https://www.scientificarchives.com/journal/journal-of-cancer-immunology

Journal of Cancer Immunology Commentary

Expanding the Cancer Neoantigen Peptide Repertoire beyond *In silico* Tools

Amit Jain[1], Jackwee Lim[2*]

[1]National Cancer Centre Singapore, 11 Hospital Crescent, Singapore 169610

[2]Singapore Immunology Network, Agency for Science, Technology and Research, 8A Biomedical Grove, Immunos, Singapore 138648

[*]Correspondence should be addressed to Jackwee Lim; lim_jack_wee@immunol.a-star.edu.sg

Received date: January 08, 2021, **Accepted date:** February 23, 2021

Commentary

CD8⁺ cytotoxic T cells recognise and kill cancer cells that present immunogenic peptides bound to the cell surface major histocompatibility complex class I (MHC-I) molecules. The immunogenicity of these peptides derives from them being recognised as non-self after their parent proteins are intracellularly processed and presented as peptide-major histocompatibility complex class I (pMHC-I) complexes. pMHC specific T cell receptor (TCR) recognition then leads to cytotoxic T cell response. Generally, robust pMHC-I binding is needed for a chance encounter with a pMHC specific TCR bearing CD8⁺ T cell derived from *in vivo* T cell evolution and thymic selection. As such, pMHC binding and stability are the key starting points towards understanding the T cell response. With current technologies, peptide and MHC interaction may be deduced from either direct pMHC binding/ stability assays or *in silico* prediction tools, and each method has its advantages and disadvantages (Table 1). Nonetheless, the ideal method should not exclude peptides that are potentially immunogenic. Hence there is continued need for more accurate high-throughput methods that assess the natural physico-biochemical interaction between peptide and MHC molecules.

Lim has recently developed the EZ MHC-I assay using the pMHC-I single chain trimer (SCT) molecule to enable a direct interrogation of the MHC ligandome predicted *in silico* or derived from patients' samples. It is an assay based on empty MHC-I protein fragmentation to rapidly characterize bound peptides for affinity and stability [1]. This will exclude predicted binders which do not stabilize the MHC-I molecule, identify missed hits, and potentially enable neoantigen discovery with better characterized peptides. Here, we describe the challenges of neoantigen selection, share missed hits identified by the EZ MHC-I assay, present the EZ MHC-I assay in greater details, and propose future SCT-based applications towards CD8⁺ T cell specific neoantigen discovery.

The hunt for immunogenic peptides

Immunogenic T cell antigens have been well characterized in the context of infectious diseases where the entire antigen from viral proteins is "non-self" and hence immunogenic. On the other hand, the identification of "immunogenic" cancer antigens or neoantigens is more challenging [2,3]. While in methylcholanthrene-induced mouse models, several immunogenic peptide sequences have been identified [4], human cancer mutations are largely patient specific and therefore bespoke, notwithstanding intratumoral heterogeneity. Broadly, cancer antigens comprise of cancer specific overexpressed proteins, viral proteins in virus associated cancers, and specific peptides derived from nonsynonymous mutations, deletions, or translocations. Several bed to bench clinical translational studies of cancer immunotherapy have unravelled that stable pMHC-T cell interactions are requisite for T cell cytotoxicity against cancer [5,6]. However, identifying stable pMHC molecules is experimentally laborious and *in silico* predictions remain under scrutiny [7-9]. Nonetheless, tremendous efforts to characterize neoantigen pools with bonafide T cell responses in patients responding to immune checkpoint inhibitors have led to clinical development of therapeutic cancer peptide vaccines by companies such as Gritstone Oncology and Genocea [10,11]. Presently, potential neoantigens are identified using paired normal

Resources	Advantages	Disadvantages
In silico algorithms e.g. NetMHCpan, NetMHCstab, MHCSeqNet and MHCflurry.	• Fairly accurate and fast • Free public access • High-throughput	• Lack of biophysical characterization • Restricted binding motifs • Numerous candidates • Missed hits
Peptide-based mass spectrometry	• Natural processing of protein antigen and presentation of peptide antigens • Limited multiplexing	• Peptide recovery challenge • Complex data analysis • Mixed results immunogenicity in humans
In vitro assays e.g. ELISA, ProImmune REVEAL and Immunitrack NeoScreen.	• Early biophysical characterization • Unrestricted peptide types • High-thoughput	• Multiple-step irreproducibility • Sample preparation challenge • Hypothetical candidates
EZ MHC-I assay	• Early biophysical characterization • Unrestricted peptide types • Direct band visualization of MHC-I protein • Shortest hands-on time among current *in vitro* assays • High-throughput	• Limited to MHC-I alleles • Production of suitable MHC-I molecule • Hypothetical candidates

Table 1: Advantages and disadvantages of different T cell epitope prediction resources.

genomic sequencing of both DNA and RNA. This creates a large pool of mutation specific peptides which are then mapped to the patient specific human leukocyte antigen (HLA). Mass spectrometry and a large literature of experimental data have enabled the development of more accurate *in silico* prediction algorithms to identify peptides that are likely to bind stably in a MHC allelic groove [12]. Furthermore, the discovery of immune checkpoint receptors, CTLA-4 and PD-1 [13] that abrogate T cell responses against cancer has led to a reinvigoration of the study of pMHC binding to identify therapeutic targets [14], especially in the context of patients treated with immune checkpoint inhibitors to relieve $CD8^+$ T cell exhaustion [15,16]. However, not all cancer mutations are similarly immunogenic, and immunogenicity is possibly driven by the duration of MHC peptide binding and pMHC-TCR bond conformation to trigger a T cell response [17,18]. Hence careful characterization of stable pMHC-I molecules can lead to the identification of neoantigens more likely to trigger tumor-specific T cell mediated antitumor immune response, and potentially drive the success of neoantigen derived cancer vaccine therapy [19-22].

An *in silico* challenge: predicting unconventional peptide binding in MHC-I groove

Early neoantigen discovery based on early proofs of peptide antigens presented on cell surfaces propelled the development of pMHC-I/ TCR related applications [23,24]. Currently, prediction of neoantigens by bioinformatics remains a popular approach prior to experimental validation. Indeed, prediction algorithms such as NetMHCpan, MHCSeqNet, MHCflurry and NetMHCstab have become highly reliable in predicting MHC-peptide binding affinity or stability [11,12,25-27]. This initially propelled the clinical development of neoantigen vaccines by both academia [28-30] and companies such as Neon, BioNTech, and subsequently also in combination with immune checkpoint inhibitors such as in the clinical trials run by Moderna and Merck [31,32]. However, the suboptimal nature of *in silico* prediction has also driven companies such as Gritstone Oncology and Genocea to develop patient data-trained deep learning tools or *in vitro* cellular methods, respectively to improve the early identification of potential neoantigens [10,11]. Hence further characterization of these neoantigens for their stability will favor more optimized therapeutics. Indeed recent studies on experimentally validated neoantigen have shown that both binding affinity and complex stability are key parameters of a pMHC molecule to stimulate patient tumor infiltrating lymphocytes and mount an immunogenic response [18,33]. However, urea denaturing methods to measure actual pMHC-I stability are tedious and predicting the energetic stability of a pMHC complex can be computationally inaccurate, a grand challenge to consider the entropic and enthalpic factors even for the protein folding community [34,35]. Indeed, a large majority of predicted neoantigens do not elicit T-cell responses as only a small fraction is capable of presentation as cell surface pMHC and subsequent recognition by the rare TCR. Therefore, actual measurement of pMHC-I affinity and stability can potentially improve the reliability of predicted peptides [7,36]. Indeed, an additional 60% of predicted epidermal growth factor receptor (EGFR) mutated peptide candidates was found using the EZ MHC-I assay, which remain to be validated in future patient studies (Figure 1).

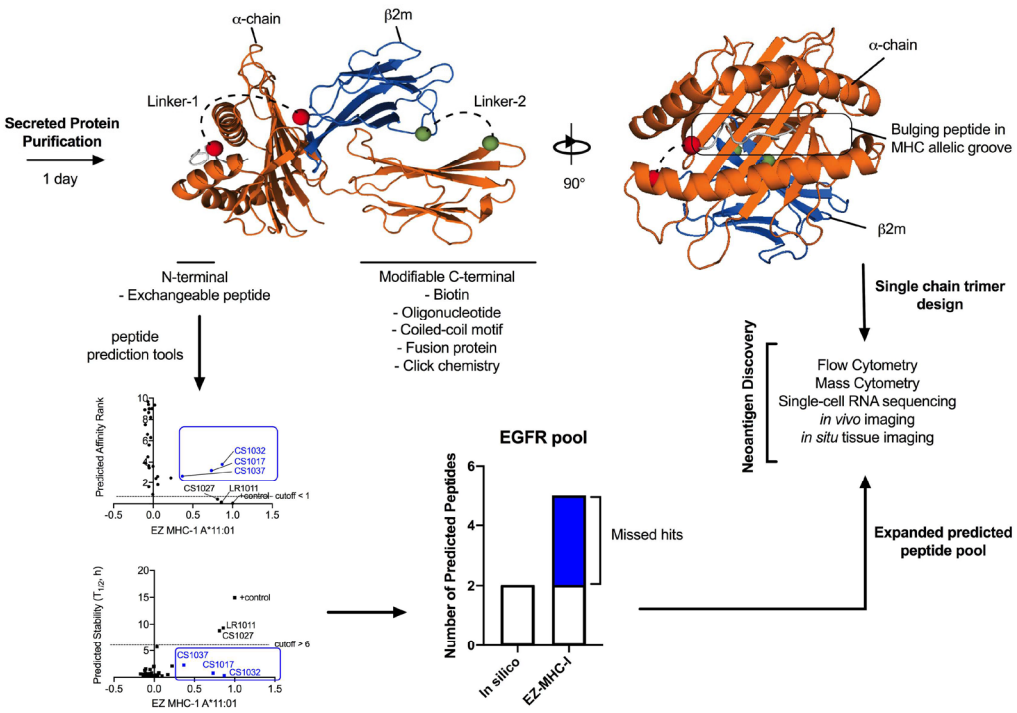

Figure 1: The pMHC-I single chain trimer protein architecture and future applications in tumor neoantigen discovery. The versatility of the pMHC-I SCT protein enables downstream innovative tools for identifying antigenic CD8⁺ T cells. The SCT is a single assembly of the peptide, β2-microglobulin and the α-chain, each separated by a linker. We have further developed the EZ MHC-I assay using the SCT format to characterize binding peptides and compare it with *in silico* prediction tools. Predicted affinity rank is calculated using NetMHCpan-4.0 algorithm and predicted stability is calculated using NetMHCstab-1.0 algorithm, shown with their recommended cutoff values. Common missed hits remain unpredicted *in silico* due to possible environmental factors such as conformational and make up 3/5 of the total EGFR predicted mutated peptide pool.

Favoring biophysical characterization with easier pMHC-I production

To date, the human MHC gene, also known as HLA has been strongly associated with hundreds of disease and thus play a pivotal role in disease susceptibility genetic testing [37]. Several structural studies have also advanced our understanding of different immunogenic peptides bound within the MHC-I/II protein groove, including the development of automated modeling albeit selected MHC-I allotypes [38]. X-ray crystallography has further revealed peptides of 10/11 amino acids, which can bind the MHC-I groove in either a zig-zag or bulging manner, while anchoring its N and C termini into the A and F pockets, respectively [39]. Moreover, several high-resolution structures have also shown non-canonical peptide lengths of up to 16-mer exiting out at the F pocket [40-43]. These suggest that longer peptide lengths can adopt unconventional binding modes within the enclosed groove of MHC-I protein, previously thought to be unique to the more opened groove of MHC-II protein. Also, longer peptide precursors of MHC-I do undergo trimming by endoplasmic reticulum aminopetidases 1 and 2 along the transporter associated with antigen processing machinery to be shorten to a more preferable 9- to 13-mer and adopt multiple bulging conformations in the MHC-I binding groove [44-46]. Taken together, the binding of peptide in the MHC-I groove is not always flat and thus unpredictable *in silico*.

To enable real biophysical characterization, different pMHC-I molecules have to be produced using recombinant methods [1,47-51]. Stable pMHC-I molecules are traditionally used in cytometry for probing CD8⁺ T cells but unsuitable reagents for peptide-exchange. Thus Bakker et al. used a photolabile peptide cleavable by UV irradiation to make empty MHC-I molecules to enable peptide exchange [51]. Similarly, advances in peptide-exchange technologies include peptide deficient MHC-I/TAPasin binding protein related complexes and thermal exchangeable pMHC-I molecules to overcome possible photodamage using UV cleavable pMHC-I molecules [49,50]. More importantly, to

encourage biophysical characterization, secreted peptide exchangeable SCT proteins were successfully made using the mellitin-based baculovius expression vector system (mBEVS, mellitin leader: MKFLVNVALVFMVVYISYIYA) [1]. A major technical advantage of a secreted SCT is the rapid purification of functional pMHC-I protein in hours instead of several days using the traditional *E. coli.* system [52,53]. Hence the time-saving mBEVS method is more likely to create more SCT fusion analogues compared to current tedious *in vitro* refolding methods, and favor biophysical characterization of pMHC-I molecules [47,54].

Challenges of developing the EZ MHC-I assay

However, identifying a peptide exchangeable SCT, which is suitable for EZ MHC-I assay can be challenging. A primary limitation is the wrong choice of a peptide can often result in insoluble SCT inclusion bodies, an indication of misfolded protein. Therefore, different peptides interacting weakly with the A and F pockets of the MHC-I groove were screened and evaluated for secreted soluble SCT protein. Additionally, these peptides unless cleaved, are covalently tethered to the N-terminal of the human β2-microglobulin chain to stabilize the original pMHC-I molecule [48]. More importantly, the tethered peptides should readily dissociate from the MHC-I molecule when cleaved as previously described [1]. For example, known HLA-A*02:01 epitopes KILGRVFFV/ KLLTKILTI and HLA-A*02:07 epitope FLPSDYFPSV were found non-exchangeable and thus unsuitable for EZ MHC-I assay. Nonetheless, suitable SCT proteins were successfully produced for EZ MHC-I assay [1].

To encourage actual physico-biochemical measurement of stable MHC-I peptide binding, we have also significantly reduced the time spent in pMHC-I binding assays by eliminating traditional enzyme-linked immunosorbent assay (ELISA) methodology. Here, blocking and washing steps in standard ELISA were removed. Instead the EZ MHC-I assay is a *de novo* approach of direct protein fragmentation [1]. EZ MHC-I assay is developed based on a combination of unfavorable observations; First, emptied MHC-I proteins were previously known to destabilize and dissociate into α–heavy and β2-microglobulin light chains [55]. Second, fusion protein when destabilized will partially unfold and become more susceptible to non-specific cleavage [56]. Third, enterokinase has been reported to be a non-specific protease in some cases [57,58]. Taken together, these unfavorable observations were successfully incorporated to make a SCT fusion protein, which results in enterokinase-induced fragments in the absence of a rescue peptide.

In search for more neoantigens

Pipeline to identify neoantigen-specific T cells in blood and tumor samples still remain challenging. Presently, neogantigens due to non-synonymous mutation can be identified using next generation sequencing techniques but still require filtering of tumor DNA against germline DNA and the subsequent identification of private neoantigens unique to different patients. However, the latter requires selecting peptides that are either naturally processed or presented in tumor cells. Moreover, to date, peptide selection using *in silico* algorithms can still generate a high number of predicted candidates, especially for cancers with high mutational load. Nonetheless, immunogenicity validation of these numerous peptide candidates has been successful in the neoantigen discovery in melanoma and glioblastoma [29,59], but is costly. Thus reducing the peptide pool while improving the quality of peptide candidates are relevant to identifying more neoantigens. However, biophysical characterization of a large number of peptides can be technically laborious and also require the MHC-I molecule. In this commentary, the EZ MHC-I assay uses the pMHC-I SCT molecules and offers a fast and hassle-free approach to screen large peptide libraries which form stable pMHC-I molecules prior to expensive patient sample screening. Besides the EZ MHC-I assay, the rapid mBEVS pMHC-I SCT protein production can also attract more users and promote SCT-based applications for detecting antigenic $CD8^+$ T cells. The C-terminal end, away from the peptide binding groove of the SCT molecule still remains highly modifiable. Possible modifications include the additional of a BirA recognition sequence for biotinylation for streptavidin tagging [60], coiled-coil motif for multimerization [61] and the incorporation of clickable chemical groups for bioconjugation to oligonucleotides [62] or as fusion protein [50,63]. Hence the feasible manipulation of the C-terminal end in the pMHC-I SCT molecule would undoubtedly create many tools for cellular cytometry, cellular assays and imaging studies to identify undiscovered tumor neoantigens (Figure 1).

Conclusion

Moving forward, the success of neoantigen discovery and cancer vaccine largely requires a pool of predicted MHC peptides with qualities of good affinity and stability. This commentary sheds light on possible missed hits, which remain unaccounted for *in silico* due to non-conventional environmental factors and opens doors for the EZ MHC-I assay or similar experimental binding/stability assays. Additionally, the use of mBEVS for rapid pMHC-I SCT protein production bearing different C-terminal modifications may create new technologies to unveil anti-tumor $CD8^+$ cytotoxic T cells.

Acknowledgements

Authors acknowledge financial support by the Singapore Immunology Network (SIgN) core grant from A*STAR. Authors also acknowledge the National Cancer Centre,

Singapore for financial support by the NCCRF Grant NCCSPG-YR2016-JAN-18.

References

1. Lim J. Destabilizing single chain major histocompatibility complex class I protein for repurposed enterokinase proteolysis. Scientific Reports. 2020 Sep 10;10(1):1-0.

2. Zhang H, Zhou X, Liu D, Zhu Y, Ma Q, Zhang Y. Progress and challenges of personalized neoantigens in the clinical treatment of tumors. Medicine in Drug Discovery. 2020 Jun 1;6:100030.

3. Garcia-Garijo A, Fajardo CA, Gros A. Determinants for neoantigen identification. Frontiers in Immunology. 2019 Jun 24;10:1392.

4. Kono K, Petersson M, Ciupitu AM, Wen T, Klein G, Kiessling R. Methylcholanthrene-induced mouse sarcomas express individually distinct major histocompatibility complex class I-associated peptides recognized by specific CD8+ T-cell lines. Cancer Research. 1995 Dec 1;55(23):5648-55.

5. Riquelme E, Carreño LJ, González PA, Kalergis AM. The duration of TCR/pMHC interactions regulates CTL effector function and tumor-killing capacity. European Journal of Immunology. 2009 Aug;39(8):2259-69.

6. Harao M, Hirata S, Irie A, Senju S, Nakatsura T, Komori H, et al. HLA-A2-restricted CTL epitopes of a novel lung cancer-associated cancer testis antigen, cell division cycle associated 1, can induce tumor-reactive CTL. International Journal of Cancer. 2008 Dec 1;123(11):2616-25.

7. Rasmussen M, Fenoy E, Harndahl M, Kristensen AB, Nielsen IK, Nielsen M, et al. Pan-specific prediction of peptide–MHC class I complex stability, a correlate of T cell immunogenicity. The Journal of Immunology. 2016 Aug 15;197(4):1517-24.

8. Abella JR, Antunes DA, Clementi C, Kavraki LE. Large-scale structure-based prediction of stable peptide binding to class i hlas using random forests. Frontiers in Immunology. 2020 Jul 22;11:1583.

9. Serçinoğlu O, Ozbek P. Sequence-structure-function relationships in class I MHC: A local frustration perspective. PloS One. 2020 May 18;15(5):e0232849.

10. Lam H, McNeil LK, Starobinets H, DeVault VL, Cohen RB, Twardowski P, et al. An Empirical Antigen Selection Method Identifies Neoantigens That Either Elicit Broad Antitumor T-cell Responses or Drive Tumor Growth. Cancer Discovery. 2021 Jan 27.

11. Bulik-Sullivan B, Busby J, Palmer CD, Davis MJ, Murphy T, Clark A, et al. Deep learning using tumor HLA peptide mass spectrometry datasets improves neoantigen identification. Nature Biotechnology. 2019 Jan;37(1):55-63.

12. Jørgensen KW, Rasmussen M, Buus S, Nielsen M. Net MHC stab–predicting stability of peptide–MHC-I complexes; impacts for cytotoxic T lymphocyte epitope discovery. Immunology. 2014 Jan;141(1):18-26.

13. Gubin MM, Zhang X, Schuster H, Caron E, Ward JP, Noguchi T, et al. Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. Nature. 2014 Nov;515(7528):577-81.

14. Høydahl LS, Frick R, Sandlie I, Løset GÅ. Targeting the MHC ligandome by use of TCR-like antibodies. Antibodies. 2019 Jun;8(2):32.

15. Pfannenstiel LW, Diaz-Montero CM, Tian YF, Scharpf J, Ko JS, Gastman BR. Immune-checkpoint blockade opposes CD8+ T-cell suppression in human and murine Cancer. Cancer Immunology Research. 2019 Mar 1;7(3):510-25.

16. Grywalska E, Pasiarski M, Góźdź S, Roliński J. Immune-checkpoint inhibitors for combating T-cell dysfunction in cancer. OncoTargets and Therapy. 2018;11:6505.

17. Sasmal DK, Feng W, Roy S, Leung P, He Y, Cai C, et al. TCR–pMHC bond conformation controls TCR ligand discrimination. Cellular & Molecular Immunology. 2020 Mar;17(3):203-17.

18. Wells DK, van Buuren MM, Dang KK, Hubbard-Lucey VM, Sheehan KC, Campbell KM, et al. Key parameters of tumor epitope immunogenicity revealed through a consortium approach improve neoantigen prediction. Cell. 2020 Oct 29;183(3):818-34.

19. De Plaen E, Lurquin C, Van Pel A, Mariamé B, Szikora JP, Wölfel T, et al. Immunogenic (tum-) variants of mouse tumor P815: cloning of the gene of tum-antigen P91A and identification of the tum-mutation. Proceedings of the National Academy of Sciences. 1988 Apr 1;85(7):2274-8.

20. Robbins PF, El-Gamil M, Li YF, Kawakami Y, Loftus D, Appella E, et al. A mutated beta-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. Journal of Experimental Medicine. 1996 Mar 1;183(3):1185-92.

21. Brändle D, Brasseur F, Weynants P, Boon T, Van den Eynde B. A mutated HLA-A2 molecule recognized by autologous cytotoxic T lymphocytes on a human renal cell

carcinoma. The Journal of Experimental Medicine. 1996 Jun 1;183(6):2501-8.

22. Peng M, Mo Y, Wang Y, Wu P, Zhang Y, Xiong F, et al. Neoantigen vaccine: an emerging tumor immunotherapy. Molecular Cancer. 2019 Dec;18(1):1-4.

23. DeWeerdt S. Calling cancer's bluff with neoantigen vaccines. Nature. 2017 Dec 21;552(7685).

24. Jiang T, Shi T, Zhang H, Hu J, Song Y, Wei J, et al. Tumor neoantigens: from basic research to clinical applications. Journal of Hematology & Oncology. 2019 Dec;12(1):1-3.

25. O'Donnell TJ, Rubinsteyn A, Laserson U. MHCflurry 2.0: Improved pan-allele prediction of MHC class I-presented peptides by incorporating antigen processing. Cell Systems. 2020 Jul 22;11(1):42-8.

26. Phloyphisut P, Pornputtapong N, Sriswasdi S, Chuangsuwanich E. MHCSeqNet: a deep neural network model for universal MHC binding prediction. BMC Bioinformatics. 2019 Dec;20(1):1-0.

27. Reynisson B, Alvarez B, Paul S, Peters B, Nielsen M. NetMHCpan-4.1 and NetMHCIIpan-4.0: improved predictions of MHC antigen presentation by concurrent motif deconvolution and integration of MS MHC eluted ligand data. Nucleic Acids Research. 2020 Jul 2;48(W1):W449-54.

28. Ott PA, Hu Z, Keskin DB, Shukla SA, Sun J, Bozym DJ, et al. An immunogenic personal neoantigen vaccine for patients with melanoma. Nature. 2017 Jul;547(7662):217-21.

29. Carreno BM, Magrini V, Becker-Hapak M, Kaabinejadian S, Hundal J, Petti AA, Ly A, Lie WR, Hildebrand WH, Mardis ER, Linette GP. A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. Science. 2015 May 15;348(6236):803-8.

30. Sahin U, Derhovanessian E, Miller M, Kloke BP, Simon P, Löwer M, et al. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. Nature. 2017 Jul;547(7662):222-6.

31. Burris III HA, Patel MR, Cho DC, Clarke JM, Gutierrez M, Zaks TZ, et al. A phase 1, open-label, multicenter study to assess the safety, tolerability, and immunogenicity of mRNA-4157 alone in subjects with resected solid tumors and in combination with pembrolizumab in subjects with unresectable solid tumors (Keynote-603). J. Glob. Oncol. (2019).

32. An Efficacy Study of Adjuvant Treatment With the Personalized Cancer Vaccine mRNA-4157 and Pembrolizumab in Patients With High-Risk Melanoma (KEYNOTE-942). Case Med. Res. (2019) https:/doi.org/10.31525/ct1-nct03897881.

33. Harndahl M, Rasmussen M, Roder G, Dalgaard Pedersen I, Sørensen M, Nielsen M, et al. Peptide-MHC class I stability is a better predictor than peptide affinity of CTL immunogenicity. European Journal of Immunology. 2012 Jun;42(6):1405-16.

34. Caro JA, Harpole KW, Kasinath V, Lim J, Granja J, Valentine KG, et al. Entropy in molecular recognition by proteins. Proceedings of the National Academy of Sciences. 2017 Jun 20;114(25):6563-8.

35. Senior AW, Evans R, Jumper J, Kirkpatrick J, Sifre L, Green T, et al. Improved protein structure prediction using potentials from deep learning. Nature. 2020 Jan;577(7792):706-10.

36. Schmidt J, Guillaume P, Dojcinovic D, Karbach J, Coukos G, Luescher I. In silico and cell-based analyses reveal strong divergence between prediction and observation of T-cell–recognized tumor antigen T-cell epitopes. Journal of Biological Chemistry. 2017 Jul 14;292(28):11840-9.

37. Gao J, Zhu C, Zhu Z, Tang L, Liu L, Wen L, et al. The human leukocyte antigen and genetic susceptibility in human diseases. Journal of Bio-X Research. 2019 Sep 1;2(3):112-20.

38. Rigo MM, Antunes DA, De Freitas MV, de Almeida Mendes MF, Meira L, Sinigaglia M, et al. DockTope: a Web-based tool for automated pMHC-I modelling. Scientific Reports. 2015 Dec 17;5(1):1-3.

39. Guo HC, Jardetzky TS, Garrettt TP, Lane WS, Strominger JL, Wiley DC. Different length peptides bind to HLA-Aw68 similarly at their ends but bulge out in the middle. Nature. 1992 Nov;360(6402):364-6.

40. McMurtrey C, Trolle T, Sansom T, Remesh SG, Kaever T, Bardet W, et al. Toxoplasma gondii peptide ligands open the gate of the HLA class I binding groove. Elife. 2016 Jan 29;5:e12556.

41. Collins EJ, Garboczi DN, Wiley DC. Three-dimensional structure of a peptide extending from one end of a class I MHC binding site. Nature. 1994 Oct;371(6498):626-9.

42. Guillaume P, Picaud S, Baumgaertner P, Montandon N, Schmidt J, Speiser DE, et al. The C-terminal extension landscape of naturally presented HLA-I ligands. Proceedings of the National Academy of Sciences. 2018 May 15;115(20):5083-8.

43. Josephs TM, Grant EJ, Gras S. Molecular challenges

imposed by MHC-I restricted long epitopes on T cell immunity. Biological Chemistry. 2017 Aug 28;398(9):1027-36.

44. Ayres CM, Corcelli SA, Baker BM. Peptide and peptide-dependent motions in MHC proteins: immunological implications and biophysical underpinnings. Frontiers in Immunology. 2017 Aug 7;8:935.

45. Howarth M, Williams A, Tolstrup AB, Elliott T. Tapasin enhances MHC class I peptide presentation according to peptide half-life. Proceedings of the National Academy of Sciences. 2004 Aug 10;101(32):11737-42.

46. Ekeruche-Makinde J, Miles JJ, Van Den Berg HA, Skowera A, Cole DK, Dolton G, et al. Peptide length determines the outcome of TCR/peptide-MHCI engagement. Blood. 2013 Feb 14;121(7):1112-23.

47. Garboczi DN, Hung DT, Wiley DC. HLA-A2-peptide complexes: refolding and crystallization of molecules expressed in Escherichia coli and complexed with single antigenic peptides. Proceedings of the National Academy of Sciences. 1992 Apr 15;89(8):3429-33.

48. Yik YL, Netuschil N, Lybarger L, Connolly JM, Hansen TH. Cutting edge: single-chain trimers of MHC class I molecules form stable structures that potently stimulate antigen-specific T cells and B cells. The Journal of Immunology. 2002 Apr 1;168(7):3145-9.

49. Luimstra JJ, Franken KL, Garstka MA, Drijfhout JW, Neefjes J, Ovaa H. Production and thermal exchange of conditional peptide-MHC I multimers. Current Protocols in Immunology. 2019 Sep;126(1):e85.

50. Overall SA, Toor JS, Hao S, Yarmarkovich M, O'Rourke SM, Morozov GI, et al. High throughput pMHC-I tetramer library production using chaperone-mediated peptide exchange. Nature Communications. 2020 Apr 20;11(1):1-3.

51. Bakker AH, Hoppes R, Linnemann C, Toebes M, Rodenko B, Berkers CR, et al. Conditional MHC class I ligands and peptide exchange technology for the human MHC gene products HLA-A1,-A3,-A11, and-B7. Proceedings of the National Academy of Sciences. 2008 Mar 11;105(10):3825-30.

52. Kim S, Zuiani A, Carrero JA, Hansen TH. Single chain MHC I trimer-based DNA vaccines for protection against Listeria monocytogenes infection. Vaccine. 2012 Mar 9;30(12):2178-86.

53. Hansen TH, Lybarger L. Exciting applications of single chain trimers of MHC-I molecules. Cancer Immunology, Immunotherapy. 2006 Feb 1;55(2):235.

54. Schmittnaegel M, Hoffmann E, Imhof-Jung S, Fischer C, Drabner G, Georges G, et al. A new class of bifunctional major histocompatibility class I antibody fusion molecules to redirect CD8 T cells. Molecular Cancer Therapeutics. 2016 Sep 1;15(9):2130-42.

55. Parker KC, DiBrino M, Hull L, Coligan JE. The beta 2-microglobulin dissociation rate is an accurate measure of the stability of MHC class I heterotrimers and depends on which peptide is bound. The Journal of Immunology. 1992 Sep 15;149(6):1896-904.

56. Kühnel B, Alcantara J, Boothe J, van Rooijen G, Moloney M. Precise and efficient cleavage of recombinant fusion proteins using mammalian aspartic proteases. Protein Engineering. 2003 Oct 1;16(10):777-83.

57. Shahravan SH, Qu X, Chan IS, Shin JA. Enhancing the specificity of the enterokinase cleavage reaction to promote efficient cleavage of a fusion tag. Protein Expression and Purification. 2008 Jun 1;59(2):314-9.

58. Liu Q, Lin J, Liu M, Tao X, Wei D, Ma X, et al. Large-scale preparation of recombinant human parathyroid hormone 1–84 from Escherichia coli. Protein Expression and Purification. 2007 Aug 1;54(2):212-9.

59. Johanns TM, Dunn GP. Applied cancer immunogenomics: leveraging neoantigen discovery in glioblastoma. Cancer Journal (Sudbury, Mass.). 2017 Mar;23(2):125.

60. Fairhead M, Howarth M. Site-specific biotinylation of purified proteins using BirA. InSite-Specific Protein Labeling 2015 (pp. 171-184). Humana Press, New York, NY.

61. Burkhard P, Stetefeld J, Strelkov SV. Coiled coils: a highly versatile protein folding motif. Trends in Cell Biology. 2001 Feb 1;11(2):82-8.

62. Hara S, Nojima T, Seio K, Yoshida M, Hisabori T. DNA-maleimide: an improved maleimide compound for electrophoresis-based titration of reactive thiols in a specific protein. Biochimica et Biophysica Acta (BBA)-General Subjects. 2013 Apr 1;1830(4):3077-81.

63. Dahotre SN, Chang YM, Romanov AM, Kwong GA. DNA-barcoded pMHC tetramers for detection of single antigen-specific T cells by digital PCR. Analytical Chemistry. 2019 Jan 18;91(4):2695-700.