Letter to the Editor

Detection of clinically relevant T-cell receptors requires tailored approaches, and TCR gene therapy carries inherent risks

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Köksal and Wälchli (1) highlight important issues regarding the isolation of clinically relevant T-cell receptors (TCRs) and their use in TCR gene therapy for the treatment of cancer. Although we see overlap in the opinions held by them and ours, we would like to take this opportunity to resolve some misrepresentations of our data and methodology (2). We will also attempt to show that the discussion concerning the safety of TCR gene therapy is more nuanced than described by Köksal and Wälchli.

Köksal and Wälchli describe our platform as artificial based on the assumption that software prediction algorithms identified T-cell epitopes derived from BOB1. However, they neglect the fact that all BOB1-derived peptides were identified from the HLA ligandome of B-lymphocytes (3); peptides bound to HLA complexes of cultured cells were eluted and analyzed using mass-spectrometry. This database encompasses peptides that are endogenously processed and presented in HLA molecules on the cell surface of B cells. The clinical relevance of this approach is further underscored by the fact that we have identified novel BOB1 peptides in the HLA ligandome of primary samples of B-cell leukemia and lymphoma, and multiple myeloma (unpublished data). We have previously shown that the empirical data contained in the database greatly enhances the efficacy of isolating T-cell clones targeting biologically relevant T-cell epitopes (4). Prediction algorithms were only used to assign peptides to a specific HLA allele since the database was generated by using B-lymphocytes expressing multiple HLA alleles.

Köksal and Wälchli state that our approach may underestimate the availability of targetable T-cell epitopes. Alternatively, they propose a methodology based on the overexpression of therapeutic targets in antigen-presenting cells (APCs) to stimulate T-cell responses *in vitro* (5). This is an interesting and highly complementary approach to identify T-cell epitopes that may be below the detection limit of strategies based on peptide elution combined with analysis by mass spectrometry. However, expression of target-antigens at abnormally high levels may increase the detection of reactive T cells which in turn require unphysiologically high antigen-density for activation.

Interestingly, the strategy mentioned by Köksal and Wälchli also relies on the use of pMHC-tetramers for clinical translation. However, the isolation of T cells binding to pMHC-tetramers was preceded by laborious generation of APCs and repeated rounds of *in vitro* stimulation of PBMCs over the course of several weeks. In contrast, our protocol resulted in the identification of promising T-cell clones within 2 weeks following pMHC-tetramer guided enrichment with hands-on laboratory work on only a few days: pMHC-tetramer guided isolation and an initial screening process. In addition, our protocol can be easily adapted for the use of robotics (6). The efficiency of our platform can be further increased due to the multiplex nature of the pMHC-

tetramer technology; T-cell clones with specificity for different antigens can be simultaneously isolated by pools of pMHC-tetramers composed of distinct peptide and HLA molecules. We have used this approach in published and unpublished data demonstrating the high-throughput capacity of our platform.

Although the use of pMHC-tetramers resulted in the isolation of a majority of T-cell clones exhibiting undesired reactivity, we also demonstrated that the majority of these T-cell clones can be excluded through the initial screen that rapidly assessed peptide specificity and sensitivity. This screen limited the in-depth analysis to 11 promising T-cell clones, an amount that is well feasible for a laboratory experienced in cell culture.

Köksal and Wälchli interpreted the isolation of a single T-cell clone from the starting material of $1{,}000\times10^6$ as a sign of poor efficiency. However, these data speak to the immense sensitivity of our approach resulting in the isolation of a clinically relevant TCR with an apparently very low precursor frequency. We believe that immediate single-cell sorting and clonal expansion permitted the detection of this rare cell event. In contrast, several rounds of *in vitro* stimulation of bulk PBMCs as suggested by Köksal and Wälchli may have occulted the detection of this T-cell clone due to preferential expansion of T-cell clones with greater precursor abundance or stronger proliferative capacity. We believe that the methodologies put forward by Köksal and Wälchli are specifically powerful in the isolation of neoantigen/frame-shift-mutation-derived specific T-cell responses; a setting in which the stimulation of unspecific T-cell responses can be expected to be low.

TCR gene transfer carries the risk of potential off-target and/or on-target off-tumor toxicity. Due to the promiscuous nature of TCR-peptide-MHC interaction, these toxicities are currently impossible to fully predict before applying promising TCRs in humans. Köksal and Wälchli argue that TCRs isolated from the HLA-mismatched setting may be more cross-reactive since these TCRs have not undergone proper thymic selection. However, in the publication by Kumari *et al.* (5), Wälchli supports the view that peptide-specific recognition of T cells directed against allogeneic HLA molecules is rather the rule than the exception. We have also demonstrated that recognition of peptides in the HLA-mismatched setting occurs in a peptide-specific manner (7,8). Furthermore, using a synthetic peptide library (9), we have evaluated the peptide-promiscuity of PRAME-specific T-cell clones isolated from the HLA-matched and mismatched setting. T cells expressing PRAME-specific TCRs derived from either the allogeneic or autologous setting showed overlapping requirements for position and type of amino acids in an array of peptides (unpublished data).

It is noteworthy, that TCRs reactive to tumor-expressed self-antigens that have been isolated from patients can also cause unexpected off-target toxicity when used in TCR gene transfer. After all, even a patient-derived TCR has only been subjected to negative and positive selection based on the HLA genotype of the patient from which it was isolated. Peptide promiscuity and cross-reactivity towards allogeneic HLA molecules present in another patient may still cause harmful toxicities. Additional risk of unexpected toxicity can also stem from genetic disparities caused by polymorphisms and person-specific mutations, also noted by Köksal and Wälchli (1).

Fatal toxicity was observed for a MAGE-A3-specific TCR that showed cross-reactivity with a Titin-derived peptide (10). Interestingly, the MAGE-A3-specific TCR was isolated in an autologous setting. To the conclusion presented by Köksal and Wälchli, we would like to add, that the MAGE-A3-specific TCR had been affinity-enhanced by extensively modifying the complementary determining regions (CDRs), specifically the CDR2 of the TCR alpha chain. The CDR2 region is known to primarily interact with the MHC molecule and not the peptide. Therefore, cross-reactivity may be rather the result of stronger overall interaction between the modified TCR and its respective HLA molecule. Of note, extensive evaluation for off-target specificity of the affinity-enhanced TCR was performed after human fatalities were observed. However, it remains unclear whether pre-clinical evaluation could have identified this specific case of cross-reactivity with a Titin-derived peptide. Therefore, we agree with Köksal and Wälchli's assessment that cross-reactivity of TCRs can only be insufficiently mapped before applications in humans. Additionally, we embrace Köksal and Wälchli's stance that TCR-engineered T cells should be equipped with safety mechanism to be able to exert control following administration. However, we argue that such safety mechanism should be employed irrespective of the origin of the TCR.

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Footnote

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Ethical Statement: The authors are 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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