture protocols and rapid identification of high-affinity TCRs would be desirable.

Screening for the ability of candidate neoantigenic peptides to induce responses in CD8 T cells from healthy, donor-derived T-cell repertoires was combined with a novel, flow cytometry-based assay to measure peptide–MHC stability (15). The results demonstrated that immunogenic neoantigens had a significantly longer half-life than the non-immunogenic ones. In fact, addition of measured peptide-HLA stability to predicted binding-affinity of the peptide to HLA significantly improved the precision levels by which the immunogenic peptides could be identified. These data corroborate well with previous studies of microbial peptides (54, 55), indicating that peptide–MHC stability is a better predictor of immunogenic peptides than peptide–MHC binding-affinity. Thus, development of assays which facilitate high-throughput stability measurements are called upon.

CONCLUDING REMARKS

Tumors implement several immunosuppressive mechanisms to evade the immune defense of the cancer patient. These peripheral tolerance mechanisms can either reversibly or irreversibly impede the effector function of the patient's tumor-targeting T-cell repertoire. Immunotherapies with expanded TILs or checkpoint blockade rely on specificities present in the patient's own T-cell repertoire. Although clinical benefits are remarkable, they are often transient.

Transplantation of the immune system from an HLA-matched donor, which has not been compromised by tumor-induced peripheral tolerance, can induce cures in patients with hematological malignancies. The beneficial and powerful GvT effect of alloHSCT is mainly driven by donor T cells recognizing single amino acid differences in polymorphic peptides. However, since the targets are unknown and unpredictable, the desired GvT effect may be accompanied by potentially detrimental GvHD.

Personalized immunotherapies aim to explicitly target tumor-specific neoantigens, minimizing the risk of T-cell attack on healthy tissues. However, several hurdles have to be overcome to make genome-based approaches a treatment option for large groups of patients. Whole-exome sequencing can rapidly identify possible neoantigens in individual tumors, but defining those neoantigens that are immunogenic and clinically applicable remains a time-consuming, demanding task. Here, the unlimited source of donor T-cell repertoires can prove very informative. Identification of immunogenic neoantigens can guide the design of personalized vaccination and adoptive T-cell transfer therapies, and educate algorithms to become more accurate in predicting neoantigen immunogenicity. Neoantigen-reactive donor T cells can also provide TCRs, which can be used to retarget patient's naïve T cells to attack the tumor. Simultaneous targeting of multiple neoantigens expressed homogeneously in the tumor and essential for maintaining the tumorigenic phenotype, thus unlikely to be lost, might be ideal to achieve durable clinical responses. Off-the-shelf TCRs targeting neoantigens derived from driver mutations recurrent in large patient groups and in prevalent tumor types would be most practical. Such neoantigens are, however, scarce and appear poorly immunogenic when presented on patient HLA (56). Thus, there is a high demand for strategies to rapidly define clinically applicable personal neoantigens, a challenge that potentially can be answered by donor T-cell repertoires.

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REVIEW
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Neoantigen Targeting—Dawn of a New Era in Cancer Immunotherapy?

Thomas C. Wirth and Florian Kühnel**

Clinic for Gastroenterology, Hepatology and Endocrinology, Medical School Hannover, Hannover, Germany

During their development and progression tumors acquire numerous mutations that, when translated into proteins give rise to neoantigens that can be recognized by T cells. Initially, neoantigens were not recognized as preferred targets for cancer immunotherapy due to their enormous diversity and the therefore limited options to develop "one fits all" pharmacologic solutions. In recent years, the experience obtained in clinical trials demonstrating a predictive role of neoantigens in checkpoint inhibition has changed our view on the clinical potential of neoantigens in cancer immunotherapy. Technological advances such as sequencing of whole cancer genomes, the development of reliable algorithms for epitope prediction, and an increasing number of immunotherapeutic options now facilitate the development of personalized tumor therapies directly targeting a patient's neoantigenic burden. Preclinical studies in mice that support the excellent therapeutic potential of neoantigen-directed immunotherapies have provided blueprints on how this methodology can be translated into clinical applications in humans. Consistently, very recent clinical studies on personalized vaccinations targeting *in silico* predicted neoepitopes shed a first light on the therapeutic potential of personalized, neoantigen-directed immunotherapies. In our review, we discuss the various subtypes of tumor antigens with a focus on neoantigens and their potential in cancer immunotherapy. We will describe the current methods and techniques of detection as well as the structural requirements for neoantigens that are needed for their recognition by T cells and for tumor destruction. To assess the clinical potential of neoantigens, we will discuss their occurrence and functional relevance in spontaneous and hereditary cancers and their prognostic and predictive value. We will present in detail the existing immunotherapeutic options that exploit the neoantigen burden of tumors encompassing both preclinical efforts that provided convincing technological proof-of-concept and the current clinical studies confirming the potential of neoantigen-directed immunotherapies.

Keywords: neoantigens, vaccination, personalized cancer immunotherapy, adoptive transfer, mutations

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María Marcela Barrio, Fundación Cáncer, Argentina
Per thor Straten, Herlev Hospital, Denmark

****Correspondence:***
Thomas C. Wirth
wirth.thomas@mh-hannover.de;
Florian Kühnel
kuehnel.florian@mh-hannover.de

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INTRODUCTION

Designing tumor therapies which effectively destroy tumors but spare healthy tissues is considered the Holy Grail in clinical oncology. Conventional chemotherapies target tumors but also dividing cells in healthy organs and are therefore frequently associated with significant toxicity. Promising antitumor activity without detrimental side effects, the advent of targeted therapies as a novel class of more tumor-selective oncology drugs initially raised a lot of enthusiasm. Indeed, such targeted

therapies led to remarkable remissions in hematologic malignancies as observed with the introduction of imatinib for the treatment of chronic myeloid leukemia (1). In most solid tumors, however, targeted therapies have yielded only limited benefit for cancer patients. Despite the improvement of progression-free survival of cancer patients undergoing palliative treatments, the ultimate goal of significantly improved overall survival could not be achieved.

For more than a century, immunotherapy has been postulated at times as a promising alternative to conventional cancer therapy although clinical proof of its therapeutic efficacy in large patient cohorts was lacking. The perception of immunotherapy as an alternative therapeutic means was mainly driven by case reports of immune-mediated tumor control in cancer patients. Upon occasional observations of tumor regressions in patients in the context of erysipela and high fever, it was William Coley who in late nineteenth century inoculated sarcomas with bacteria (2). Since this was probably the first documented attempt to engage the patient's immune system in the fight against cancer, William Coley has been referred to as the "father of cancer immunotherapy" (3). Although he reported remarkable outcomes in individual patients, his results were frequently questioned and his methods were later abandoned in favor of the upcoming and "more modern" chemo- and radiotherapy that promised convenient handling and better reliability.

At the beginning of the twentieth century, Paul Ehrlich came up with the suggestion that the immune system is involved in carcinogenesis and in the control of tumor growth during progression (4). Some decades later, these concepts were further corroborated by mechanistic studies in mice. Several groups found that after surgical removal of methylcholanthrene-induced tumors, mice were immune against a second challenge with the same tumor material further supporting the idea of the existence of antitumor immunity (5–7). The discovery of dendritic cells as the relevant cell population for the expansion of T cells in mixed leukocyte reactions (8) and the characterization of the major histocompatibility complexes (9, 10) laid the foundation for a better understanding of the mechanisms of antigen presentation and the mechanisms that govern the induction of cancer-specific cellular immune responses. Supported by methodological advances regarding *in vitro* cultivation of antitumoral cytotoxic T lymphocytes, T cells were suggested as the major effector cell population that specifically responds to tumor antigens in humans (11, 12). Correspondingly, it had been recognized in several clinical studies that the abundance of tumor-infiltrating lymphocytes (TILs) correlates with improved survival of cancer patients (13, 14) indicating that the cytotoxic activity of lymphocytes indeed interferes with tumor growth. The antitumoral potential of T-lymphocytes in patients was later confirmed in a more direct manner. After isolation of TILs, readministration into patients in combination with IL-2 resulted in objective responses in metastatic melanoma (15).

More recently, a number of mechanistic studies in mice have confirmed that the immune system recognizes and attacks tumor cells at all stages of carcinogenesis in a process referred to as immune surveillance. Even premalignant senescent cells are detected and cleared by a process that involves both macrophages and CD4 cells (16). The role of T cells in recognition of tumor cells and control of tumor growth was convincingly shown by Shankaran et al. (17). By comparing the immunogenicity of carcinogen-induced tumors in wild-type and immunodeficient mice, the authors demonstrated that T cell reactivity is the critical determinant of the immunogenicity of mature tumors. How T cells shape the antigenic profile of a tumor in a process referred to as immunoediting was later described in detail in two studies by the groups of Schreiber and Jacks (18, 19). The fundamental influence of the immune system on cancer progression at all stages of cancer development and progression has been acknowledged and consequently designated a hallmark of cancer (20).

However, despite the extensive knowledge of the mechanisms involved in immune-mediated tumor control, successful translation of immunotherapies into the clinic lagged significantly behind these scientific advances. Targeted immunotherapies using peptide- or cell-based vaccines were astonishingly ineffective in clinical trials. Even when the first DC-vaccine targeting prostate cancer (Sipuleucel-T) provided evidence of clinical efficacy the gain in median survival was, similar to the advances achieved with targeted therapies, rather modest without evidence for long-term progression-free survival (21).

Surprisingly, it was a generic approach of T cell stimulation that finally succeeded and initiated the recent success story of cancer immunotherapy. Instead of eliciting a target-antigen-directed immune response in the context of a cancer vaccine, the pharmacologic interference with inhibitory immune checkpoints such as CTLA-4 or the PD-1/PD-L1 axis restored cytotoxicity of preexisting, exhausted cancer-specific T cells. It has to be pointed out that these therapies for the first time in clinical oncology resulted in long-term remissions in advanced cancers (22, 23) that are regarded as complete cures, so far. However, this excellent outcome is limited to a relatively small number of patients, a striking reminiscence of what William Coley observed more than 120 years ago. While the scientific proof of the exceptional therapeutic efficacy of immunotherapy in cancer treatment has been overwhelming, it is also becoming increasingly evident that these immunotherapies are not the long sought "magical bullet" applicable to all cancers. In some tumor entities such as melanoma and Hodgkin lymphoma, over all response rates to either single or combined PD-1/CTLA-4 checkpoint inhibition are encouragingly high (24–26). However, other important cancer entities such as liver cancer and pancreatic cancer are much more resistant to this therapy. Despite all progress, the majority of patients will not experience complete responses, at least when treated with the present options of immunotherapy. Owing to these limitations of current immunotherapies, there is a lot of space for novel therapies that specifically target tumor antigens with well-defined molecular characteristics, thus fulfilling the promise of an individualized immunotherapeutic approach.

CLASSIFICATION OF TUMOR ANTIGENS

The initial discovery of the interaction between tumor and immune cells was followed by intensive research to identify the target antigens that were recognized by the adaptive immune system. In 1989, a cell surface glycoprotein of the mucin

family, MUC1, which is expressed in tumors in an aberrantly glycosylated form, was described as a tumor antigen that can be recognized by cytotoxic T cells (27). The melanoma antigen family A1 (MAGE-A1) was found to be expressed not only in melanoma but also in other tumor entities whereas it could not be detected in normal tissue except the testis (28). Tumor-specificity of MAGE-A1 is due to the fact that germ line cells lack MHC class I molecules for presentation of the corresponding peptides on their cell surface. MAGE-A1 was therefore a prototypic example of tumor antigens termed cancer testis antigens (CTAs). Another class of tumor antigens are tumor-associated antigens (TAAs) derived from proteins that are overexpressed in cancer but also occur in normal cells. These proteins are frequently involved in transformation-related mechanisms as exemplified by the human epidermal growth factor receptor 2 (Her2/neu) and have also been using as immunotherapeutic targets (29). Since TAAs are also expressed by normal cells, their role as a target antigen for tumor therapy is solely based on their preferential expression in cancers. Their basal expression in normal tissues subjects these antigens to central and peripheral tolerance mechanisms, leading to selection of low-avidity T cells. However, TAA-directed immunotherapy using T cell receptor (TCR)-transgenic, high-avidity T cells may cause severe autoimmunity (30). Compared with TAAs, CTAs such as MAGE-A1 or New York esophageal squamous cell carcinoma-1 (NY-ESO-1) have attracted more attention due to their broad abundance in several tumor entities and their restriction to tumor tissue. Expression CTAs has been shown to be associated with intratumoral lymphocyte infiltration and improved prognosis of cancer patients though these lymphocytes might be functionally impaired (31, 32). It has also been established that adoptive transfer of lymphocytes genetically engineered with an NY-ESO-1-directed TCR is able to induce tumor regression (33).

A highly promising class of tumor antigens are tumor-specific antigens (TSAs). These proteins are not encoded in the normal genome and encompass antigens derived from viral oncogenic proteins (e.g., SV40 from Epstein Barr Virus, or E6/E7 from human papilloma virus) or from proteins that are the result of somatic mutations or gene rearrangements. Whereas the presence of virus-derived proteins is mostly limited to tumors originating from a viral infection process, tumors in general acquire mutations during carcinogenesis and progression, resulting in altered proteins that may serve as neoantigens (34). Neoantigens may be either directly linked to the transformation process (driver mutations) or may occur as a byproduct of increasing genetic instability (passenger mutations) (35). Interestingly, mathematic modeling of the accumulation of mutations during tumor progression suggests that the number of driver mutations may correlate with the total number of mutations in the tumor (36). Neoantigens are probably the most interesting targets for immunotherapies since neoepitopes are not subject to thymic selection and central tolerance. Therefore, the existence of high-avidity T cells is very likely. Furthermore, it has been shown that failure of intrathymic gene expression can give rise to immunogenicity comparable with neoantigens as demonstrated for the melanoma antigen MART-1 (37).

Depending on the position of the mutated amino acid in the sequence of the MHC-bound peptide the non-synonymous mutations differentially impact the quality of the neoantigen. While mutations in anchor positions primarily affect peptide affinity, mutations outside the anchor positions preferentially influence the interaction of the peptide/MHC complex with the TCR. As a consequence, mutations in anchor positions potentially create high affinity epitopes while mutations in the TCR-interacting positions may lead to the recognition of naive T cells which specifically recognize the mutated neoepitope.

First evidence that neoepitopes resulting from non-synonymous mutations are recognized by the immune system as "altered self" was provided by Wölfel et al. The authors identified a p16INK4a-insensitive cyclin-dependent kinase 4 (CDK4)-R24C mutation in melanoma patients as a neoantigen that was a target of CTL responses (38). This mutation of CDK4 disrupts the cell-cycle regulation exerted by the tumor suppressor p16INK4a and is therefore closely associated with carcinogenesis. Consistent with the assumption that neoantigen-directed T cell responses may play a significant role in tumor growth control, it was subsequently demonstrated by the Wölfel group that the antitumor response of autologous T cells in a melanoma patient was predominantly driven by T cells recognizing mutated neoantigens (39). Additionally, neoantigen-directed T cells could be detected in *ex vivo* expanded TILs that had been adoptively transferred in melanoma patients who subsequently experienced a complete tumor regression (40). Together, these findings shed a first light on the use of neoantigen-directed immunotherapies and their clinical potential.

IDENTIFICATION OF POTENTIAL NEOANTIGEN TARGETS FOR IMMUNOTHERAPY

According to the results of high throughput cancer genome sequencing it has been firmly established by now that all tumors contain a significant number of somatic mutations (34). However, since neoantigens are the result of sporadic mutations caused by DNA damaging agents and/or random errors of the DNA repair machinery, the set of neoantigens of a tumor is believed to be highly individual ("private"). This feature discriminates neoantigens from tissue-specific, tumor-associated, or other tumor-selective antigens which are considered shared "public" antigens due to their expression in specific organs, their overexpression in cancer or their selective expression in defined tumor entities, respectively. As an exception from this rule, some cancers with high mutational load including microsatellite-instable tumors have been shown to possess a set of shared neoantigens owing to the preferential mutation of distinct genetic regions termed microsatellites (41).

As a consequence of the mostly private nature of neoantigens, potential neoantigen targets can only be identified after analysis of a tumor mutanome by means of whole exome and/or next generation RNA sequencing (42, 43). Depending on the tumor entity and the underlying cause of cancer development these analyses have revealed a wide range for the number of neoantigens detected, ranging from only few mutations in some forms of astrocytoma to several thousands in some melanomas and lung cancers (44).

Of the vast number of non-synonymous mutations detected in tumors only a tiny fraction may be suited for tumor treatment. To enhance the chances for successful immunotherapy a number of critical features of neoantigens have been described that impact on their quality as immunotherapeutic target.

Expression of Neoantigens in Tumor Cells

As one of the more simple requirements, the target antigen has to be expressed inside the tumor cells. Following transcription from genomic DNA into mRNA, the non-synonymous mutations are translated into the corresponding mutated proteins. Since most studies targeting tumor neoantigens perform next generation mRNA sequencing, mRNA quantity has been used most frequently as a surrogate marker for target gene expression although direct protein measurement is likely to be more accurate. Nevertheless, studies have supported the notion that higher mRNA quantites correlate with both protein quantity and the number of peptide/MHC complexes presented on the tumor cell surface (45). This correlation, however, is rather weak since post-translational regulation of protein expression and proteasomal processing of the target antigen are neglected.

Formation of Stable Neoepitope/MHC Complexes

It is known from reductionist antigen models using adoptive T cell transfer that binding affinities between antigenic peptides and MHC class I as well as the binding affinity of the peptide/MHC complex to the corresponding TCR are critical determinants of tumor-directed T cell reactivity and the capability of T cells to reject a tumor (46, 47). It is therefore mandatory to thoroughly assess the ability of neoantigen-derived peptides to form stable peptide/MHC complexes that are able to tightly bind to their cognate TCRs. Following synthesis of the mutated protein, a fraction of the resulting protein is processed by the proteasome, loaded onto MHC class I molecules after transport into the endoplasmatic reticulum by the TAP transporter and presented on the cancer cell surface. The process of proteasomal degradation can be predicted from a number of computational algorithms (e.g., NetChop) but the accuracy of prediction remains to be improved. The expression of neoantigen epitopes with potential clinical relevance has successfully been demonstrated by mass spectrometry (48) but the sensitivity of the method is often limited to epitopes with high expression on MHC. It has been recently shown that the sensitivity of neoepitope detection by mass spectrometry can be significantly increased by monoallelic analysis (49) after retroviral transduction of tumor cells with a specific HLA allele. Due to the limitations of mass spectrometry for less abundant proteins, many studies have not quantitated expression of neoepitopes on tumor MHC complexes but instead focus on *in silico* prediction of neoepitope affinity for a given MHC molecule (50–52). This is justified by the fact that high affinity peptides are more likely to form stable complexes with MHC resulting in increased expression on MHC complexes on the cell surface. Nevertheless, it has to be taken into account that despite high affinity some neoepitopes may never be generated by the proteasome.

To predict the strength of the peptide/MHC binding, the affinity of potential MHC class I epitopes is calculated based on the patient's MHC haplotype. For this purpose, a number of software programs (e.g., NetMHC and SYFPEITHI) are available that allow accurate prediction of peptide affinity for a neoepitope if the binding properties of the MHC allele are sufficiently characterized (18). Although the reliability of these *in silico* analyses has been questioned (53) the *in silico* binding prediction still represents the first and most important step to identify potential neoantigen targets.

Activation of Neoantigen-Specific T Cells by Stable Peptide/MHC/TCR Interactions

To generate neoantigen-specific adaptive immune responses the peptide–MHC complexes must be presented on the surface of antigen-presenting cells and interact with neoepitope-specific T cells. In endogenous tumor-specific immune responses, tumor cells undergoing cell death are taken up by dendritic cells which then process endocytosed neoantigens and present the class II neoepitopes on their MHC class II molecules. In parallel, MHC class I neoepitopes are presented on DCs by cross-presentation, a process by which endocytosed proteins after proteasomal cleavage gain access to MHC class I molecules inside the endoplasmatic reticulum (54). This dual requirement for MHC class II molecule presentation and efficient cross-presentation of CD8 epitopes on MHC class I molecules is almost exclusively limited to dendritic cells and among these most prominent to the BATF3-driven lineage (55).

For vaccinations with soluble, short peptides containing MHC class I epitopes (typically 8–10 amino acids in length) or MHC class II epitopes the exogenously administered peptides have to compete with endogenous MHC class I and class II peptides that are already present on antigen-presenting cells. If the neoepitopes are of higher affinity than the endogenous MHC class I, they are able to replace the endogenous peptides directly on the APC surface. For vaccinations in the form of DNA, RNA, or polypeptides/proteins, the target antigen must undergo cross-presentation since these antigen carriers are usually taken up and endocytosed by antigen-presenting cells, thus underlying the same restrictions mentioned above for the endogenous tumor cell-specific T cells responses.

To elicit robust immune responses, the neopeptide–MHC complexes must form an immunological synapse with TCRs on either CD4 or CD8 T cells. Since neoantigens represent "*de novo*" antigens it has been postulated that neoantigen-specific T cells are not subject to central tolerance. As a consequence, neoantigen-specific T cells may not only be of higher functional avidity but may also be more abundant than T cells recognizing autoantigens.

Currently, the methodology for the detection of naive neoantigen-specific T cells in peripheral blood is limited, both in preclinical models and in humans. As an alternative, the number of potentially neoantigen-responsive T cells has been assessed by selective screening of PD-1 expressing, circulating CD8 T cells for their potential to recognize neoantigens (56). However, PD-1 expression on neoantigen-specific T cells indicates beginning exhaustion that could prevent an accurate identification of tumor-specific T cells if intracellular cytokine stainings are performed

for T cell detection. Therefore, researchers have advocated the use of tetramers/pentamers to reliably identify neoantigen-specific T cells (57). Using peptide–MHC multimers with DNA barcodes this technology has recently been adapted to allow the large-scale detection of cancer-specific T cells, including T cells specific for neoantigens (58).

However, these methods have a number of caveats to consider. First, detectable neoantigen-specific T cells in patients are likely to be antigen-experienced and more terminally differentiated (59). It is unknown how the quality of these cells compares to truely naive T cells which are endowed with potent replicative capacities. In fact, the number of naive T cells may be reduced in patients with detectable immune responses due to the prior stimulation of the naive T cell pool. Second, systemic immune responses have shown only limited correlation with intratumoral immune responses. Systemic neoantigen-specific T cells may be present due to the lack of trafficking of the neoantigen-specific T cells to the tumor site or because their cognate antigen is not expressed on the tumor cells. Third, neoantigen-specific immune responses detected in the circulation of cancer patients have undoubtedly failed to clear the tumor and their cognate antigen might therefore not represent a favorable target antigen. More studies are needed to assess whether this failure to reject a tumor is primarily due to tumor-derived immunosuppressive mechanisms which could be restored with checkpoint inhibitors or whether this is a T cell intrinsic failure.

Once the neopeptide is sufficiently expressed on MHC and the MHC/peptide/TCR synapse is formed (providing the so-called signal I) the robustness of the ensuing immune response is dependent on additional costimulation (signal II) and secretion of immunostimulatory cytokines such as IFNα and IL-12 (signal III) (60). Since these signals are provided primarily by dendritic cells, efficient T cell priming usually requires signaling through costimulatory molecules and toll-like receptors to induce optimal DC maturation. How this is best achieved in tumor vaccinations remains a matter of debate and much effort is currently devoted to developing strategies that selectively target and activate dendritic cells *in vivo*.

The final step of the neoantigen-directed therapy requires the trafficking of the activated T cell into the tumor tissue and the recognition of the peptide–MHC complex on the surface of the cancer cell by the TCR (61). The T cell must interact with the peptide/MHC complex on the cancer cell and the net result of the TCR/peptide–MHC complex interaction and the activation state of the T cell must result in the production of cytolytic granules. The exact requirements for efficient tumor cell killing currently remain elusive but the affinity of the peptide again seems to play a major role. Interestingly, visualization of the interaction of T cells and tumor cells suggest that the process of tumor cell killing *in vivo* may take much longer than the same process *in vitro*, possibly requiring multiple consecutive hits from cytotoxic T cells (62).

ROLE AND FREQUENCIES OF NEOANTIGENS IN SPONTANEOUS CANCERS

For the most part neoantigens have been considered random, spontaneous mutations with little overlap between individual patients. Of a wide spectrum of tumors analyzed for their total mutational burden, only few have demonstrated a mutation frequency above 10/megabase DNA. In these tumors, the few neoantigens are randomly distributed throughout the genome which has led to a view of neoantigens as entirely "private" antigens. At second sight, however, different classes of mutagens have been shown to induce non-random changes in genomic DNA sequences. As an example, UV light induces C to T transitions in dipyrimidine contexts whereas tobacco smoke preferably induces G to T transitions. For tumors with low mutational load this bias in DNA alterations is not sufficient to result in recurrent non-synonymous mutations. In tumors with a high mutational load like melanoma, however, a C to T transition in the gene RQCD1 has been shown to result in a recurrent P131L mutation with a prevalence of 4% in a population of 715 melanomas (63). Similarly, large scale whole-exome sequencing in 619 colorectal cancer patients revealed preferential mutations in BCL9L, RBM10, CTCF, and KLF5 (64). Of interest, some of these genes are known driver genes in other tumor entities pointing toward a preferential selection of genetic alterations that promote tumor growth. These results suggest that although most neoantigens in sporadic tumors are indeed "private," both the type of mutagen and a selection for driver mutations can result in recurrent neoantigens whose frequencies are currently underestimated. These results warrant further large-scale whole exome analyses in other tumor entities to corroborate the findings from melanoma and colorectal cancer patients.

ROLE OF NEOANTIGENS IN HEREDITARY CANCERS WITH DNA REPAIR DEFICIENCIES

Cancers with hereditary defects in genes involved in DNA repair are characterized by high frequencies of non-synonymous mutations. As an example, patients with Lynch syndrome harbor mutations in DNA mismatch repair genes resulting in thousands of neoantigens per tumor. In patients with Lynch syndrome, the mismatch repair deficiency does not only induce DNA base exchanges but results in the accumulation of insertions or deletions at mutation-prone DNA hot spots with repetitive base pair sequences [referred to as microsatellite instability (MSI)]. As a consequence, whole exome analyses of tumor samples from patients with Lynch syndrome have revealed a number of recurrent frameshift mutations in genes with microsatellite sequences. Similar to the reported genetic alterations in sporadic tumors some of these frameshift mutations presumably target genes involved in tumor development, particularly genes with tumor-suppressor function including TGFBR2, BAX (65, 66), CRTC1, BCL9, JAK1, and PTCH1 (67). The preferential mutation of genes with microsatellite sequences in patients with Lynch Syndrome has led to the identification of a set of genes with high mutation frequencies in MSI patients (TGFBR2, AIM2, HT001, and TAF1B) which have been used as a vaccine in a clinical trial (68). Although prototypic, colorectal MSI cancers represent only one example of tumors with mismatch repair deficiencies. Highly immunogenic mutations have, for example, been reported for other MSI tumor entities including gastric cancer, ovarian cancer, glioblastoma and

others (69), Polymerase ε-mutant glioblastoma (70), colorectal, and endometrial cancers (71, 72), as well as BRCA-mutated ovarian cancer (73). For these tumor entities, recurrent neoantigens that are shared between patients may represent prime targets for immunotherapy, in particular frameshift mutations which typically harbor multiple novel epitopes that are recognized across various MHC haplotypes.

As a potential caveat, the large mutational burden in patients with mismatch repair deficiencies seems to greatly accelerate the formation of immune escape variants. In patients with MSI-tumors, defects in antigen presentation have been detected in MHC molecules and in molecules associated with MHC expression at high frequencies (74). These MSI cancers may exhibit greatly reduced sensitivity to T cell-mediated killing, a potential caveat that has to be considered for the appropriate design and timing of vaccines targeting MSI cancers.

PROGNOSTIC AND PREDICTIVE VALUE OF NEOANTIGENS

Microsatellite instable tumors are increasingly recognized as a subset of tumors with distinct prognostic and predictive features. In patients with colorectal cancers, MSI tumors are overrepresented in early stage cancers but underrepresented in metastatic disease. This feature of MSI tumors has been attributed to the presence of high numbers of immune cells in MSI tumor specimens which may limit local tumor recurrence and systemic spread. Patients with MSI-H colorectal cancers UICC stage II have been shown to have a favorable prognosis compared to patients with MSI-L or microsatellite stable (MSS) tumors (75, 76), depending on the individual mutation and concomitant allelic losses (77, 78). In metastatic stage IV colon cancer the prognosis of patients with MSI colorectal cancer is similar to patients with MSS tumors but associated with better survival in patients with peritoneal metastases and lower survival in patients with lymphatic or blood-borne metastases (79).

The better prognosis of patients with MSI tumors has a direct impact on the treatment of this patient population after tumor resection. Owing to the lower frequency of local recurrence and systemic spread after resection, adjuvant therapy for UICC stage II MSI colon cancer is not recommended. For UICC stage III patients, the usefulness of adjuvant therapy in MSI patients is still a matter of debate, with some studies arguing in favor of adjuvant therapy (80) and others against it (81).

More recently, the clinical success of checkpoint inhibitors in melanoma patients has revealed an additional predictive role of the mutational load in patients treated with either anti-PD-1 or anti-CTLA-4 antibodies (82–84). These clinical effects have been suggested to be due to neoantigen-specific immune responses which are restored upon administration of checkpoint inhibitors (85, 86). In patients with MSI tumors, both PD-L1 expression on cancers and PD-1 expression on TILs is increased thus providing a molecular basis for the better clinical response to anti-PD-1 therapy (87–89). The predictive role of neoantigens may extend to other immunological treatments including adoptive T cell therapy. Although this has not yet been demonstrated convincingly, neoantigen-specific immune responses have been detected in TILs (90, 91). In these patients, neoantigen-specific immune responses show evidence for robust clonal expansion indicating that the quantity and quality of neoantigens in the expanded T cell pool could influence the therapeutic efficacy of TIL transfer.

NEOANTIGEN-DIRECTED TUMOR THERAPIES

The notion that neoantigen-specific T cell responses are involved in tumor growth control in patients raised significant interest in identifying specific neoantigens as suitable targets to facilitate the design of tumor-directed vaccines. A particularly attractive kind of neoantigens are those that represent relevant mutation in tumor driver genes. It has been widely assumed that immunotherapies targeting noepitopes originating from oncogenic driver mutations may induce antitumor responses in a most effective manner since they are most likely essential for tumor survival and are homogenously expressed throughout the tumor tissue. Consequently, investigations initially focused on neoepitopes derived from well-known mutations in prominent oncogenes such as KRAS mutated at codon 12, or mutated p53 (92, 93). An oncogenic alteration that frequently occurs in melanoma is the V599E missense mutation in the kinase domain of BRAF giving rise to a mutation-specific epitope that can be recognized by T cells (94, 95). Furthermore, in particular hematological malignancies mutations in either JAK2 (JAK2V617F) or mutations in exon 9 of calreticulin are abundant incidents giving rise to spontanoues T cell responses (96, 97). These mutations could be interesting targets for immunotherapy as well as recently described amino acid exchange in the histone H3 gene (K27M) that is frequent in glioma (98, 99). A peptide vaccine against this mutation was capable to effectively induce mutations-specific immune-responses in a MHC-humanized mouse model (100). Also in humanized mice, it has been demonstrated that a vaccine targeting mutant isocitrat dehydrogenase-1 (IDH1R132H) induced mutation-specific T cells and was able to control the growth of preestablished tumors (101). A corresponding vaccine is currently investigated in clinical trials in glioma patients.

Although the abovementioned mutations represent rather frequent genetic events the abundance of shared neoepitopes is significantly reduced by the huge HLA diversity rather low and inter-individual overlap is limited. The feasibility to develop broadly applicable vaccines has been recently estimated by a genomic analysis and epitope prediction of more than 63,000 tumors across multiple tumor entities and for the most common HLA A/B subtypes (102). Hypothesizing that sets of carefully selected neoantigens could allow for development of broadly applicable vaccines these calculations revealed that neoantigen targets still remain highly diverse even when regarding major and frequent driver mutations. Nevertheless, the fact that shared neoepitopes are not fully private compared to other mutation-derived epitopes is an important technical advantage, and, once established corresponding vaccines could function as a valuable

backbone in more complex multiepitope targeting approaches to prevent the rise of escape mutants.

Several technological advances in parallel opened up new avenues for the discovery of neoantigens and their potential use as target antigens in cancer immunotherapy. Next generation sequencing facilitated the exploitation of whole tumor exomes and revealed that in all tumors the mutated genome encodes for a variable but significant number of non-synonymous mutations and thus potential neoantigenic epitopes (34). Furthermore, raw DNA sequencing data can be rapidly processed *in silico* and algorithms are available that help to predict neoepitopes. These technologies therefore promise to achieve the identification of suitable neoepitope candidates for patient-specific immunotherapy within acceptable time, being one of the most critical requirement for patients with progressive tumor growth. The correlation of therapeutic efficacy of checkpoint inhibitors and the neoantigenic load clearly demonstrated that neoepitopes could play a prominent role also in more target-selective approaches of cancer immunotherapy. Several preclinical studies developed and simulated workflows including tumor exome mining and neoepitope prediction, eventually followed by methods to confirm truely immunogenic neoepitopes within the predicted pool, aiming at the development of a personalized immunotherapy. Just recently, the first results from clinical studies which applied these preclinically established methods to real-life therapeutic settings in humans have been reported. The promising clinical results will be described in more detail in the following subchapters. Furthermore, the full spectrum of immunotherapies targeting neoantigens in cancer patients is summarized in **Figure 1**.

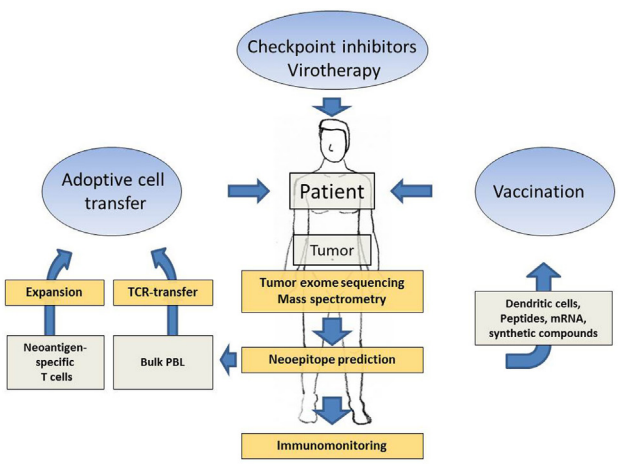

FIGURE 1 | Schematic presentation showing how patient-specific tumor sequencing data can be translated into several options of neoantigen-directed immunotherapies. Next generation sequencing of tumor tissue, neoepitope prediction and mass spectometry analysis, supported by immune activating interventions such as checkpoint inhibition and virotherapy, provide the methodology to delineate promising neoantigenic patterns as targets for tailored immunotherapies. Neoantigen-directed immunotherapies include adoptive cell transfer approaches and tailored vaccines.

Checkpoint Inhibitors As a Systemic Approach to Activate Neoantigen-Directed T Cell Responses

Antagonizing coinhibitory molecules has shown great success in treatment of some cancer entities even at advanced stage (22, 23). Checkpoint inhibitors are generic stimulators of T cell responses and part of their activity is therefore directed against neoantigens that can be detected by T cells. A potential involvement of neoepitopes in therapeutic efficacy in melanoma has early been assumed since the mutational load in this particular tumor entity is rather high. To prove the relevance of neoantigens as immunotherapeutic targets the contribution of neoantigens to the observed therapeutic responses following application of checkpoint inhibitors has been assessed by a number of studies. First evidence that there is indeed a positive correlation between the mutational burden in tumors and the observed response came from checkpoint inhibitor studies using either PD-1/PD-L1 or CTLA-4 inhibitors. After tumor exome sequencing and data processing with NetChop and NetMHC algorithms, van Rooij et al. showed the expansion of a T cell response directed against a mutated version of ATR (ataxia teleangiectasia and rad3 related) in a melanoma patient after therapeutically effective ipilimumab treatment (85). Snyder et al. directly investigated the correlation of the mutational load in melanoma and therapeutic response to CTLA-4 inhibitors ipilimumab and tremelimumab (82). They found that the mutational load was indeed associated with the degree of clinical benefit. More detailed investigations by genome-wide neoepitope analysis and patient-specific HLA-typing allowed the description of specific neoantigenic "landscapes" that are present in tumors responding to this therapy. In parallel, Rizvi et al. correlated the clinical benefit of the PD-1 inhibitor pembrolizumab with the mutational load in a patient cohort with non-small cell cancer patients with a wide range of neoantigen frequencies due to cigarette smoking. They found that the non-synonymous mutational load in tumors was associated with improved objective response, durable clinical benefit and progression-free survival (83). Therapeutic efficacy correlated with several parameters resulting from the mutational load such as a mutation-rich molecular smoking signature, higher neoantigenic burden, and DNA repair pathway mutations. Consistent results were described by a phase 2 study in colorectal cancer patients with mismatch repair deficiency which harbor hundreds to thousands of mutations (103). Tumors with mismatch repair deficiency showed a significantly higher progression-free survival after pembrolizumab therapy compared to mismatch-proficient tumors. These observations confirmed the role of mutational burden as a predictive marker in checkpoint therapy of MSI patients and suggest an important role for neoantigen-directed immune reponses in patients with highly mutated tumors. Nevertheless, checkpoint inhibitor studies have also demonstrated that accurate prediction of immunoresponsiveness remains challenging since a significant number of patients failed to respond to checkpoint inhibition despite a high mutational load. Future studies are therefore required to reliably discriminate predictive from non-predictive mutations in patients undergoing checkpoint inhibition and to convincingly demonstrate the role of neoantigen-directed adaptive immune responses.

Preclinical Studies Targeting Neoantigens

Several studies in mouse models delivered blueprints how neoantigen-directed immunotherapies can be applied in the future for effective immunotherapy of cancer. Through investigations in mouse models it was demonstrated that neoepitopes are important targets of the immunoediting processes during carcinogenesis (18, 19) which decisively shape the immunogenic profile of a mature tumor. The Schreiber group identified and validated neoepitopes in highly immunogenic carcinogen-induced sarcomas including neoantigens that enabled tumor rejection such as spectrin-β R913L, or were involved in tumor rejection in response to checkpoint inhibition such as neoepitopes derived from mutated LAMA4 or mutated ALG8 (104). In parallel, investigations were undertaken to find out how these neoantigen-derived immunogenic profiles can be determined and translated into suitable targeted immunotherapies. The Sahin group used next generation sequencing to identify 962 non-synonymous point mutations in B16F10 melanoma cells from which 563 were found in expressed genes (42). Next, the researchers investigated the actual immunogenicity of 50 selected peptides harboring most promising candidate mutations according to prediction with NetMHC. One third of these peptides were indeed immunogenic with 60% preferential activity against the mutated peptide compared with the wild-type equivalent. In transplant tumor models, vaccinations with these peptides conferred antitumor activity in protective as well as therapeutic settings. Together with the findings by the Schreiber group described above these observations clearly demonstrated the feasibility of bioinformatic evaluations of entire neoepitope spectra that can be used as raw material to identify therapeutically relevant immune responses targeting neoepitopes. In a different approach, Yadav et al. further optimized the accuracy of immunogenic neoantigenic peptide identification by including mass spectrometry of peptides present on MHC class I molecules (105). For their purpose, the authors investigated two widely used tumor models including the murine colon carcinoma cell line MC38. Using whole-exome and transcriptomic sequencing as well as MHC binding prediction, they found 1,300 amino acid changes of which 13% were potential MHC class I binders. A small fraction of these candidates were indeed confirmed by mass spectrometry. The circle of candidates was further narrowed down by molecular modeling of the peptides bound in the groove of MHC class I. Only those peptides that exposed the mutation to the exterior were considered immunogenic. Those included the strong H-2D_b epitopes of mutated Reps1 and Adpgk, and the H-2K_b epitope of Dpagt1. Remarkably, vaccinations with peptides predicted by this combined *in silico* prediction/mass spectrometry approach yielded therapeutically active T cell responses thus impressively confirming its excellent accuracy. Central aims of these strategies were not only to show the feasibility of *in silico* prediction of neoepitopes within acceptable time but also to demonstrate the accuracy and reliability of the chosen *in silico* approach in identifying immunogenic neoepitopes. Mass spectrometry was a first powerful analytical step to narrow down the number of therapeutically relevant neoepitodes with an additional validation step. However, the more stringent the selection criteria are *in silico* or *in vivo*, the higher the risk to omit relevant neoepitope candidates for targeted therapies. As an example, the nature of the chosen immunotherapy may impact on the quality of neoepitope responses as Gubin et al. demonstrated in their study when showing treatment-specific transcriptional alterations in neoepitope-specific CD8 T cells after CTLA-4, or PD-1 checkpoint inhibition, respectively (104). As an alternative means to identify neoepitope-specific CD8 T cell with potential relevance in immunotherapeutic treatments, we have pursued an alternative strategy including neoepitope prediction and confirmation of immunogenicity using intratumoral application of oncolytic viruses. To this end, we analyzed the spectrum of candidates for neoepitope-specific CD8 T cell responses in murine CMT64 lung cancer cells that are highly resistant to immunotherapy such as systemic PD-1 blockade (106). Similar to the aforementioned studies, next generation sequencing and data processing facilitated the detection of 274 non-synonymus mutations. The corresponding peptide sequences were analyzed by the SYFPEITHI algorithm for CD8 T cell epitope prediction to yield 44 neoepitope candidates that potentially bind to MHC class I with high affinity. Among those, five neoepitope-specific responses directed against the mutations H2Q2-D244E, Ndufs1-V491A, Rab13-K196N, Ppat-I208M, and Gsta2-Y9H were identified in peripheral blood following intratumoral application of an oncolytic adenovirus in all investigated individuals. Interestingly, when intratumoral virotherapy was administered together with systemic PD-1 checkpoint inhibition, a strong broadening of the neoepitope spectrum with improved antitumor efficacy was observed including neoepitope-specific responses that were neither detectable after PD-1 blockade nor after virotherapy, when applied as monotherapies. The use of tumor selectively replicating viruses is therefore not only an effective means to lyse tumors. Our observations also demonstrate that viral oncolysis mimics the effect of a vaccine that covers the complete antigenic spectrum of the target tumor, including neoantigens. Consequently, application of oncolytic virotherapy may not only be used as direct tumor therapy but may also serve as a method to validate the responsiveness of tumor-specific T cell clones to a predicted neoepitope, either for tracking and assessing the success of therapy or for facilitating the design of additional immunotherapeutic means to further enhance responding neoepitope-specific T cell responses. Yet another alternative prediction method considered the difference in NetMHC score between a neoepitope and the unmutated counterpart together with the overall affinity of the peptide bound to MHC class I. By applying this method, Duan et al. were able to detect unique neoepitopes that provided substantial tumor protection (107). Interestingly, the authors also found neoepitopes with rather weak affinities that were lower than the affinity threshold that is usually considered sufficient for effective interaction. Though mechanistic studies have suggested that high affinity neoepitopes are mandatory for tumor rejection, it will need further investigations to discriminate how several weak or moderate avidity CD8 T cell responses may cooperate in tumor rejection. Although responses against a single, low-affinity neoepitope might be insufficient for tumor rejection, multipronged responses may develop enough cumulative antitumor efficacy required for rejection and at the same time prevent the generation of escape variants.

A further critical aspect is the contribution of neoepitope-specific immune responses by CD4 T cells. The contribution of CD4 T cells to antitumor effects is known from depletion studies and the presence of antitumoral antibodies (104, 108). The observed control of tumor growth in experimental tumor models harboring transposons for CD4 and CD8 neoepitopes also suggest a mechanistical role of CD4 and CD8 T cell interaction in cancer immunosurveillance (109). The relevance of CD4 T cell neoepitopes has been shown in humanized mouse models and in patients. Schumacher et al. demonstrated that after peptide vaccination of mice transgenic for human MHC class I and II with a mutated peptide of isocitrate dehydrogenase 1 (IDH1), mice developed effective MHC class II-restricted, mutation-specific antitumor immune responses resulting in growth control of tumors expressing mutated IDH1 in a CD4 T cell-dependent manner (101). The Rosenberg group confirmed the therapeutic relevance of a CD4 T cell neoepitope in a patient with metastatic cholangiocarcinoma following adoptive cell transfer (110). Linnemann et al. investigated and validated neoantigen-specific CD4 T cell responses in melanoma patients and found that these responses are indeed present but rather rare events (90). In a study in mice, the Sahin group found surprisingly that vaccinations using long peptides containing predicted CD8 T cell neoepitopes resulted in effective, tumor-directed T cell responses that were vastly dominated by neoepitope-specific CD4 T cell responses (111). These findings might be due to the method used for prediction or to species-specific effects. Also, it has to be taken into consideration that prediction of MHC class II epitopes is in general more error-prone than prediction of MHC class I epitopes. This is due to the fact that peptides are more loosely bound to the groove of MHC class II and a more variable size is tolerated making *in silico* prediction more demanding compared with prediction of CD8 T cell epitopes.

Whereas multiple studies support the usefulness of neoepitope prediction for the design of immunotherapies the limitations of this method have been far less defined. A study by Martin et al. suggest that this could be the case in tumors with moderate to low mutational burden. This has been assessed by whole exome and transcriptome sequencing on ID8-G7 cells (112). The authors identified 39 transcribed missense mutations and applied corresponding peptide vaccines in mice. Whereas 7 of 17 neoepitope-specific vaccines, directed against predicted MHC class I binding mutations, induced robust mutation-specific T cell responses, none of the vaccines yielded a therapeutic benefit in tumor-bearing mice illustrating the limits of neoantigen-directed immunotherapy.

A specific future requirement for neoepitope response prediction in immunotherapy should include the reliable coverage and definition of a neoepitope-specific T cell responses capable of tumor rejection. This remains a challenging task when only relying on *in silico* approaches. Certainly, a stringent immunomonitoring is required to detemine neoepitope-specific T cells that actually respond to therapy (113). Much of the preclinical work using *in silico* epitope prediction that has been presented up to now has been performed in inbred animal models with relatively little pathogen exposure reflecting rather unexperienced "naive" individuals. It is therefore a general question in how far the obtained data reflects the situation in humans patients considering the vast diversity of the "immunome" in immunologically experienced cancer patients. Therefore, additional analytical steps are urgently needed that take into consideration how the human immune system is altered in aged and immunologically experienced cancer patients. This should facilitate the design of a tailored therapy that fits the needs of a truly personalized neoantigen vaccine.

Adoptive Cell Transfer Strategies

Next generation sequencing techniques and neoepitope prediction have also facilitated more precise investigations of the specificities of TILs and the design of neoantigen-directed T cells for adoptive transfer immunotherapies. Adoptive transfer of tumor-directed immune effector cells such as TILs represents a classical approach to target tumor antigens for cancer immunotherapy. A striking advantage compared with active immunization or checkpoint inhibition is that tumor-reactive cells can be identified and then expanded *in vitro* to large numbers before giving them back to the patient in combination with IL-2 (114). As a potential limitation, the method requires an invasive procedure to obtain material for isolation and growth of the desired TILs. Furthermore, TILs may contain exhausted, terminally differentiated populations that limit their use in adoptive T cell therapy approaches or T cells that do not recognize tumor antigens. An alternative is to redirect peripheral blood lymphocytes to tumors by transduction with heterologous TCRs to facilitate tumor recognition. First clinical trials with ACTs using genetically engineered TCRs against MART-1 or NY-ESO1 showed objective tumor responses, but also "off-target" toxicities (33, 115). Since neoantigens are bona fide TSAs, the adoptive transfer of neoantigen-directed T cells promises antitumoral activity without off-target effects and thus reduced adverse events. Correspondingly, various therapeutic approaches have been reported that successfully translate these principles to adoptive transfer of neoepitope-directed T cells. The Blankenstein group (116) generated transgenic T cells expressing a TCR directed against a known immunogenic mutation in CDK4 which results in two mutant isoforms of CDK4. In an MHC class I humanized mouse tumor model, the authors showed effective expansion of T cells and IFN-γ expression. Interestingly, the response to these two isoforms was dramatically different indicating the highly variable quality of neoantigens to serve as T cell targets. Using transcriptomic sequencing of a UV-induced tumor, Leisegang et al. identified a mutation in p68, a coactivator of p53. This mutation turned out to be a well suited neoepitope since it reflects a trunk mutation and binds to MHC with high affinity. TCR-transgenic T cells recognizing this neoepitope were capable of eradicating established tumors. However, when the antigen was autochthonously expressed, T cell pressure promoted the emergence of escape variants (117). Immune escape was prevented when expression of the neoantigen was warranted in all tumor cells or when additional immunotherapeutic means such as irradiation were applied. The emergence of escape variants parallels clinical experience with molecular targeted therapies and strongly recommends the development of multi-targeted immunotherapies to prevent immunotherapy failure.

To engage a significant number of functional neoantigen-directed T cell specificities, the Rosenberg group first enriched

neoantigen-specific TILs prior to isolation of the corresponding TCRs. The information from tumor exome sequencing and epitope prediction was used to generate tandem minigene constructs harboring the corresponding mutated sequences for expression of the corresponding neoepitope peptides in dendritic cells. Coincubation of these DCs with TILs resulted in enrichment of neoantigen-reactive T cells facilitating the isolation of neoantigen-reactive TCRs for later transduction of peripheral blood lymphocytes (118). The authors thus presented a feasible method to generate functional and effective neoantigen-reactive T cells for future adoptive cell transfer immunotherpies.

This group also proposed the TCR transfer into peripheral blood lymphocytes by electroporation of sleeping-beauty transposons encoding patient-derived TCRs reactive against particular neoantigens. In a murine context, these T cells harboring TCR encoding transposons were able to rapidly expand and to mount polyfunctional responses against the cognate neoantigens suggesting sleeping beauty mediated transposition of mutation-specific TCRs as a suitable method to generate personalized adoptive T cell therapies (119).

A limiting factor of neoantigen-directed immunotherapy appears to be fact that only a minority of predicted neoepitopes is recognized by autologous TILs. To address this bottelneck, Strønen et al. suggested strategies to complement the spectrum of T cell responses in individual patients using the TCR repertoire of healthy donors. In these heterologous T cell repertoires they discovered neoantigen-recognizing T cells responding to predicted neoepitopes in tumor patients that were neglected by the patients autologous T cell repertoire. T cells redirected with the TCRs from donor-derived T cells were then able to effectively recognize the patient-derived melanoma cells (120).

A generally attractive method to redirect T cells to cancer cells is the use of chimeric antigen receptors (CARs). In CARs, the ligand for the molecular target is usually a single chain variable fragment (scFv) derived from a target-binding antibody. The use of CARs circumvents some problems associated with the use of TCR transfer such as mixed chimerism, unwanted off-target specificities and MHC downregulation in target cells. Posey et al. have developed a CAR that recognizes the tumor-specific glycoform of MUC1, a TSA already described in the introduction (121). Anti-MUC1 CAR T cells demonstrated effective cytotoxicity and tumor growth control in xenograft models of leukemia and pancreatic cancer. However, the glycosylated form of MUC1 is present in various cancers and is therefore not fully representative of mutation-derived neoantigens as described in the previous chapters. It remains an open question whether the CAR approach can be reasonably translated into highly personalized immunotherapies targeting mutation-derived neoantigens.

Neoepitope-Directed Vaccination and Current Clinical Trials

Although the history of clinical success of cancer vaccines has so far been rather disappointing, vaccines remain a promising tools for targeted immunotherapy. It is currently unknown whether vaccines with neoantigens are able to augment pre-existing responses in patients which have failed to reject a tumor. It has been shown by Carreno et al. in melanoma patients that a dendritic cell vaccine directed against a number of predicted neoepitopes indeed led to an increase in naturally occurring neoantigen-specific responses. Most importantly, the vaccination was able to induce epitope spreading by triggering *de novo* neoantigen-specific responses with diverse TCR usage (52). These observations showed the clear benefit of vaccinations with regard to the breadth of the immune response and the clonal diversity of neoantigen-directed immunity.

Two recent clinical studies have provided further proof-of-concept to translate neoepitope prediction into personalized cancer vaccine formulations which induce effective tumor responses in patients with advanced cancer (50, 51). Sahin et al. have administered a highly personalized RNA-based vaccine in 13 patients with advanced melanoma. The personalized vaccines were set up by an RNA reflecting five connected 27mer peptides harboring MHC class I and class II neoepitopes with high binding prediction scores. The researchers showed that vaccination led to rapid expansion of neoepitope-specific responses with central and effector memory phenotypes. Vaccine-dependent T cell infiltration and neo-epitope-specific tumor cell killing was confirmed in resected tumor material. With regard to the clinical outcome, the authors observed a reduction in metastases and an objective response in two out of five patients. One of the two responding patients later relapsed due to the loss of β2-microglobulin indicating an adaptive immune escape of the tumor. Strikingly, the authors found a complete response when vaccination was combined with checkpoint inhibition. In a second study, Ott et al. subcutaneously applied a personalized, peptide-based vaccine with polyIC:LC (Hiltonol) in six patients with advanced melanoma (51). Here, up to 20 neoepitopes were selected, based on previous determination of the neoepitope binding affinity to HLA molecules. After vaccination, the patients developed multifunctional CD4 and CD8 T cell responses. Of these patients, four had no recurrence at month 25 after treatment. The two patients with recurrent disease were additionally treated with the PD-1 blocking antibody pembrolizumab and experienced complete regressions while significant expansion of the neoepitope-specific T cell repertoire was observed. Although these findings need to be further corroborated in larger clinical studies, they prove feasibility and safety of this approach and promise excellent synergy when combined with subsequent checkpoint blockade.

RESISTANCE MECHANISMS

The development and systemic spread of cancer represents a failure of both the innate and the adaptive immune system. Immune cells have been shown to control even the earliest events of malignant transformation by induction of senescence in pre-malignant cells in a CD4-dependent manner (16). Due to the constant interaction of malignant and immune cells the tumor undergoes a process called "immunoediting" consisting of the three distinct phases elimination, editing and escape (17, 122). During the elimination phase, innate and adaptive immune cells recognize malignant cells and eliminate them to

prevent the formation of tumors. In case this early elimination fails, tumor cells may enter a state of dormancy in which both systemic spread and complete elimination of the malignant cells is prohibited. Eventually, cancer cells may escape from this equilibrium phase by evading recognition of the immune system, resulting in local formation of cancers, tumor recurrence and eventually systemic spread. In clinically apparent tumors a number of escape mechanisms have been shown that prevent recognition by the immune system.

First, tumors may silence the expression of the recognized antigen. Similar to tumors with only heterogeneous expression of the target antigen, this escape mechanism results in the development of tumor cells devoid of the target antigen. Loss of the target antigen has been convincingly shown in mice and cancer patients undergoing immunotherapy (123, 124). This phenomenon of immune escape is of particular importance in mono- or oligoclonal immune responses and in immunotherapeutic approaches that target bystander mutations. Under these circumstances, cancer cells can easily escape recognition by the T cells without detrimental effects on cancer cell growth by downregulating the expression of target genes if their functions are dispensable for cell viability.

Second, antigen presentation can be negatively affected. Tumors may downregulate the expression of MHC molecules, either by allelic loss or downregulation of protein transcription or translation (125). However, the frequency of MHC-downregulation is difficult to estimate since MHC expression appears to be extremely heterogenous when stained in biopsy material, also when regarding primary tumors and metastases (126, 127). A further reason for reduced antigen presentation could be the loss or reduced expression of genes that are part of the antigen-processing machinery such as the transporter associated with antigen processing (TAP) (128). The impact of these mechanisms of immune evasion has been convincingly shown in cancer patients and may result in the formation of cancer cells that are no longer subject to surveillance by CD4 or CD8 T cells (129). However, these cancer cells can still be targeted by natural killer cells or CAR T cells which are able to recognize cells devoid of MHC molecule expression or can be eliminated indirectly in a processes referred to as bystander killing (130).

As a third escape mechanism tumors may create a local milieu of immunosuppression that prevents the formation of an immunological synapse between cytotoxic T cells and the tumor cells. This may be achieved simply by preventing access of T cells to tumor cells inside the affected organ, for example by extensive proliferation of tumor-associated stromal structures (131). Alternatively, tumors may orchestrate the accumulation of immunosuppressive cell populations (e.g., regulatory T cells and myeloid-derived suppressor cells) into the tumors that negatively affect T cell trafficking, expansion or differentiation of T cells into functional cytotoxic T cells, leading to functional exhaustion and ultimately deletion of cancer-specific T cell clones (132). This resistance mechanism is of particular importance in solid tumors with a strong stromal component which often takes an active part in the suppression of adaptive immune responses (133, 134). Upregulation of PD-1 on T cells has been shown to be one of the phenotypic hallmarks of cancer-induced T cell exhaustion thus laying the foundation for the ground-breaking checkpoint inhibitor studies in patients with solid tumors (135).

CONSIDERATIONS ON DESIGN OF THERAPEUTICALLY EFFICIENT NEOANTIGEN VACCINES

The mechanisms of immune escape described above have to be considered in the design of immunotherapy trials targeting neoantigens. To minimize the chance for the development of escape variants current vaccination trials aim at inducing polyclonal immune responses against multiple epitopes, in some cases up to 20 neoepitopes in a single vaccination (51). However, these epitopes are typically derived from bystander rather than driver mutations due to the limited number of functionally activating somatic mutations in driver genes. The outgrowth of tumor cell clones without MHC surface expression poses a yet unsolved problem to neoantigen-targeting vaccination approaches. One of the most promising strategies to prevent the formation of MHC-negative clones could be to minimize the time for the tumor to adapt to the adaptive immune response by mounting rapid, polyclonal T cell responses ("hit hard and early" strategy). However, this strategy does not take into account that MHC-negative cancer clones could be present even before the vaccination. This has to be considered since MHC downregulation appears to be a rather frequent event in response to tumor immune recognition. If loss of MHC expression represents a stochastic event, reduction of tumor mass before the vaccination or adjuvant vaccinations after tumor resection could represent a possible solution.

FUTURE OUTLOOK

In the past years, immune responses targeting neoantigens have gained considerable attraction due to a number of clinical reports that have demonstrated the potent clinical effect of adaptive immune responses against these TSAs in cancer patients. As summarized above, the impact of neoantigen targeting extends from a predictive role in checkpoint inhibition to convincing clinical effects in individual patients after adoptive cell transfer and culminates in the recent success of personalized neoantigen vaccines in melanoma patients (50, 51). After a series of disappointing vaccination attempts, these results currently spur the hope that the goal of personalized immunotherapy is finally within reach. However, for a broad application of neoantigen-targeting immunotherapies in humans there are still a significant number of obstacles that have to be addressed and solved in the near future. Cancers treated by personalized immunotherapies in the form of adoptive CTL transfer or vaccinations are exposed to a high selection pressure favoring the evolution of escape variants. In fact, some of the very first reports of neoantigen-directed vaccines have already demonstrated a number of resistance mechanisms of solid tumors. As one example, loss of neoantigens with heterogeneous expression inside the treated tumor has been shown to result in the selection of subclones devoid of the target neoantigen (136) by means of chromosomal deletion. In another study, expression of the target was not only reduced by loss of the mutant alleles but

also by a global downregulation of target gene expression (137). Some tumor entities including checkpoint-inhibition refractory pancreatic duct adenocarcinoma may escape neoantigen-targeted vaccination therapy simply by inducing a potent local immunosuppressive milieu (138) that prevents activation of neoantigen-specific T cells. The most frequent escape mechanism, however, has been shown to be the loss of global MHC expression, both after adoptive transfer of T cells and after neoantigen-directed vaccination (139). Loss of MHC class I expression may represent the most challenging escape mechanism resulting in complete abrogation of tumor recognition by cytotoxic T cells.

The evolution of escape mechanisms calls for the careful design of neoantigen-directed immunotherapies to avoid the selection of resistant subclones and to ensure successful vaccination. As an example, tumors characterized by a strong immunosuppressive micromilieu may be treated by combining neoantigen-targeting vaccines with chemotherapeutic regimens that deplete immunosuppressive Tregs or MDSC (e.g., cyclophosphamide and gemcitabine, respectively). To enhance the efficacy of the vaccine and to break local immunotolerance, checkpoint inhibitors have already been used either in combination or after neoantigen vaccination, resulting in complete tumor regression in a number of treated patients (50). The combination of neoantigen vaccines and checkpoint inhibitors seems in many ways ideal since these novel vaccines induce high numbers of tumor-specific T cells whose cytotoxic function can be restored by coadministration of checkpoint inhibitors. To prevent the selection of tumor clones with downregulated target antigen the choice of the neoantigen targets seem critical. In contrast to monoclonal immune responses, polyclonal immune responses against multiple neoantigens have been shown in murine tumor models to reduce the formation of escape variants (140). Ideally, the spectrum of neoantigens is to include driver mutations in genes with essential functions in tumor cell vitality, proliferation or metastasis. However, since driver mutations are not only rare but also rarely immunogenic in the context of a given MHC haplotype these mutations have so far not been used frequently in the context of neoantigen vaccines.

The emergence of tumor subclones devoid of MHC class I expression represents the most challenging resistance mechanism to vaccines so far. Allelic loss of MHC molecules prevents recognition of the tumor cells by CD8 T cells and, in some cases, even CD4 T cells. In contrast, downregulation of MHC molecules can be counteracted by small molecules such as cobimetinib which is currently under clinical investigation in combination with the PD-L1 targeting antibody atezolizumab for the treatment of colorectal cancer (trial number: NCT01988896). A critical question to be considered in the design of vaccines is whether MHC-negative tumor clones are already present at the beginning of the vaccination or if the resistant clones emerge during vaccination. If resistant clones emerge during vaccination, then vaccination should be designed to inflict maximum damage in a short period of time to avoid the equilibrium and the escape phase of the tumor-immune cell interaction. According to this hypothesis, an ideal vaccination regimen would consist of a limited number of vaccinations with a maximized magnitude of the ensuing T cell response ("hit hard and early"). Even in the case that MHC-negative tumor subclones are already present at the beginning of the vaccination, a "hit hard and early" vaccination might have advantages since high magnitude immune responses may favor the influx of natural killer cells which preferentially recognize and eliminate MHC-negative tumor cells. MHC-negative tumor cells should be preferred targets of natural killer cells. Consequently, it should be considered to combine neoantigen-directed immunotherapy with systemic NK cell activators. In preclinical studies, antibodies targeting NK cell checkpoints, such as CD96 have demonstrated the ability to control metastasis (141). In addition to the engagement of natural killer cells, CAR T cells could be used to overcome MHC-restriction and restore sensitivity to immunotherapy. A more simple approach to newly emerging MHC-negative tumor subclones or metastases may be to perform surgical resection whenever possible. Since the risk for the formation of MHC-negative tumor cells may correlate positively with tumor mass. This is consistent with results of mathematic models on targeted therapies which suggest that the likelihood of resistance following targeted therapy is a straight correlate of the number of tumor cells present at therapy start (142). Therefore, the combination of neoantigen vaccines with surgery or alternative cytoreductive means seems to be critical to minimize the risk of resistant tumor cells. The use of vaccines as an adjuvant treatment following surgery seems ideal since removal of the tumor abrogates the tumor-mediated immunosuppression and minimizes the number of post-operative tumor cells and therefore the chance for the survival of MHC-negative clones. In contrast to current chemotherapeutic regimens the combined treatment of surgery and tumor vaccinations may be a valuable option even in patients with advanced/metastatic disease since resistance to vaccinations is frequently a local instead of a systemic challenge. Finally, strategies targeting neoantigens could be of therapeutic value even in neoadjuvant settings. Consistent with this assumption, it has been shown in murine models with resectable tumors that neoadjuvant T cell stimulation using antibodies targeting PD-1 and CD137 was more effective in preventing metastasis compared with the same treatment when applied after tumor resection (143). These results suggest that under certain circumstances the tumor may serve as an important source of immunogenic antigens that can be exploited to induce neoantigen-specific immune responses.

In summary, the advent of novel therapies targeting neoantigens will revolutionize the treatment of cancer patients in the decades to come by fulfilling the promise of a personalized, individual treatment. Vaccinations are ideally suited for combination therapies, particularly in combination with checkpoint inhibitors, but also in combination with surgery, radiation therapy, chemotherapy, and locoregional and locally ablative procedures.

AUTHOR CONTRIBUTIONS

TW and FK planned the manuscript and wrote sections of the manuscript. Both authors read and approved the submitted version of the manuscript.

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Computational Pipeline for the PGV-001 Neoantigen Vaccine Trial

*Alex Rubinsteyn[1], Julia Kodysh[1], Isaac Hodes[1], Sebastien Mondet[1], Bulent Arman Aksoy[1,2], John P. Finnigan[1,3], Nina Bhardwaj[1,3] and Jeffrey Hammerbacher[1,2]**

[1]Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [2]Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC, United States, [3]Icahn School of Medicine at Mount Sinai, Tisch Cancer Institute, New York, NY, United States

This paper describes the sequencing protocol and computational pipeline for the PGV-001 personalized vaccine trial. PGV-001 is a therapeutic peptide vaccine targeting neoantigens identified from patient tumor samples. Peptides are selected by a computational pipeline that identifies mutations from tumor/normal exome sequencing and ranks mutant sequences by a combination of predicted Class I MHC affinity and abundance estimated from tumor RNA. The personalized genomic vaccine (PGV) pipeline is modular and consists of independently usable tools and software libraries. We hope that the functionality of these tools may extend beyond the specifics of the PGV-001 trial and enable other research groups in their own neoantigen investigations.

Keywords: neoantigens, personalized vaccine, immunoinformatics, genomics, computational pipeline

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Sine Reker Hadrup, Technical University of Denmark, Denmark

****Correspondence:***
Jeffrey Hammerbacher
correspondence@hammerlab.org

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INTRODUCTION

Cancer neoantigens are antigens presented on tumor cells which, due to either mutation or modification, are not presented on normal cells. Neoantigens generated by tumor DNA mutations have been shown to play a significant role in mediating the destruction of tumor cells by the adaptive immune system (1–3). Several groups have used therapeutic vaccines targeting neoantigens to clear tumors in murine models (4–6). Consequently, many human neoantigen vaccine trials are now under way and several have published promising early results (7, 8). Since very few cancer mutations are recurrent between patients, the identification of neoantigens requires a personalized genomic approach (9). We describe the sequencing protocol and immunogenomic pipeline of PGV-001, a neoantigen vaccine trial at the Mount Sinai Hospital (10).

The personalized genomic vaccine (PGV) computational pipeline takes tumor/normal sequencing data as an input and generates a ranked list of mutated peptide sequences. The steps along the way of determining a personalized vaccine's contents are implemented as configurable independent tools.

OVERVIEW OF THE PGV-001 PERSONALIZED VACCINE TRIAL

PGV-001 is a phase I clinical trial at Mount Sinai Hospital, studying the safety and immunogenicity of a multipeptide personalized genomic vaccine for the treatment of cancers. A PGV dose consists of 10 synthetic long peptides (11), each containing a somatic mutation from the patient's tumor, as well as an immunostimulatory adjuvant: poly-ICLC (12). In the PGV-001 trial, the personalized vaccine is administered in the adjuvant setting, for patients who undergo a complete resection and have no evidence of residual disease.

When a new patient enrolls in the trial, their tumor and normal samples are collected and processed to isolate and sequence DNA and RNA. The computational pipeline of PGV-001 is then used to select the peptide contents of the vaccine. The major steps between surgery and vaccination are shown in **Figure 1**, whereas details of the computational pipeline are shown in **Figure 2**.

The candidate vaccine peptides generated by the computational pipeline are ranked by abundance and predicted MHC affinity, which both contribute to immunogenicity. The manufacturer attempts to synthesize the top 15 ranked candidate peptide sequences and delivers 10 lyophilized peptides which they are able to purify to sufficient quality and quantity. The peptides are dissolved in DMSO and mixed with poly-ICLC immediately before use. The personalized vaccine is administered as an intracutaneous injection and is given to the patient 10 times over a span of 6 months.

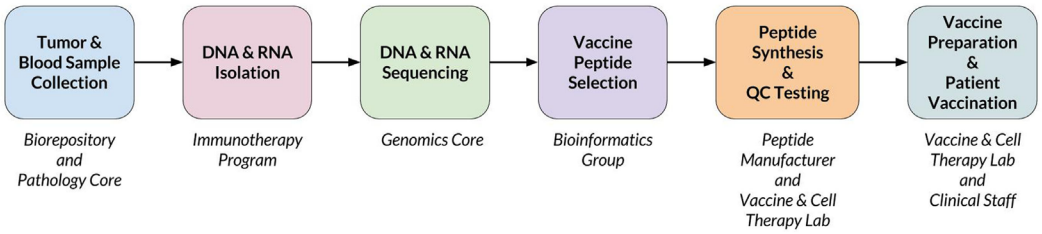

FIGURE 1 | Overview of PGV-001 trial.

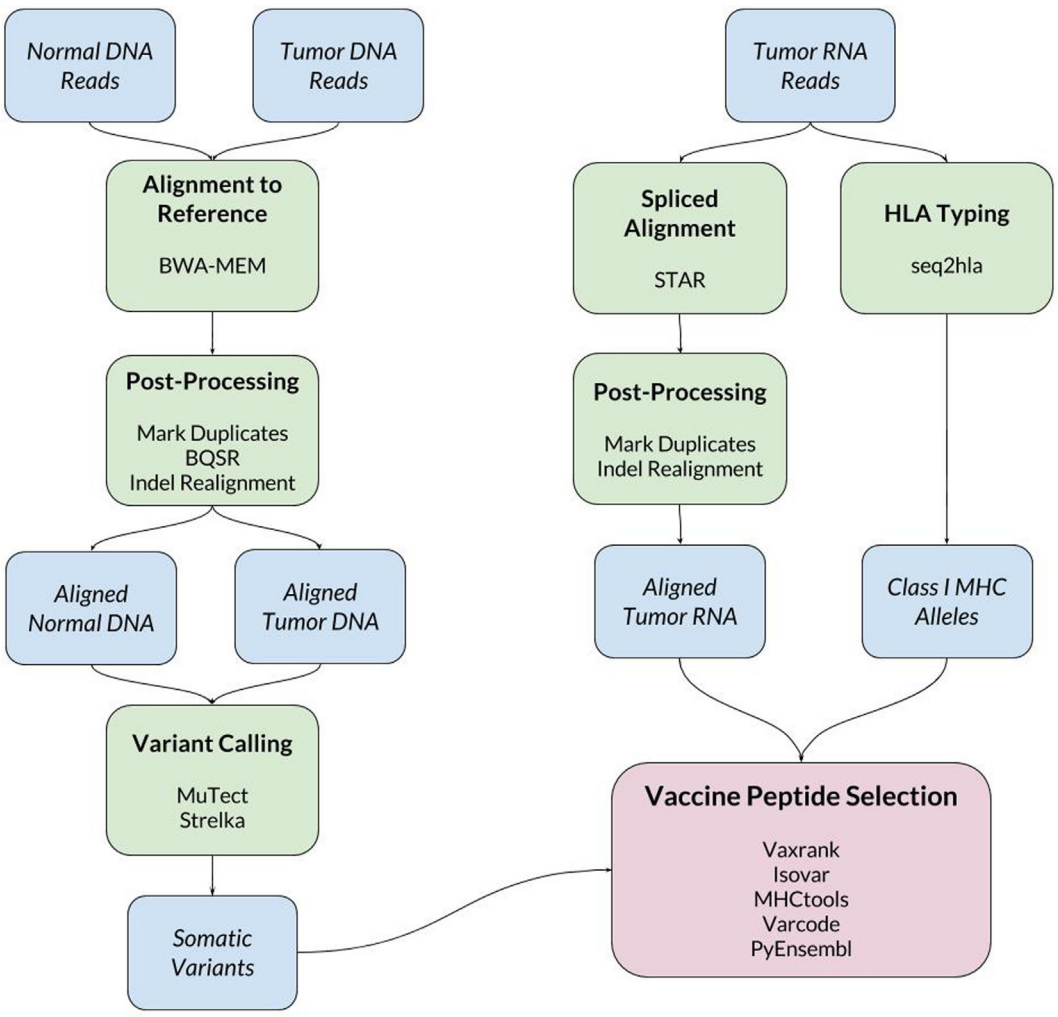

FIGURE 2 | Schematic of bioinformatics tools used in PGV-001 pipeline.

SEQUENCING PROTOCOL FOR DNA AND RNA

The sequencing protocols used for both DNA and RNA can dramatically affect the sensitivity of variant detection, and thus ultimately change the results of the vaccine pipeline. The largest determinants of sensitivity are the sample quality, method of sequencing library preparation, and quantity of sequenced reads. Whenever possible, PGV uses fresh frozen tumor tissue samples, which results in significantly improved variant detection accuracy as compared with sequencing of formalin-fixed (FFPE) samples (13). An additional benefit of using fresh frozen samples is that mRNA can be enriched using poly-A capture, whereas the fragmented RNA of FFPE samples can only be prepared with less efficient methods such as ribosomal depletion (14). For patients with solid tumors, normal DNA is extracted from peripheral blood rather than potentially contaminated adjacent tissue (15).

Fragmentation by sonication was preferred to transposase-based methods (16) due to significant sequence bias, leading to lost coverage after marking duplicate reads (17). Among the exome enrichment techniques which use sonication, we chose Agilent's SureSelect XT kit due to its efficient rate of capturing on-target reads (18).

We chose to target 150× mean coverage for the normal DNA (exome) sequencing since this was found to be the point of diminishing sensitivity across different variant calling pipelines (19). Several of the cancer types allowed in the PGV-001 trial (particularly lung and head/neck cancers) have been shown to result in systematically low purity samples (20). To accommodate a significant degree of non-cancerous DNA, we assume 50% tumor purity and consequently target 300× exome coverage for the tumor DNA sample.

A final consideration is the choice of read length, which does not significantly impact variant discovery from DNA but does impact variant phasing in RNA. Since a 25mer vaccine peptide is translated from 75 bp of coding sequence, PGV could theoretically use any read length above that minimum. To allow for many distinct aligned positions overlapping the same region of coding sequence, the PGV protocol uses 125-bp reads. These provide a good compromise between length and base quality on the HiSeq 2500 instrument.

OVERVIEW OF THE COMPUTATIONAL PIPELINE

The inputs to the computational pipeline are unmapped sequencing data from tumor DNA, tumor RNA, and normal patient DNA. The tumor and normal DNA samples are aligned against the human GRCh37 reference genome using BWA-MEM (21). The tumor RNA is aligned using STAR (22), which has been found to have particularly high sensitivity for detecting indel variants (23). Both DNA and RNA alignment files are processed according to GATK Best Practices (24). One noteworthy deviation from the standard GATK pipeline is our use of assembly based indel realignment on tumor RNA data (in addition to the DNA samples). This is done to improve the sensitivity of detecting RNA reads which support indel somatic variants.

Somatic Variant Calling

Somatic variant calling is performed using MuTect (25) and Strelka (26), whose results are combined by taking a union of called variants. In cases where the final pipeline yields an insufficient number of vaccine peptides (fewer than 15), we rerun the pipeline including MuTect2 among the set of variant callers to increase sensitivity.

HLA Typing

To make predictions about epitope presentation to T-cells, it is necessary to know the patient's HLA type. This can be determined computationally from exome or bulk RNA sequencing or validated externally using HLA-specific amplicon sequencing (27). The PGV pipeline currently uses seq2hla (28) for HLA typing from tumor RNA while also using amplicon sequencing of normal DNA for validation. Across 10 patients, the two methods have only disagreed on a single allele, where HLA-C*07:02 was mistyped as HLA-C*07:01. This high degree of concordance matches our previous experience with HLA typing of fresh frozen tissue samples; formalin-fixed tissue is more likely to give discordant results between different sequencing methods.

Vaccine Peptide Selection

The bulk of the custom software developed for this trial is related to vaccine peptide selection. The results of the above steps are a set of somatic variants, aligned tumor RNA reads, and the patient's HLA type. These data are then used to determine mutant protein sequences, estimate mutation abundance, predict MHC ligands overlapping mutations, and finally to generate a ranked list of candidate vaccine peptides.

Some of the tools used in vaccine peptide selection include:

- *Vaxrank* (29): overall vaccine selection tool with ranking logic.
- *Isovar* (30): determines mutant protein sequence from somatic variants and tumor RNA.
- *Varcode* (31): predicts variant effects for filtering out silent mutations.
- *PyEnsembl* (32): provides reference genome annotations that are used by Varcode to determine exon boundaries and transcript sequences.
- *MHCtools* (33): common interface to peptide–MHC-binding predictors.

Due to their importance, Isovar and Vaxrank are both described in greater detail in the following two sections.

ISOVAR: DETERMINING THE MUTANT PROTEIN SEQUENCE

There are several different software packages that predict the protein-level effect of a coding mutation (31, 34, 35). However, for the purposes of selecting a vaccine peptide's sequence, it is not sufficient to predict a DNA mutation's protein effect without considering the transcripts in which it occurs. A

somatic mutation can be associated with selective splicing of particular RNA isoforms (36) and can also cooccur with other genomic variants. Thus, in the PGV pipeline, the tumor RNA sequencing data are also used to determine a mutant coding sequence.

For each mutation, it is possible to infer multiple coding sequences from supporting RNA reads due to sequencing error, splicing diversity, and tumor heterogeneity. To account for these potentially complicating factors, we developed a tool called Isovar (30), which can be downloaded from https://github.com/hammerlab/isovar. Isovar uses RNA to assemble the most abundant coding sequence for each mutation. An overview of the algorithm is given in **Figure 3**.

One advantage of using RNA to determine the coding sequence is that it phases adjacent (germline or somatic) variants. Examples of the impact of adjacent variants on a coding sequence are shown in **Figures 4** and **5**. A further advantage is that Isovar, by using mutation-supporting RNA reads to determine each mutant protein sequence, naturally estimates allele-specific expression. If the PGV pipeline, on the other hand, used bulk expression it would potentially overestimate how much of a mutant protein is being made. In an extreme case, all of the RNA reads aligning to a particular gene could be wild type, with none supporting the somatic variant of interest.

```
function MutantProteinSequences(v)
    Reads(v) = tumor RNA reads which contain somatic variant v
    Seqs(v) = longer sequences assembled from Reads(v)
    Transcripts(v) = reference transcripts overlapping variant v
    for s in Seqs(v) do
        for t in Transcripts(v) do
            if s matches t then
                i = offset to first codon in matching portion of t
                p = translate s_{i:} into amino acid sequence
                yield p
```

FIGURE 3 | Overview of Isovar algorithm for determining mutant protein sequences.

VAXRANK: VACCINE PEPTIDE SELECTION

Once we have determined the amino acid sequences containing somatic mutations and estimated their abundance in the tumor, the final step is to rank them according to desirability of inclusion in personalized vaccine.

There are many potential correlates of immunogenicity that can be used to prioritize neoantigens, such as expression, MHC-binding affinity, peptide–MHC complex stability, proteasomal cleavage, and other antigen-processing steps. Of those, the PGV pipeline optimizes for high expression and predicted strong Class I MHC binding. There are several published computational predictors of Class I MHC-binding affinity which have demonstrated high accuracy (37–39). PGV uses NetMHCpan (37) due to its extensive coverage of patient alleles.

The final ranking of candidate vaccine peptides according to predicted MHC binding and expression is performed by a tool called Vaxrank (29). Vaxrank identifies high-affinity mutant MHC ligands within each peptide and combines these predictions into a single MHC-binding score. This score is then scaled according to the expression of that mutation in the tumor. The formula for computing these MHC and expression scores is given in **Figure 6**. The scale and offset for MHC affinity normalization was determined by a logistic fit of affinity versus immunogenicity from the dataset used to determine the classical 500-nM affinity threshold (40). There is no rigorous justification for the multiplicative scoring function, other than the intuition that epitope abundance and MHC affinity are independent prerequisites for immunogenicity.

Since some peptides cannot be manufactured using solid-phase synthesis, our vaccine peptide selection algorithm includes manufacturability heuristics, such as minimization of cysteine content.

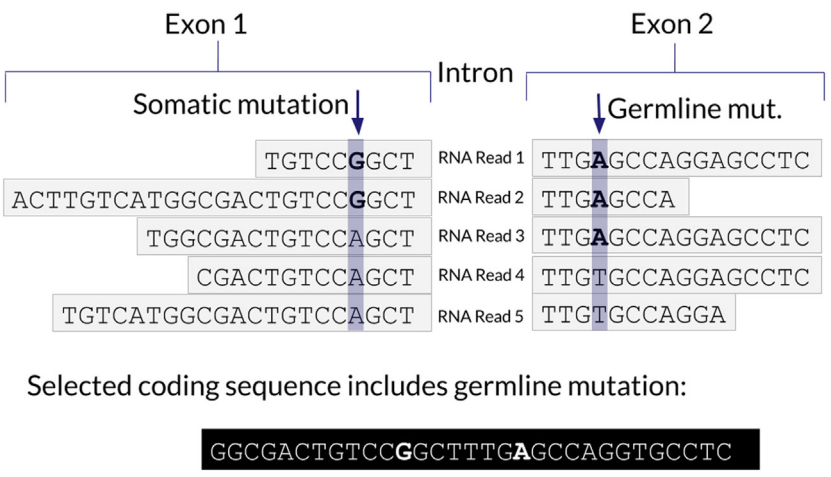

FIGURE 4 | Schematic representation of a somatic mutation co-occurring with a germline mutation.

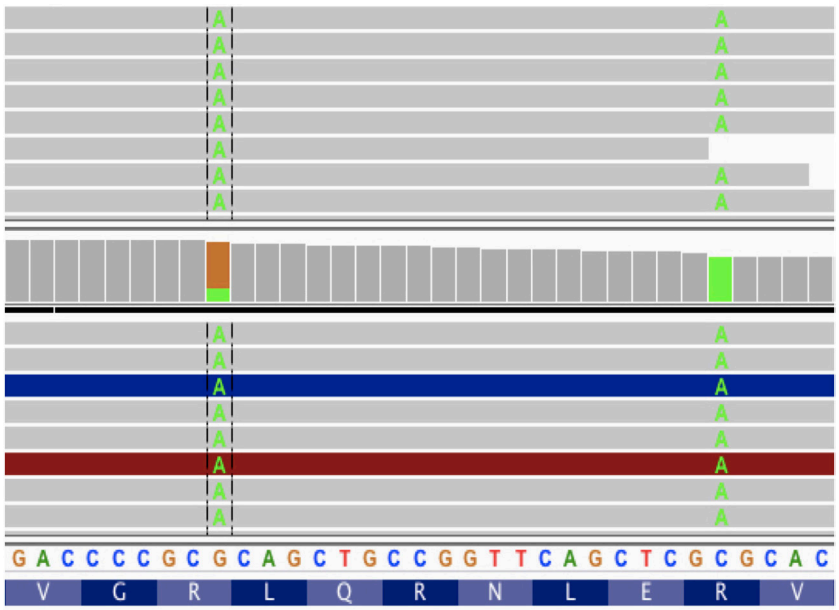

FIGURE 5 | Screenshot from IGV with tumor DNA on top and tumor RNA on bottom. The two somatic variants from patient data 7 amino acids apart. If these mutations were considered without phasing, we would get two different vaccine peptides, neither of which would match the protein sequence produced by tumor cells.

$$\text{TotalScore} = \text{ExpressionScore} \cdot \text{BindingScore}$$

$$\text{ExpressionScore} = \sqrt{\#\text{ supporting reads}}$$

$$\text{BindingScore} = \sum_{p}^{\text{mutant peptides}} \sum_{mhc}^{\text{alleles}} \sigma(\text{IC50}(p, mhc))$$

$$\sigma(x) = \exp(-\frac{x - 150}{350})$$

FIGURE 6 | TotalScore used to rank somatic variants in a way that attempts to balance predict MHC binding and abundance. ExpressionScore uses read count (instead of a normalized measure like FPKM) since these scoring criteria are not meant to be compared between patient samples. BindingScore sums normalized binding affinities of mutant peptides across all patient alleles and lengths between 8 and 11.

Vaxrank can be downloaded from https://github.com/hammerlab/vaxrank.

EPIDISCO: PARALLEL IMPLEMENTATION OF THE PGV PIPELINE

The workflow management tool that orchestrates the execution of the PGV pipeline is called Epidisco (41). Epidisco is used to set up a local or cluster compute environment, install all relevant bioinformatics tools (external software such as GATK, as well as our own tools including Isovar and Vaxrank), and coordinate the execution of these different tools on the input data.

Epidisco accelerates portions of the genomics pipeline on two levels. Independent computational tasks such as QC checks, the processing of the RNA-sequencing data, and the joint analyses of the normal-tumor DNA-sequencing data are all run in parallel. Within the invocation of each tool, when possible, sequencing data are split into multiple segments, partial results are computed in parallel and then merged.

Epidisco supports local computation, traditional HPC schedulers such as LSF, and cloud-based resources from Google Cloud and AWS. On a typical machine, running the complete PGV pipeline for a single patient can take up to 4 days; but making use of five or more computers for parallelization reduces the overall running time down to a single day.

Epidisco also makes the PGV pipeline tolerant to failures of intermediate steps and allows resuming the pipeline from the point of failure with a simple restart request. By handling such failures in an automated way, carrying out cleaning procedures, and restarting only the tasks that need to be rerun, the workflow makes it easier for researchers to operate such complex computational tasks. Epidisco provides command line and web-based utilities to facilitate starting a new workflow, collecting the results, and troubleshooting specific parts of a pipeline.

The individual infrastructure tools used by the PGV pipeline are implemented as an OCaml stack and include:

- *Ketrew*: custom workflow manager who handles dependency management, parallelization, and smart restarts of failed tasks.
- *Biokepi*: wraps bioinformatics tools so they can be used with Ketrew and statically ensures the absence of common mistakes during pipeline construction.
- *Secotrec*: cluster management tool that allows deployment on cloud services such as the Google Cloud Platform and Amazon Web Services.
- *Epidisco*: the actual implementation of the PGV-001 pipeline.

DISCUSSION

The PGV pipeline is a modular, highly configurable, freely available method for selecting the contents of a therapeutic neoantigen vaccine. The PGV pipeline has been used to predict vaccine peptides for several mouse models (LLC, B16 F1/F10), five "dry run" patients whose samples were processed according to the PGV protocol but did not participate in the trial, and five patients who were being considered for enrollment in the trial. Of the patients eligible for enrollment, one has been treated so far and another enrolled. The remainder did not enroll due to progression of disease or low-quality tumor samples.

Several other groups have released pipelines for neoantigen vaccine prediction, most notably pVAC-seq (42) and MuPeXI (43). A deep comparison between neoantigen pipelines likely requires evaluating T-cell response and antitumor activity after vaccination, which is beyond the scope of this paper. There are, however, a few obvious differences between the PGV pipeline and others which have been published:

- *Modularity*: the PGV pipeline has been developed as a collection of flexible standalone tools, rather than a single monolithic script. These tools can be repurposed for other immunogenomics analyses and have already been used for retrospective analyses of checkpoint blockade clinical trials (44).
- *Inputs are FASTQ files*: MuPeXI and pVAC-seq both require the implementation of separate genomics pipeline to infer patient HLA type, call somatic variants, and quantify expression. The PGV pipeline, by contrast, is self-contained in the sense that its inputs are raw FASTQ files and its outputs are vaccine peptide predictions.
- *Dependence on tumor RNA*: the PGV pipeline relies on tumor RNA reads to determine the mutant protein coding sequence. MuPeXI and pVAC-seq, by contrast, only consider expression data after predicting a mutant protein sequence from a variant in isolation. PGV's approach has potential benefits in capturing altered patterns of splicing and phasing somatic variants with other nearby variants. These potential benefits, however, have yet to be evaluated systematically.
- *Liberal software license*: all of the software components that comprise the PGV pipeline are freely available under the Apache software license. MuPeXI does not yet appear to have a fully open source license, while pVAC-seq uses the more restrictive non-profit open software license.
- *Optimization of peptide sequence for solid-phase synthesis*: PGV appears to be unique among freely available neoantigen pipelines in attempting to choose peptides whose sequence content is more likely to be successfully manufactured. We have found this to be an important step, especially when using longer peptides, due to the significant delays introduced by failed synthesis or purification attempts.

The PGV-001 trial is the first in a series of planned neoantigen vaccine investigations. Several improvements to the PGV pipeline are planned, including the use of genomic fusions and other structural variants as neoantigen sources, clonality as a consideration for variant prioritization, and additional immunological predictions such as proteasomal cleavage and Class II MHC binding. As immune response data from ongoing preclinical work and PGV-001 becomes available, our method for combining correlates of immunogenicity into a single ranking will require extensive evaluation.

AUTHOR CONTRIBUTIONS

The first draft of this paper was originally written by IH and substantially revised by AR and JK. Genomics and immunoinformatics tools were implemented by AR, JK, and other lab members. The workflow libraries were written mostly by SM. The actual pipeline (connecting all the individual tools and running them in parallel) was written mostly by IH with help from BA. The overall design of the software was determined through extensive conversation with JH. The PGV trial was designed by NB, JF, AR, and several others in the Bhardwaj lab.

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Mutation-Derived Neoantigens for Cancer Immunotherapy

John C. Castle, Mohamed Uduman, Simarjot Pabla, Robert B. Stein and Jennifer S. Buell**

Agenus Inc., Lexington, MA, United States

Mutation-derived neoantigens distinguish tumor from normal cells. T cells can sense the HLA-presented mutations, recognize tumor cells as non-self and destroy them. Therapeutically, immunotherapy antibodies can increase the virulence of the immune system by increasing T-cell cytotoxicity targeted toward neoantigens. Neoantigen vaccines act through antigen-presenting cells, such as dendritic cells, to activate patient-endogenous T cells that recognize vaccine-encoded mutations. Infusion of mutation-targeting T cells by adoptive cell therapy (ACT) directly increases the number and frequency of cytotoxic T cells recognizing and killing tumor cells. At the same time, publicly-funded consortia have profiled tumor genomes across many indications, identifying mutations in each tumor. For example, we find basal and HER2 positive tumors contain more mutated proteins and more TP53 mutations than luminal A/B breast tumors. HPV negative tumors have more mutated proteins than HPV positive head and neck tumors and in agreement with the hypothesis that HPV activity interferes with p53 activity, only 14% of the HPV positive mutations have TP53 mutations vs. 86% of the HPV negative tumors. Lung adenocarcinomas in smokers have over four times more mutated proteins relative to those in never smokers (median 248 vs. 61, respectively). With an eye toward immunotherapy applications, we review the spectrum of mutations in multiple indications, show variations in indication sub-types, and examine intra- and inter-indication prevalence of re-occurring mutation neoantigens that could be used for warehouse vaccines and ACT.

Keywords: cancer, mutations, neoantigens, immunotherapy, therapeutic vaccine, TCR

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Nejat K. Egilmez, University of Louisville, United States

****Correspondence:***
John C. Castle
john.castle@agenusbio.com
Jennifer S. Buell
jennifer.buell@agenusbio.com

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INTRODUCTION

Cells of the immune system recognize and lyse tumor cells. Mutation neoantigens are critical for tumor control: T cells recognize mutant peptides bound to MHC alleles on tumor cells both in mice and humans (1) and tumor mutational burden (TMB) predicts tumor response to anti-CTLA4 (2) and anti-PD1 treatment (3). Tumors that become resistant to pembrolizumab, an anti-PD1-therapy immunotherapy, often contain mutations in immune-related genes, including in interferon-receptor–associated Janus kinases and the antigen-presenting protein beta-2-microglobulin, suggesting that anti-PD1 therapeutic activity is mediated through neoantigen presentation and recognition (4). Individualized vaccination using autologous tumor lysate (5) or autologous tumor-derived heat shock protein-peptide complexes (6) imparted tumor-specific T-cell responses and vaccination with synthetic mutation-encoding peptides or nucleic acids imparted mutation-specific responses in humans and mice (7–9). T cells have been discovered that recognize mutant KRAS

neoantigens; transfer of these T cells led to tumor regression in multiple lesions followed by escape in a separate lesion after genetic deletion of the peptide presenting HLA locus (10). Together, these demonstrate that neoantigens encoding mutations can mediate the tumor-focused immune response and can be exploited as an exquisitely tumor-specific therapeutic target.

The Cancer Genome Atlas (TCGA) is a comprehensive effort to understand the molecular basis of cancer (11). TCGA member organizations have profiled hundreds of individual tumors in each of many indications, including the identification of somatic mutations present in each tumor. The mutation profiles are available for public download for further analysis. In addition to analysis of individual tumors, intra- and inter-indication analyses pinpoint re-occurring mutations (12). Mutation frequencies can be divided into sub-populations and the immunogenicity of each mutation can be predicted (13–16). Here, we examine mutations in cancer populations, compare subgroups such as smokers and non-smokers, identify re-occurring mutations, and predict HLA binding of mutation-containing peptides.

MATERIALS AND METHODS

TCGA datasets: protein mutations, gene expression, and medical annotation including lung cancer smoking status, head and neck cancer HPV status, colorectal microsatellite status and breast cancer PAM-50 assignment were downloaded from the UCSC Cancer Genomics Browser (17) on April 24, 2015. TCGA tumor mutations were downloaded from the GDC Data Portal (https://portal.gdc.cancer.gov/) on May 3, 2017. Missense mutations were mapped to human reference genome GRCh37 and filtered for those mutations present in at least two tumor samples.

The number of samples for each type: AML (197), thyroid (427), renal chromophobe (66), ovarian (142), breast (982), prostate (425), lower grade glioma (527), glioblastoma (291), renal papillary cell (113), uterine carcinosarcoma (57), renal clear cell (213), colon (224), rectum (81), endometrial (194), stomach (91), head and neck (509), cervical (40), bladder (238), pancreatic (144), lung adenocarcinoma (543), lung squamous cell (178), and melanoma (369).

HLA affinity calculation: for each mutation, we calculated the binding of all possible 8, 9, and 10 amino acid mutation-containing peptides to 23 common HLA alleles using NetMHCpan version 3.0 (14).

RESULTS

Tumor mutational burden by indication: rather than examine mutation rates (18), **Figure 1** shows the TMB as the number of proteins with non-synonymous point mutations in a tumor, grouped by cancer indication, along with the indication-specific median. As expected, AML and thyroid tumors have few mutated proteins, with medians of 9 and 16, respectively. Melanoma and lung cancers have the most, with medians of 276, 244, and 197, respectively.

The intra-indication TMB burden varies considerably. The pancreatic tumors profiled in this dataset, for example, contain at least 30 mutated proteins. Conversely, there are melanoma, lung adenocarcinoma, stomach, and head and neck tumors with very few mutations. From the organs known to develop microsatellite instability (MSI) related tumors, including colon, stomach, and endometrial (19), are many tumors with extremely high numbers of mutations. Other indications with high median mutational

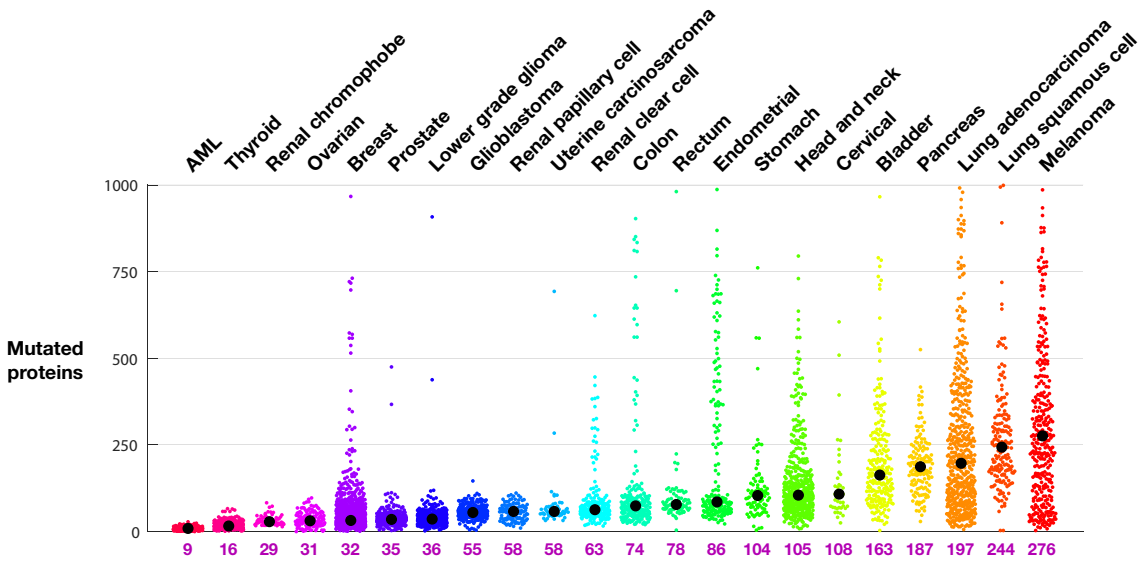

FIGURE 1 | Tumor mutational burden (TMB) in each tumor from TCGA profiles. Tumors are grouped according to indication. Each colored dot represents one tumor. The indication median is indicated by a black circle and listed below the plot. TMB is defined as the number of proteins with a non-synonymous mutation.

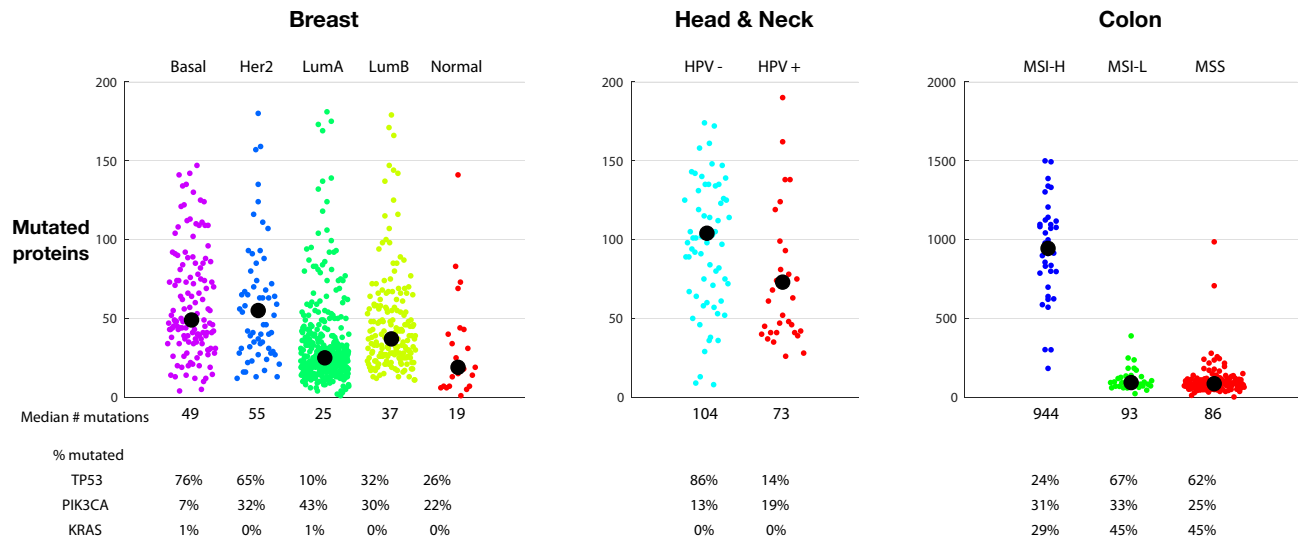

FIGURE 2 | Tumor mutations burden (TMB) in sub-classes of breast tumors **(Left)**, head and neck tumors **(Middle)**, and colon tumors **(Right)**. Each colored dot represents one tumor. Medians are listed and indicated by a black circle. The percentage of tumors with TP53, PIK3CA, and KRAS mutations is listed. The vertical scale of the colon tumor plot is different from the scale in the breast and head and neck tumor plots. TMB is defined as the number of proteins with a non-synonymous mutation.

burden show long tails (populations of tumors with many mutations), in particular melanoma and lung adenocarcinoma, but also lung squamous, bladder, and head and neck tumors. While MSI tumors are uncommon in breast cancer (20), there is curiously a clear population of breast tumors with significantly more mutations.

Figure 2 shows tumor mutations from three indications, each divided into subclasses. Breast tumors can be subdivided into five categories using TCGA gene expression to assign each tumor to the PAM-50 classes: basal, HER2 positive, luminal A, luminal B, and normal (21). The median and spread of mutations across each class varies considerably. Basal tumors, a class highly overlapping with the triple negative classification, and HER2 positive tumors have the highest median number of mutated proteins, 49 and 55, respectively, and a similar broad distribution extending to almost 200 mutations. Normal breast tumors have the lowest median, 19 mutations, while luminal A tumors show a tight symmetrical distribution around the median, 25 mutations. Interestingly, the percentage of tumors containing p53 mutations roughly tracks the median number of mutations in each class, highest in basal and HER2 positive tumors and lowest in luminal A tumors. The percentage of tumors with PIK3CA tumors is by far lowest in the basal group and highest in the luminal A group.

Head and neck tumors comprise a large tumor class, including tumors in the oropharyngeal, oral cavity, oropharynx, hypopharynx, and larynx. Some head and neck tumors, particularly oropharyngeal tumors located in the tonsils and base of the tongue, are caused by HPV infection (22). HPV16 virus can integrate into the genome, and the resulting HPV16 E6 and E7 proteins interfere with activity of endogenous p53 and RB1 protein activity, respectively. **Figure 2**, middle, clearly shows a marked difference in mutation number: HPV negative tumors have, on average, more mutations than HPV positive tumors: 104 vs. 73 median proteins with non-synonymous point mutations. In agreement with the hypothesis that HPV activity interferes with p53 activity, only 14% of the HPV positive mutations have p53 mutations, vs. 86% of the HPV negative tumors. This suggests that the presence of HPV removes the need to mutate p53.

Figure 2, right, shows colon tumors sub-classified into microsatellite instability high (MSI-H), microsatellite instability low (MSI-L), and microsatellite stable (MSS) classes. Endometrial and stomach tumor sub-classes have similar distributions (not shown). As expected, the MSI-H tumors have a much higher mutation number than MSI-L and MSS tumors. In colon tumors, the MSI-H tumors contain a median of 944 mutations vs. 93 and 86 in the MSI-L and MSS tumors, respectively. The number of tumors with PIK3CA mutations is similar across the three sub-groups. However, similar to the HPV positive tumors, the percentage of MSI-H tumors with p53 mutations is much lower, here suggesting that the MSI-H status lessens the need of p53 mutations for oncogenesis. Likewise, the frequency of KRAS mutations is lower in the MSI-H group, suggesting that the MSI-H phenotype also lessens the need for the KRAS driver activity.

Figure 3, left, examines the relationship between TMB and smoking in lung adenocarcinoma as reported in the TCGA dataset. There is a dramatic and clear correlation between smoking and TMB. Tumors from never-smokers have a median of 61 mutated proteins; tumors from patients who stopped smoking over 15 years ago have a median of 166, tumors from patients who stopped smoking <15 years ago have a median of 212, and tumors from current smokers have a median of 248 mutated proteins. Recent clinical trials have shown increased

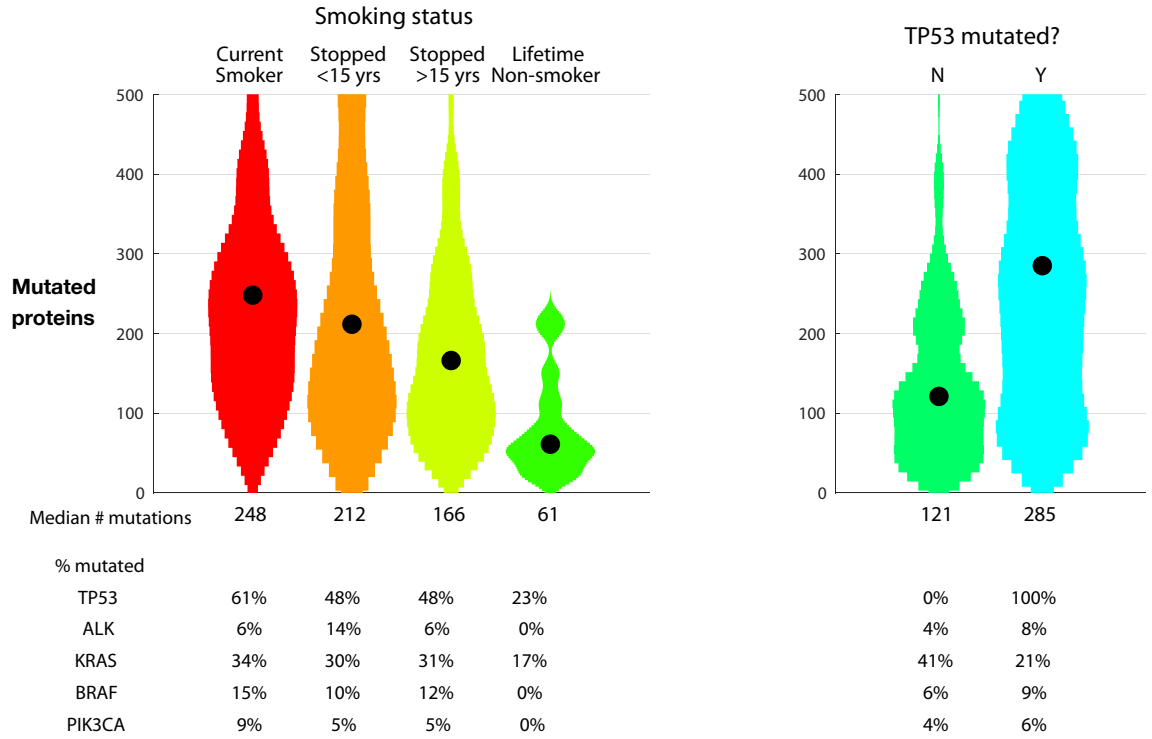

FIGURE 3 | Tumor mutational burden (TMB) in lung adenocarcinoma. Plots show distributions as violin plots with medians indicated as a point. **(Left)** TMB vs. smoking status. **(Right)** TMB vs. TP53 mutational status. TMB is defined as the number of proteins with a non-synonymous mutation.

benefit of anti-PD1 and anti-CTLA4 antibodies for the treatment of non-small-cell lung cancer (NSCLC) tumors with high TMB, defined as tumors with >10 mutations per megabase or as tumors with great than a median of 158 mutations (23, 24). In the TCGA dataset, these cutoffs eliminate most never-smokers, enriching for current and recent smokers.

The percentage of tumors with TP53 protein mutations is almost three-fold higher in current smokers than in never-smokers, 61% vs. 23%, respectively. Further examining the association of TP53 mutations, **Figure 3**, right, shows the relationship between TP53 mutations and TMB. The tumors with TP53 mutations have over twice the number of mutated proteins compared to tumors with non-mutated TP53, 285 vs. 121 mutated proteins, respectively. Conversely, the rate of mutated KRAS is almost twice as large in the TP53 non-mutated tumors, 41% vs. 21%, respectively. This occurs despite the lowest rate of mutated KRAS in never-smoker tumors, which have lower TMB and TP53 mutation rates. This suggests that oncogenic dependency on TP53 and KRAS mutations is orthogonal.

For a warehouse ("off-the-shelf" or "prêt-à-porter") approach for mutation-targeting neoantigen vaccines and TCRs, the mutation frequency is critical. The more frequent a mutation occurs, intra- and inter-indication, the larger the candidate patient population. Further, as immune recognition is dependent in part on presentation of the mutation by patient HLA alleles, identifying which HLA molecules bind the mutation neoantigen predicts the patient population. While, fascinatingly, there are re-occurring synonymous mutations (25), we focus here on common non-synonymous point mutations. **Figure 4** shows non-synonymous mutations found in at least 10% of the samples in any one indication.

HLA allele B08, for example, is predicted to strongly bind (<10 nM) the peptide containing mutation NBPF10 p.E3455K, a mutation found in uterine carcinosarcoma and prostate tumors. Allele B15 is predicted to strongly bind mutation TVP23C p.C51Y, a frequent mutation in renal papillary cell tumors. Most of the re-occurring mutations are predicted to bind one or more common HLA allele with binding affinity 500 nM or stronger, suggesting candidate patient subsets for investigation of each re-occurring mutation.

Some mutations are found in a specific indication. The IDH1 p.R132H mutation is found primarily in lower grade glioma, and found in 42% of these tumors. Other mutations, such as PIK3CA p.E545K, KRAS p.G12D, and KRAS p.G12V, occur in many indications. We examined the cumulative sum of the five most frequent mutations within an indication (**Figure 5**), as important for warehouse approaches. With exceptions, hot-spot mutations typically do not co-occur in one tumor clone; thus, here we assumed that mutations occur independently. When ranked from most to less frequent, the most common mutations in an indication occur in 50% (thyroid) to <1% (renal clear cell) of the tumors. Of the indications considered here, only thyroid, melanoma, pancreatic, and lower grade glioma tumors have a mutation found in more than 20%

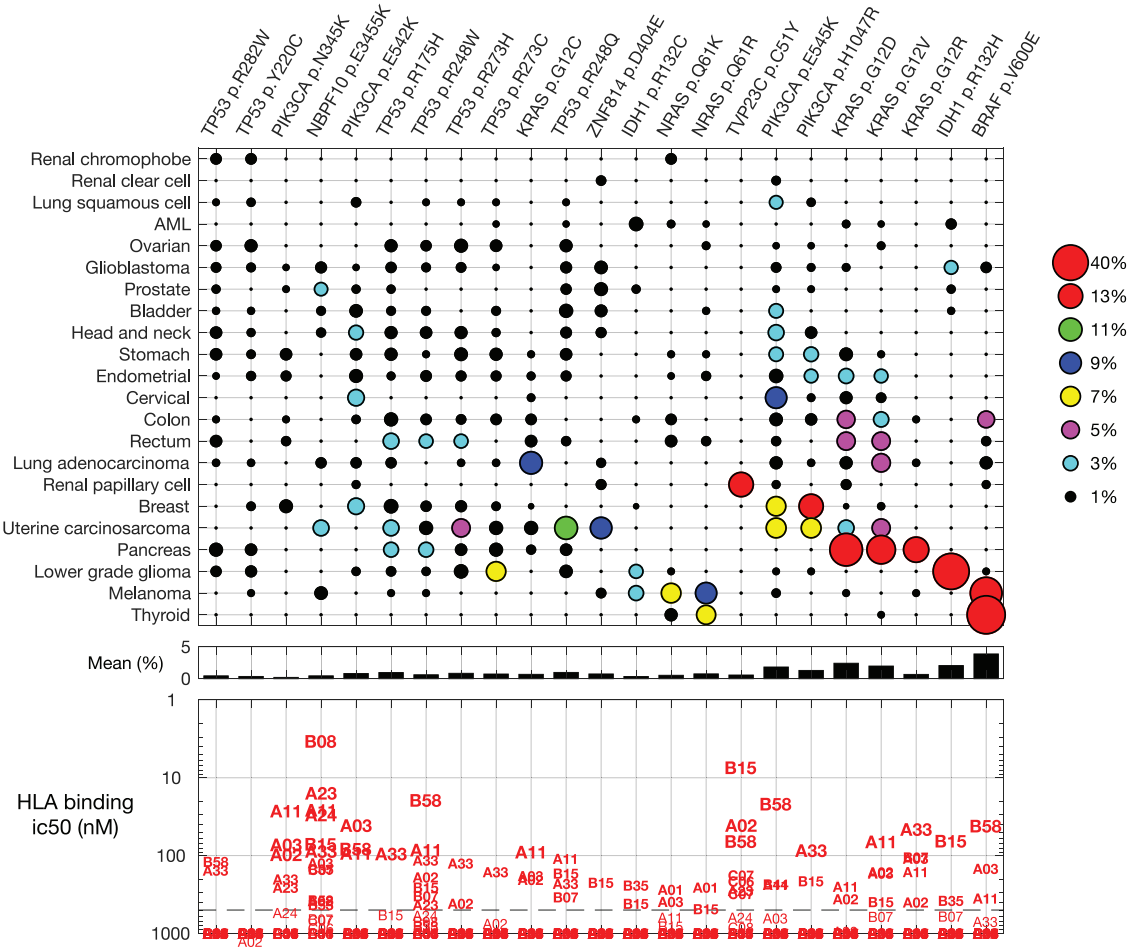

FIGURE 4 | Common mutations across all indications and their predicted HLA binding. **(Top)** Non-synonymous mutations found in at least 10% of the samples in any one indication. Indications and mutations are clustered. **(Middle)** The mean frequency across indications. **(Bottom)** Predicted binding for each mutation to common HLA class I alleles.

of the tumors. When examining the cumulative sum of the first five mutations, one finds that contributions of mutations two through five are large for the profiled pancreatic and uterine cancers: over 80 and 40% of the profiled pancreatic and uterine tumors, respectively, have one of the five most frequent mutations. The three most frequent mutations in pancreatic tumors are KRAS p.G12D, G12V, and G12R, demonstrating the importance of this aberration for pancreatic tumor oncogenicity. Conversely, the most frequent uterine tumor mutations are found in different genes, including TP53, PIK3CA, ZNF814, and KRAS, suggesting engagement of alternative pathways. While not reviewed here, there are re-occurring mutations in other indications, such as uveal melanoma and diffuse intrinsic pontine glioma (DIPG) tumors.

DISCUSSION AND CONCLUSION

Cancer mutations are found in tumor cells and absent in non-tumorous cells. Thus, as targets, mutations are, by definition, uniquely found in the tumor cells. Some of the mutations are expressed, processed, and presented on tumor HLA molecules to T cells; those mutation-containing peptides that are recognized by T cells are neoantigens. A goal of immuno-oncology is to induce recognition of these tumor-specific, non-self targets. The number of mutations, and particularly the number of clonal immunogenic mutations, predicts tumor response to immune-strengthening therapeutics, such as anti-CLTA4 and anti-PD(L)1 mAbs (26, 27). Tumors with exceptionally high mutational burden respond favorably to immune-strengthening: pembrolizumab, an anti-PD-1 mAb, has been approved to treat MSI-H or mismatch repair deficient solid tumors, regardless of tumor site or histology (28). Thus, **Figure 1**, and the subclasses in **Figures 2**, **3**, identifies the tumors and indications—those with higher mutation burden—potentially more likely to respond to general immune strengthening agents (those agents not targeting specific mutations).

Patient-specific mutations can be targeted using rapidly manufactured, individualized therapeutic vaccines. Mutations

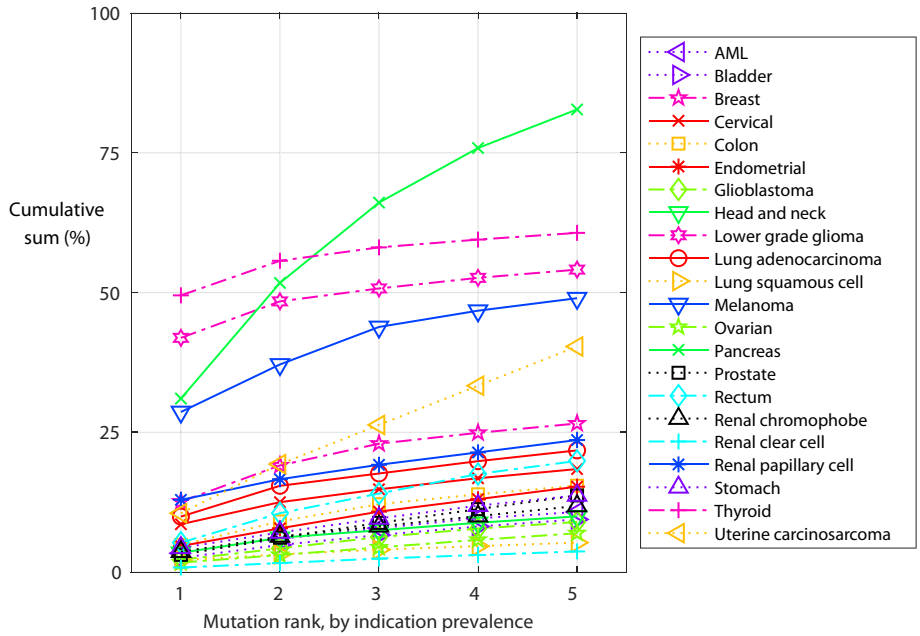

FIGURE 5 | Indication-specific cumulative sum frequency of the most frequent mutations. For each indication, the mutations are ordered by frequency and the cumulative sum calculated, assuming mutations do not co-occur.

are identified in a patient's tumor using next-generation sequencing and bioinformatics, prioritized for vaccine inclusion using criteria including mutation clonality (27) and peptide HLA binding affinity (14), manufactured, and administered with an adjuvant, potentially as part of combination therapy. Several companies have individualized clinical trials underway, including Advaxis (NCT03265080), Agenus (NCT03673020), BioNTech/Genentech (NCT03289962), Gritstone (NCT03639714), and Neon (NCT03597282).

In contrast to patient-specific individualized neoantigens, shared neoantigens provide the opportunity to create warehouse vaccines and TCRs for prêt-à-porter application. Protein post-translational modifications have been identified that are tumor-specific, shared across multiple tumors, and immunogenic (29), and thus classify as warehouse targets. Here, we examine the frequency and immunogenicity of protein-modifying DNA point-mutations found in the TCGA cohorts.

As expected, there is not a single neoantigen—no magic bullet—that is found in all tumors in all indications and for all HLA alleles. However, there are mutations found frequently in specific indications, such as IDH1 p.R132H in lower grade glioma. BRAF p.V600E, KRAS p.G12D, and KRAS p.G12V are frequent in multiple indications. Other mutations, such as PIK3CA p.E545K, are less frequently found in a single indication but are found in the tumors of many indications.

Re-occurring mutations usually do not co-occur in a single tumor clone. The five most frequent mutations in each indication typically account for more than 30% of the tumors in pancreatic, thyroid, lower grade glioma, melanoma, and uterine cancers. Presentation of each mutation-containing peptide is HLA dependent and, as such, a warehouse TCR or vaccine targeting a re-occurring mutation will be relevant for only a subset of patients. Thus, the warehouse must be stocked with multiple vaccines or TCRs, accounting for both the tumor variation and patient HLAs. Indeed, some mutations will be more visible to some patients, depending on their HLA alleles. Indeed, recent work suggests that an individual's HLA alleles shape the allowable common mutations that can occur in the individual (30), further confirming that common mutations can be seen by the immune system.

Using the impressive public domain TCGA dataset, this work shows the presence of non-synonymous single-nucleotide mutations across a broad panel of tumor indications and potential immunotherapy application. As previously described, melanoma and lung cancers have higher numbers of mutations relative to other tumors. These indications also have a long tail: a population of tumors with an exceptionally high number of mutations. Organs at risk for MSI tumors, including colon, stomach, and endometrial, show similar mutation distributions, comprising a core group of tumors with fewer mutations, typically the micro-satellite stable tumors, and the long tail of high mutation MSI tumors. Mutation rates vary among molecularly-defined tumor sub-groups: breast basal tumors have, on average, more mutations than luminal A tumors. Smoking and TP53 mutations are associated with high tumor mutation burden in lung cancers. Finally, re-occurring mutations can be found in the profiled tumors: BRAF p.V600E is found in many thyroid tumors and melanomas and mutations such as PIK3CA p.E454K can be found at appreciable levels across multiple indications. HLA binding of non-self peptides is essential for neoantigen generation; that many of these mutation-containing peptides are predicted to

bind common HLA alleles increases the likelihood that they are bona fide neoantigens suitable for warehouse vaccines and T-cell therapies.

AUTHOR CONTRIBUTIONS

JC initiated the study and generated the figures. MU, SP, and JC collected and analyzed the data. JC, RS, and JB wrote the manuscript.

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