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Spotlight

A roadmap for driving CAR T cells toward the oncogenic immunopeptidome

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A critical barrier to CAR T cell therapy is the paucity of target antigens that are broadly and stably expressed exclusively in tumors. In their comprehensive multi-omics and pre-clinical study, Yarmarkovich et al. provide proof of principle for the development and efficacy of peptide centric (PC)-CARs targeting the oncogenic immunopeptidome of neuroblastoma.

Chimeric antigen receptors (CARs), first conceived in the late 1980s, link tumor-antigen recognition, typically via a single-chain antibody variable fragment (scFv), to T cell activation (Kuwana et al., 1987). Over the past decade, CARs have proven to be a powerful tool in the engineering of T lymphocytes for the clinical treatment of various hematological malignancies. While CAR T cells are a promising strategy for attacking coldtumors that lack endogenous T cell infiltrate and/or are non-responsive to immune checkpoint blockade, to date limited clinical responses have been reported for CAR therapy of epithelial-derived solid tumors. A critical barrier is the paucity of bona fide solid tumor antigens that are broadly and stably expressed and that are not found in healthy tissues which can lead to tumor escape and severe toxicities in patients, respectively (Majzner and Mackall, 2019).

Here, Prof. John M. Maris and colleagues apply state-of-the-art technologies, including integrated genomic and immunopeptidomics, numerous computational modeling and bioinformatics tools, and a counter scFv library panning strategy, to develop novel, scFv-based, peptide-centric (PC)-CARs that exclusively engage oncogenic peptides within the human leukocyte antigen (HLA) complex at the cell-surface of neuroblastomas (Yarmarkovich et al., 2021) (overall strategy is depicted in Figure 1). Neuroblastomas are driven by epigenetically deregulated core-regulatory circuit (CRC) transcriptional networks resulting in hyperactivation of several pathways involved in growth, proliferation, etc. (Durbin et al., 2018). In addition, neuroblastomas are typically low in both mutational burden and HLA expression and hence represent a challenging target for immunotherapies (Matthay et al., 2016).

Based on the above-mentioned properties of neuroblastoma, Yarmarkovichv et al. hypothesize that the immunopeptidome may be specifically enriched with peptides derived from proteins that (1) are critical for tumorigenesis, (2) have low propensity to be downregulated, and (3) are not expressed in healthy tissues. They begin by evaluating peptides presented to T cells by performing mass spectrometry (MS)-based immunopeptidomics analysis of eluted peptides from neuroblastoma-cell-derived xenografts, patient-derived xenografts (PDX), and primary tumors. Through a series of filtering steps, including sufficient peptide affinity for HLA and HLA allele frequency, as well as evaluation of available RNA sequencing and ligandomic datasets for neuroblastoma tumors versus normal tissues, they narrow down their vast pool of peptides to 1 each from 6 different critical CRC transcription factors. They further go on to validate the presence of these peptides in tumors by measuring synthetic peptide standards with MS, and they generate crystal structures to confirm peptide binding to the predicted HLA alleles (Figure 1, left arrow). Importantly, because all 6 CRC transcription factors are further shown to bind regulatory elements at each parent gene locus within H3K27Ac super-enhancer elements, the authors postulate that transcriptional redundancy and dependency should abrogate the risk of antigen loss due to downregulation of the parent gene upon immunotherapy.

Following interrogation of the dynamics of gene expression data during development using temporal transcriptomic data, the authors focus their efforts on the neuroblastoma dependency gene and master transcriptional regulator PHOX2B, which, consistent with its function in orchestrating neural crest progenitor development, is shown to be expressed exclusively during fetal development and entirely silenced in normal tissues before birth. Notably, PHOX2B expression is one of two highly penetrant neuroblastoma susceptibility genes, according to DepMap (Dharia et al., 2021) it is the third most significant dependency in neuroblastoma, it is routinely used in neuroblastoma diagnostic assays, and its expression is not detected in normal tissues, all properties favorable for an immunotherapeutic target (Matthay et al., 2016).

The authors then proceed to build a panel of scFv-based CARs targeting the unmutated PHOX2B peptide QYNPIRTTF discovered on HLA-A*24:02. (Notably, no high-affinity T cell receptors [TCRs] were identified in multiple screens.) It is not a trivial task to develop scFv against an HLA presented peptide, due to spatial confinement of the peptide within adjacent α helices. After the failure of phage library screenings to generate scFv that are not cross-reactive with HLA, they adapt the bacterial cytoplasmic platform retained display (ReD) (Beasley et al.,

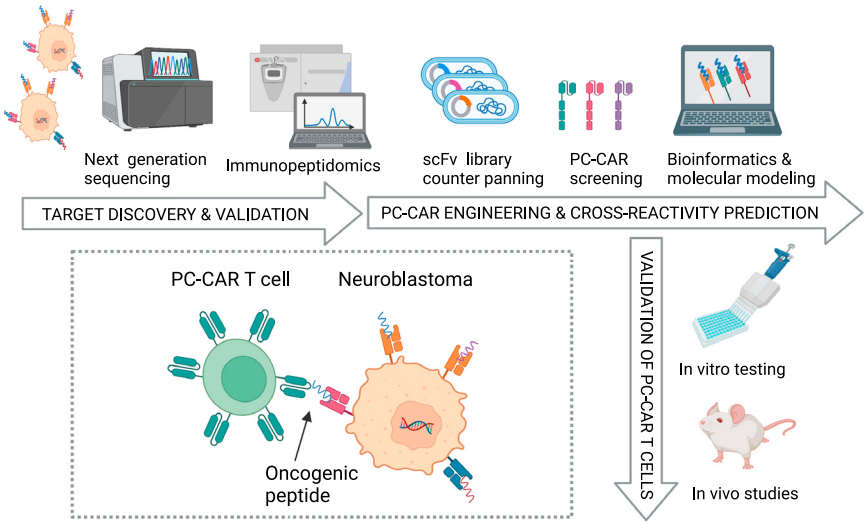

Figure 1. Schematic representing the workflow for the development of PC-CARs targeting the oncogenic immunopeptidome of neuroblastoma (depicted in box)

The strategy begins with a multi-omics approach (left arrow) in order to identify and validate oncogenic peptides presented by HLA class I proteins exclusively on the surface of neuroblastoma tumor cells. Subsequently (right arrow), library counter panning with predicted cross-reactive peptides is used to enrich scFv leads that specifically and solely bind to the target oncogenic peptide (i.e., do not engage HLA itself and do not cross-react with other peptides). CARs comprising the selected scFv are cloned and screened in experiments informed by a variety of bioinformatics and molecular modeling tools. The PC-CAR T cells are comprehensively validated (downward arrow) for specific *in vitro* activity (target cell killing and cytokine production) as well as against target and control tumors *in vivo*. Predicted cross-engagement of the PC-CARs with the same oncogenic peptide presented in alternative HLA class I molecules is also confirmed. (Figure created in BioRender.)

2015) and use a counter panning strategy with predicted potentially cross-reactive peptides to enrich from a starting Ruby scFv library comprising over 10^{11} variants (Figure 1, right arrow).

Among their panel of 25 CARs screened, one clone, 10LH, which has the highest-specificity profile (as validated based on predictions by the algorithm selective cross-reactive antigen presentation, sCRAP; https://marisshiny.research.chop.edu/sCRAP/), is chosen for deeper characterization. An alanine scan of the PHOX2B peptide reveals significant interactions of the 10LH CAR with 5 out of 7 non-anchor residues (in contrast, TCRs usually interact with 3–4 peptide residues). Additionally, the authors make the hypothesis that other HLA allotypes could present the same peptide in a binding mode similar enough to be recognized by 10LH. Indeed, using a population-scale antigen presentation tool, ShinyNAP, the authors go on to identify additional HLA allotypes (HLA-A*23:01 and HLA-A*14:02) predicted to present the PHOX2B peptide and use their own pMHC modeling software, RosettaMHC (Nerli and Sgourakis, 2020), to model the 3D conformation of the corresponding complexes and estimate their binding free energy. This approach could be a means to predict *in silico* the cross-recognition by the same scFv of a peptide presented by several HLA allotypes. The comparison of the modeled pMHC surfaces to predict those compatible with the binding of the same scFv is currently obtained by visual inspection. A more systematic application of the protocol would require the automation of this last step, notably if a large number of HLA allotypes are proposed by ShinyNAP.

Finally, the authors go on to perform several well-controlled *in vitro* and *in vivo* experiments (Figure 1, downward arrow). For example, they show potent on-target ($PHOX2B^+$ and HLA-A*24:02$^+$ or HLA-A*23:01$^+$) neuroblastoma cell line killing and IFN-γ production by 10LH PC-CAR T cells, but no reactivity against cell lines that do not express PHOX2B. Moreover, the PC-CAR T cells demonstrate potent tumor killing in mice engrafted with target-expressing neuroblastoma PDX tumors as well as aggressive cell lines. Interestingly, *in vivo* treatment is associated with HLA upregulation in PDX tumors, presumably due to potent IFN-γ release by the PC-CAR T cells as had been observed *in vitro*.

Taken together, this impressive study by Yarmarkovich et al., which pairs genomic, transcriptomic, epigenomic, and immunopeptidomics datasets and utilizes a range of cutting-edge bioinformatic and molecular modeling tools, represents a detailed roadmap toward the development of CARs that can be specifically targeted against the oncogenic immunopeptidome. An important advantage of PC-CAR T cells is the potential for treating a broader patient population with a given receptor as compared to TCR T cells which face HLA-restriction (i.e., the PC-CARs can recognize the oncogenic peptides in the context of several different HLA complexes), or neo-antigen targeting strategies which are usually patient specific. Indeed, if this innovative approach of redirecting CAR T cells exclusively against nonmutated, oncogenic peptides can be demonstrated for other cancer types, and if PC-CAR T cells prove to be non-toxic and efficacious in the clinic, this work would represent a major breakthrough for the field.

For future directions, to counter target downregulation, it may prove beneficial to treat patients with PC-CARs against peptides derived from multiple oncogenic proteins. If, however, downregulation of critical components of the HLA class I presentation pathway such as β2 m occurs, the target oncogenic peptides will no longer be presented, and the tumor will escape treatment. Hence, exploring the development of PC-CARs targeting HLA class II presented oncogenic peptides is warranted. One can further envision an allogeneic PC-CAR T cell product. In addition, the development of PC ON-switch or STOP-switch-CARs (Giordano-Attianese et al., 2020) could enhance patient safety. Finally, combinatorial therapies or co-engineering strategies to target barriers in the tumor microenvironment and harness endogenous immunity can be evaluated for improved patient responses to PC-CAR T cells (Lanitis et al., 2020). It is evident that we have entered a new era of interdisciplinary multi-omics data generation and analysis, as well as of innovative computational synthetic biology engineering approaches, which together, as demonstrated in this study,

can enable important advances in cancer treatment.

DECLARATION OF INTERESTS

V.Z. is a consultant for Cellestia Biotech. G.C. has received grants or research support or is coinvestigator in clinical trials by Bristol-Myers-Squibb, Celgene, Boehringer Ingelheim, Roche, Tigen Pharma, Iovance, and Kite. The institution of G.C. (CHUV) has received honoraria for consultations or presentations by G.C. from Roche, Genentech, BMS, AstraZeneca, Sanofi-Aventis, Nextcure, and GeneosTx. G.C. has patents in the domain of antibodies and vaccines targeting the tumor vasculature as well as technologies related to T cell expansion and engineering for T cell therapy. G.C. receives royalties from the University of Pennsylvania.

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