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Targeting of cancer neoantigens with donor-derived T cell receptor repertoires

Erlend Strønen,[1,2] Mireille Toebes,[3] Sander Kelderman,[3] Marit M. van Buuren,[3] Weiwen Yang,[1,2] Nienke van Rooij,[3] Marco Donia,[4] Maxi-Lu Böschen,[1,2] Fridtjof Lund-Johansen,[2,5] Johanna Olweus,[1,2]*† Ton N. Schumacher[3]*†

[1]Department of Cancer Immunology, Oslo University Hospital Radiumhospitalet, Oslo, Norway. [2]K. G. Jebsen Centers for Cancer Immunotherapy and for Inflammation Research, Institute for Clinical Medicine, University of Oslo, Oslo, Norway. [3]Division of Immunology, Netherlands Cancer Institute, Amsterdam, Netherlands. [4]Center for Cancer Immune Therapy, Department of Hematology, Herlev Hospital, University of Copenhagen, Herlev, Denmark. [5]Department of Immunology and Transfusion Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway.

***Corresponding author. E-mail: johanna.olweus@medisin.uio.no (J.O.); t.schumacher@nki.nl (T.N.S.)**

†These authors contributed equally to this work.

Accumulating evidence suggests that clinically efficacious cancer immunotherapies are driven by T cell reactivity against DNA mutation-derived neoantigens. However, among the large number of predicted neoantigens, only a minority is recognized by autologous patient T cells, and strategies to broaden neoantigen specific T cell responses are therefore attractive. Here, we demonstrate that naïve T cell repertoires of healthy blood donors provide a source of neoantigen-specific T cells, responding to 11/57 predicted HLA-A2-binding epitopes from three patients. Many of the T cell reactivities involved epitopes that in vivo were neglected by patient autologous tumor-infiltrating lymphocytes. Finally, T cells re-directed with T cell receptors identified from donor-derived T cells efficiently recognized patient-derived melanoma cells harboring the relevant mutations, providing a rationale for the use of such "outsourced" immune responses in cancer immunotherapy.

Accumulating data suggest that tumor regression induced by cancer immunotherapies that exploit the endogenous T cell pool (*1*, *2*) relies on recognition of neoantigens that are formed as a consequence of tumor-specific DNA mutations. A striking observation in cancer patients and in mouse models is that neoantigen specific T cell reactivity is generally limited to just a few mutant epitopes, even though the number of predicted epitopes is large (*3*–*12*). This scarcity of T cell recognized neoantigens could potentially reflect immune editing of tumors by T cells (*13*). Alternatively, an effector T cell pool toward many tumor-expressed neoantigens may be absent because of ineffective priming, or because of tolerization of these T cells. Recent work has demonstrated that vaccination with neoantigen peptide-loaded DCs can increase the breadth of mutant peptide-specific T cells in melanoma patients (*14*). In this work it could not be established whether newly induced T cells could recognize autologous tumor cells. Nevertheless, these data provide a further incentive for the development of strategies that broaden neoantigen specific T cell reactivity.

Here, we aimed to establish whether TCRs that are obtained outside of the autologous T cell repertoire can be used to engineer neoantigen-specific T cell immunity. To this purpose, we generated immune responses to HLA-A*02:01-restricted neoantigens from the non-tolerized T cell repertoires derived from donors that express this allele. Using this approach, we evaluated 1) whether donor-derived T cells can recognize relevant tumor cells, 2) whether such "outsourced" immune responses provide evidence for a neglected pool of neoantigens on human cancers, and 3) which types of mutant peptides are best seen by the T cell-based immune system.

To determine the feasibility of utilizing donor-derived T cell pools to induce neoantigen specific T cell reactivity, we initially focused on an HLA-A*02:01^{pos} stage IV melanoma patient. Whole-exome and RNA sequencing of tumor material revealed 249 non-synonymous mutations within expressed genes, and 126 mutant epitopes were predicted to bind to HLA-A*02:01 (*15*). Of these 126 neopeptides, only two were detected by T cells grown from the same tumor lesion. To investigate whether a larger fraction of predicted neoepitopes could be recognized by a healthy donor immune system, we selected 20 candidate neoepitopes based on high predicted binding affinity to HLA-A*02:01 (table S1). Non-adherent peripheral blood mononuclear cells (PBMC) from healthy donors were then co-cultured with autologous monocyte-derived dendritic cells transfected with mRNA encoding the candidate epitopes in a tandem minigene configuration, or with a control minigene encoding known epitopes from cancer/testis antigens and CD20

(*16*) that were recognized by relevant CTL (fig. S1). Analyses of resulting cell populations by MHC multimer staining revealed T cell reactivity toward 5/20 neoantigens from patient 1, whereas such reactivity was negligible in control cultures (Fig. 1, A and B). Analysis of T cell reactivity from 3 additional donors revealed 3-5 neoantigen specific T cell responses in all cases (Fig. 1C). One of the T cell responses reproducibly induced in this system, to the neoantigen $CDK4_{R>L}$, was also one of two responses detected among TIL of patient 1.

pMHC-multimer^{pos} CD8 cells were sorted from donors 2-4 to generate CTL clones. Resulting clones that stained positively with relevant pMHC-multimers (>82% of clones) were then tested for functional activity using a live-cell barcoding assay (fig. S2). Analysis of 185 CTL clones revealed reactivity of the majority of clones toward target cells pulsed with mutant peptide at concentrations down to 1 nM and below, with negligible recognition of the WT counterpart (Fig. 1D and figs. S2B and S3).

We then selected 76 CTL clones that specifically recognized target cells pulsed with neoantigens at low concentrations, to assess recognition of a short-term melanoma line of patient 1. Importantly, all $MLL2_{L>H}$-reactive CTL clones tested (n = 10) recognized the relevant melanoma cells. In contrast, no recognition of an HLA-A*02:01^{pos} third party melanoma was observed, unless pulsed with $MLL2_{L>H}$ peptide (Fig. 2, A and B). By the same token, all $CDK4_{R>L}$-reactive CTL clones tested (n = 6) showed vigorous and specific reactivity toward CDK4 mutant melanoma cells (Fig. 2B). Among CTL clones reactive with $ASTN1_{P>L}$ and $SMARCD3_{H>Y}$, 7/24 and 5/20 showed recognition of cognate melanoma (fig. S4). None of the $GNL3L_{R>C}$-reactive CTL clones tested (n = 16) recognized cognate or third party melanoma, unless pulsed with the relevant neoantigen (fig. S4).

T cell inductions were subsequently performed for predicted neoantigens from tumors of two additional patients. For patient 2, a set of 27 neopeptides (table S2) with a median predicted binding affinity to HLA-A*02:01 of 34 nM (range 2-140 nM) was selected among 154 mutant peptides predicted to bind to HLA-A*02:01. No HLA-A*02:01-restricted neoantigen specific T cell responses had been detected in TIL isolated from this patient when screening for reactivity to these 154 peptides. In contrast, responses to 6 predicted neoantigens were induced among T cells derived from 4 healthy donors (fig. S5, A and B). For patient 3, no T cell responses were detected to 10 predicted neoantigens (table S3). Predicted binding affinities of these potential neoantigens were considerably lower (median 225 nM) than those from patient 1 (median 41 nM) and 2 (median 34 nM).

From pMHC-multimer^{pos} CD8 cells from donors 5, 7 and 8 we established CTL lines reactive with the $USP28_{C>F}$, $SNX24_{P>L}$, $PGM5_{H>Y\text{-}462\text{-}470}$ and $PGM5_{H>Y\text{-}465\text{-}473}$ mutant peptides identified in the tumor of patient 2. All CTL lines responded strongly to target cells pulsed with relevant mutant peptides, whereas responses to target cells pulsed with corresponding WT peptides were generally low or negligible (fig. S5C, top row). Viable tumor material from patient 2 was scarce, and a tumor cell line for use in functional analyses could not be established. However, all but one of the CTL lines specifically recognized target cells transfected with a minigene encoding the mutant peptides $PGM5_{H>Y}$, $USP28_{C>F}$ and $SNX24_{P>L}$, flanked on both sides by 10 naturally occurring amino acids. (fig. S5C, bottom row). Together, these data demonstrate that neoantigen specific T cell responses can readily be induced in T cell repertoires from healthy donors, and that these T cells specifically recognize naturally processed neoantigens, including antigens expressed in matched tumor material.

Next, we investigated the feasibility of transferring donor-derived tumor-specific T cell reactivity by TCR gene transfer. T cell receptors from 28 CTL clones from three donors selected based on reactivity toward melanoma cells of patient 1 were sequenced (table S4), yielding 11 unique TCR sequences, with one or two TCR sequences identified per antigen-donor combination. Nine of these were reconstructed and eight were successfully expressed in peripheral blood T cells, as confirmed by anti-TCRβ staining (fig. S6), targeting all four epitopes for which anti-tumor reactivity was seen. TCR-transduced PBMCs were then tested for degranulation and IFN-γ production in response to cognate melanoma cells, with results for the five most responsive TCRs shown in Fig. 3.

The $MLL\text{-}2_{L>H}$-reactive TCR 41 strongly recognized patient-derived melanoma cells carrying the mutant MLL2 gene, with low reactivity to a third party melanoma line lacking this mutation, unless the mutant epitope was genetically introduced (Fig. 3A). Furthermore, when the mutant MLL2 ORF in the cognate melanoma was disrupted, recognition of the mutant tumor was comparable to that of the third party tumor (Fig. 3A). In addition, three $CDK4_{R>L}$-reactive TCRs were expressed in healthy donor T cells (TCR *53*, *55* and *57*), with two of these showing high recognition of both the cognate melanoma that carries the mutant CDK4 gene and of melanoma cell line Mel *526*, which carries the previously described CDK4 R24C mutation (*17*) (Fig. 3B). Notably, recognition by TCRs *53* and *57* was comparable to that seen for the patient-derived $CDK4_{R>L}$-reactive TCR 17, previously isolated from TIL of patient 1 (Fig. 3, B and C, and fig. S7). The $ASTN1_{P>L}$-reactive TCR *65* also showed specific recognition of cognate melanoma (Fig. 3D). $ASTN1_{P>L}$-reactive TCR *52* and $SMARCD3_{H>Y}$-reactive TCRs *59* and *67* did not recognize cognate or third party tumor unless the relevant neoantigen was introduced. In total,

recognition of endogenously presented neoantigen on the cognate melanoma was observed for 3 out of 4 antigens evaluated.

With neoantigens emerging as attractive targets in the development of personalized immunotherapies, strategies for the rapid identification of relevant neoantigens has become a major priority. We speculated that the use of outsourced immune responses could facilitate analysis of the rules that govern neoantigen recognition by T cells. In the current experiments, immunogenicity was evaluated for 57 peptides that had been selected based on predicted binding affinity to HLA-A*02:01. Of these, 11 generated immune responses, and T cells reactive with 10 of these epitopes recognized endogenously presented antigen. The median predicted binding affinity for this set of T cell recognized neoantigens was 28 nM (range 6-119 nM), compared to 54 nM (range 2-925 nM) for peptides that did not induce immune responses (Fig. 4A). Prior work has suggested that pMHC complex stability may form a particularly strong determinant of immunogenicity (*18*, *19*). To test the added value of experimental analysis of MHC off-rate, we developed a flow cytometry-based assay for pMHC stability (fig. S8, A and B, and tables S1, S2, S3, and S5). Analysis of pMHC off-rates for all 57 predicted neoantigens revealed that neopeptides that were recognized by donor-derived T cells displayed a significantly longer half-life as compared to neopeptides for which no responses were observed (median $t_{1/2}$ of β2M signal: 14.3 vs 4.7 hours, $p < 0.0001$) (Fig. 4B). Using a $t_{1/2}$ cut off value of 5 hours, 11 of 32 (34%) candidate neoantigens were recognized by donor T cells (Fig. 4C). Furthermore, the significant added value of measured pMHC off-rates as compared to the sole *in silico* prediction of peptide affinity is also apparent from receiver-operating characteristic (ROC) curves (fig. S8C).

In the current study, we demonstrate that T cell repertoires from healthy donors provide a rich source of T cells that specifically recognize neoantigens present on human tumors. Responses to 11 different epitopes were observed, and for the majority of evaluated epitopes, potent and specific recognition of tumor cells endogenously presenting the neoantigens was detected. The main conclusions from this work are three-fold. First, these results demonstrate the existence of a repertoire of neoantigens on human tumors to which the endogenous T cell pool has not mounted a measurable response in vivo, but that can be the target of T cells from an independent source. Specifically, among the neoantigen specific T cell populations capable of recognizing endogenously processed antigen, only one was also detected within the original TIL. This observation forms a strong incentive for the further development of immunotherapies that aim to broaden neoantigen specific T cell reactivity (*14*, *20*, *21*), either from an exogenous source, or from the endogenous T cell pool. Of note, the latter approach does rely on the presence of patient T cells that still have the capacity to respond to these neglected epitopes, an issue that remains to be addressed. Second, the ability to evaluate large series of predicted epitopes for recognition by T cells from multiple independent T cell repertoires makes it feasible to systematically examine the rules that control neoantigen recognition. Third, the current results suggest the possibility of personalized neoantigen-directed immunotherapies that are independent of the status of the patient´s own immune system.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/cgi/content/full/science.aaf2288/DC1
Materials and Methods
Figs. S1 to S8
Tables S1 to S8
References (*22*–*43*)

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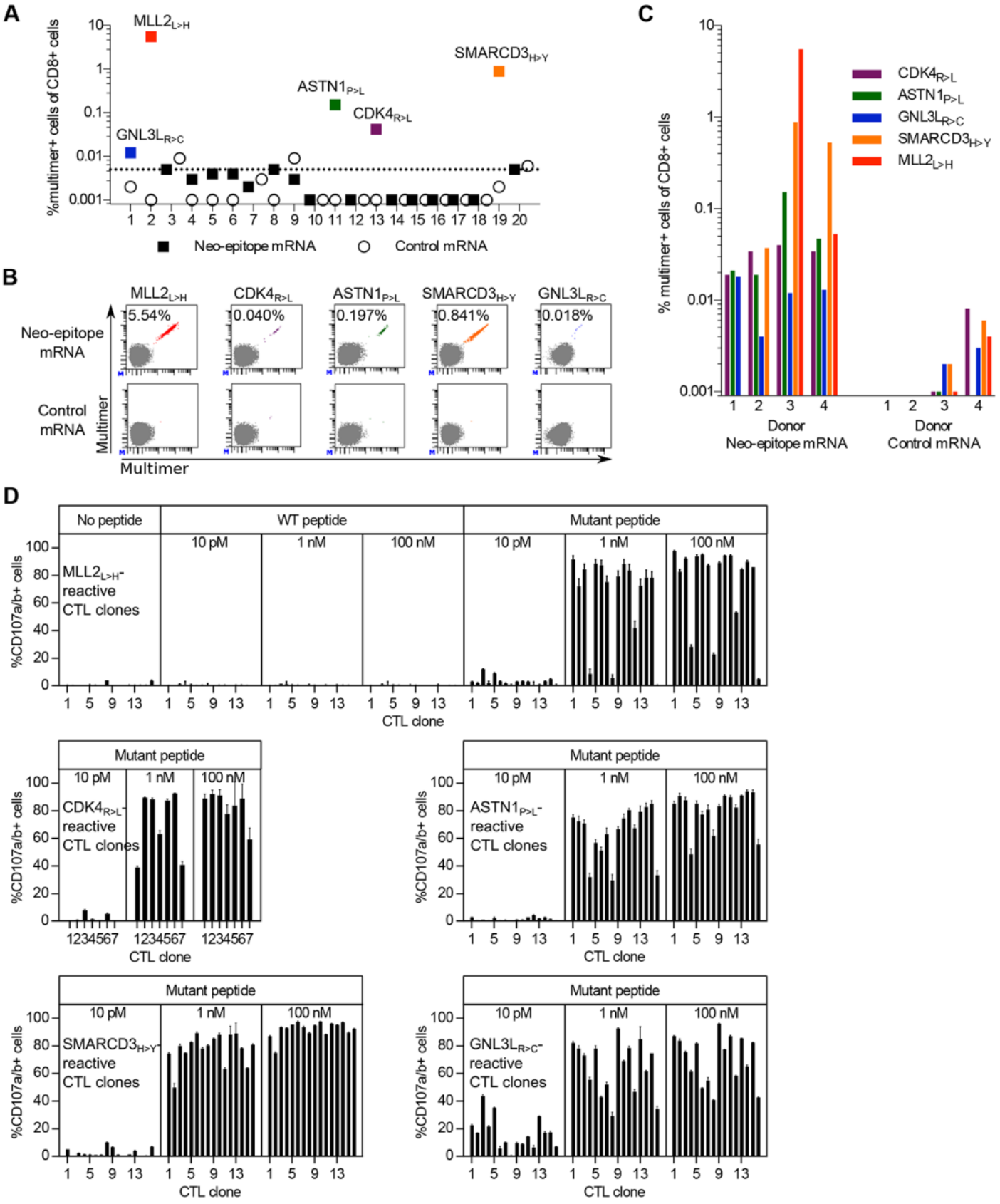

Fig. 1 (previous page). In vitro induction and functional activity of donor-derived neoantigen reactive T cells. Data in (A) to (D) depict T cell responses against predicted HLA-A*02:01-binding neoantigens from patient 1. (**A**) PBMCs (donor 3) stimulated with autologous APCs transfected with minigene mRNA encoding predicted neoantigens (filled squares) or CT/CD20 control antigens (open circles) were stained with pMHC multimers complexed with predicted epitopes. Symbols indicate percentage of live $CD8^{pos}$ cells staining positively for pMHC-multimers complexed with indicated peptides. Colored squares indicate populations sorted for further analysis, and (**B**) shows the flow cytometry analysis. (**C**) Magnitude of $multimer^{pos}$ T cell populations for the indicated predicted neoantigens induced by APCs transfected with mRNA encoded by either relevant neoepitope minigene (left) or control CT/CD20 minigene (right) from four healthy donors. (**D**) Degranulation responses of CTL clones (donor 4) analyzed as shown in fig. S2. Each graph represents the reactivity of 7-16 clones to indicated neoantigen. Controls are depicted only for $MLL2_{L>H}$-reactive CTL clones, corresponding data for remaining clones are depicted in fig. S3A. Graphs are representative for CTL clones from all donors tested and show means of triplicates. Error bars indicate SD.

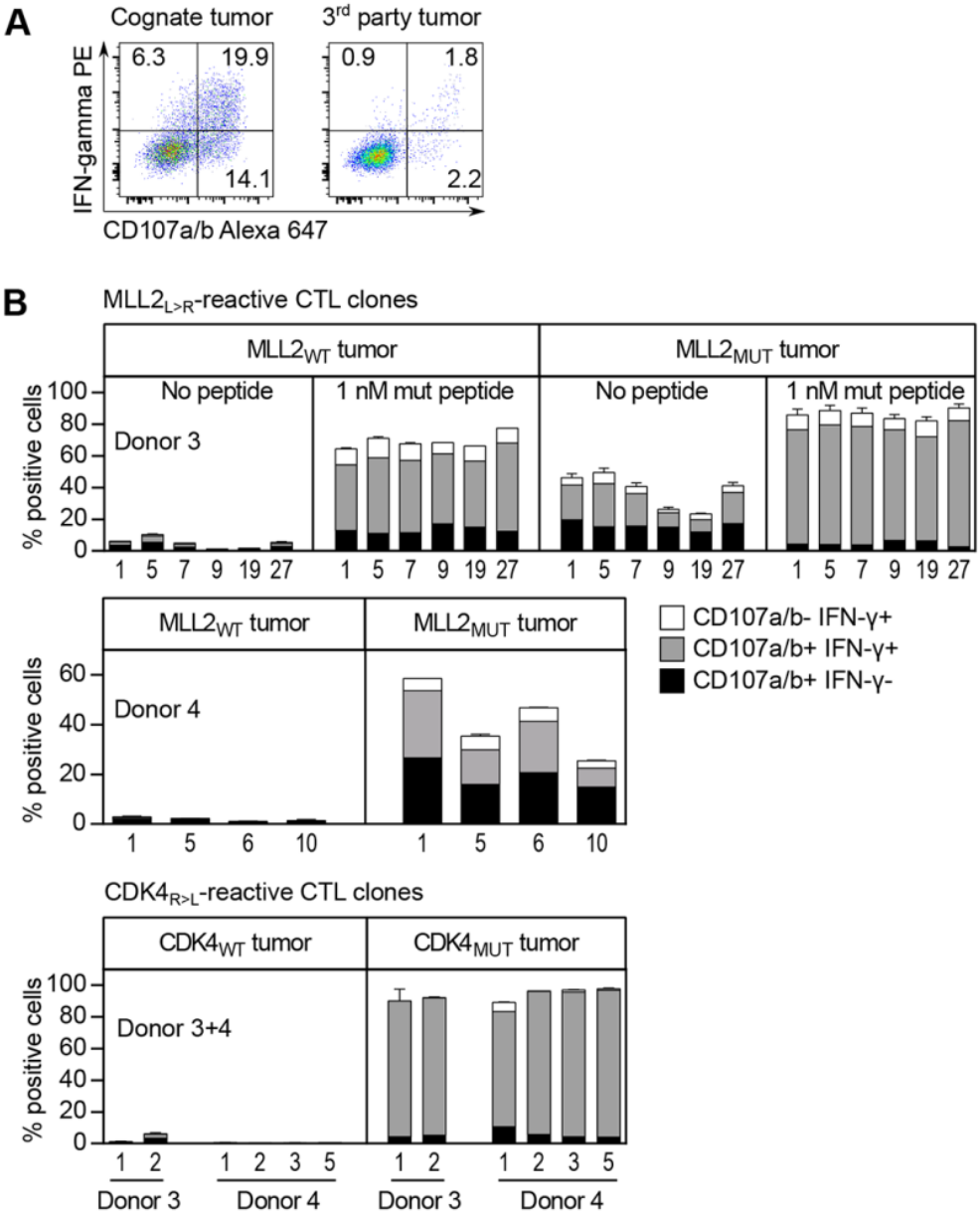

Fig. 2. Tumor recognition by donor-derived neoantigen-reactive CTL. (**A**) $MLL2_{L>H}$-reactive CTL clone #7 (donor 3) was incubated with a melanoma line derived from patient 1 that carried the mutated MLL2 gene (Cognate tumor, left), or with a third party HLA-A*02:01^{pos} melanoma line carrying the WT MLL2 gene (3rd party tumor, right). Data depict live CD8 cells and the percentage of IFN-γ^{pos} and/or CD107a/b^{pos} cells. (**B**) Neoantigen reactive CTL clones (donors 3-4) incubated with indicated melanoma lines were analyzed as described in (A). Where indicated, melanoma lines were pulsed with 1 nM of the corresponding neoantigen (shown for $MLL2_{L>H}$-reactive CTL clones from donor 3). All $MLL2_{L>H}$- and $CDK4_{R>L}$-reactive CTL clones were selected for TCR sequencing. Graphs show means of duplicates, error bars indicate SD.

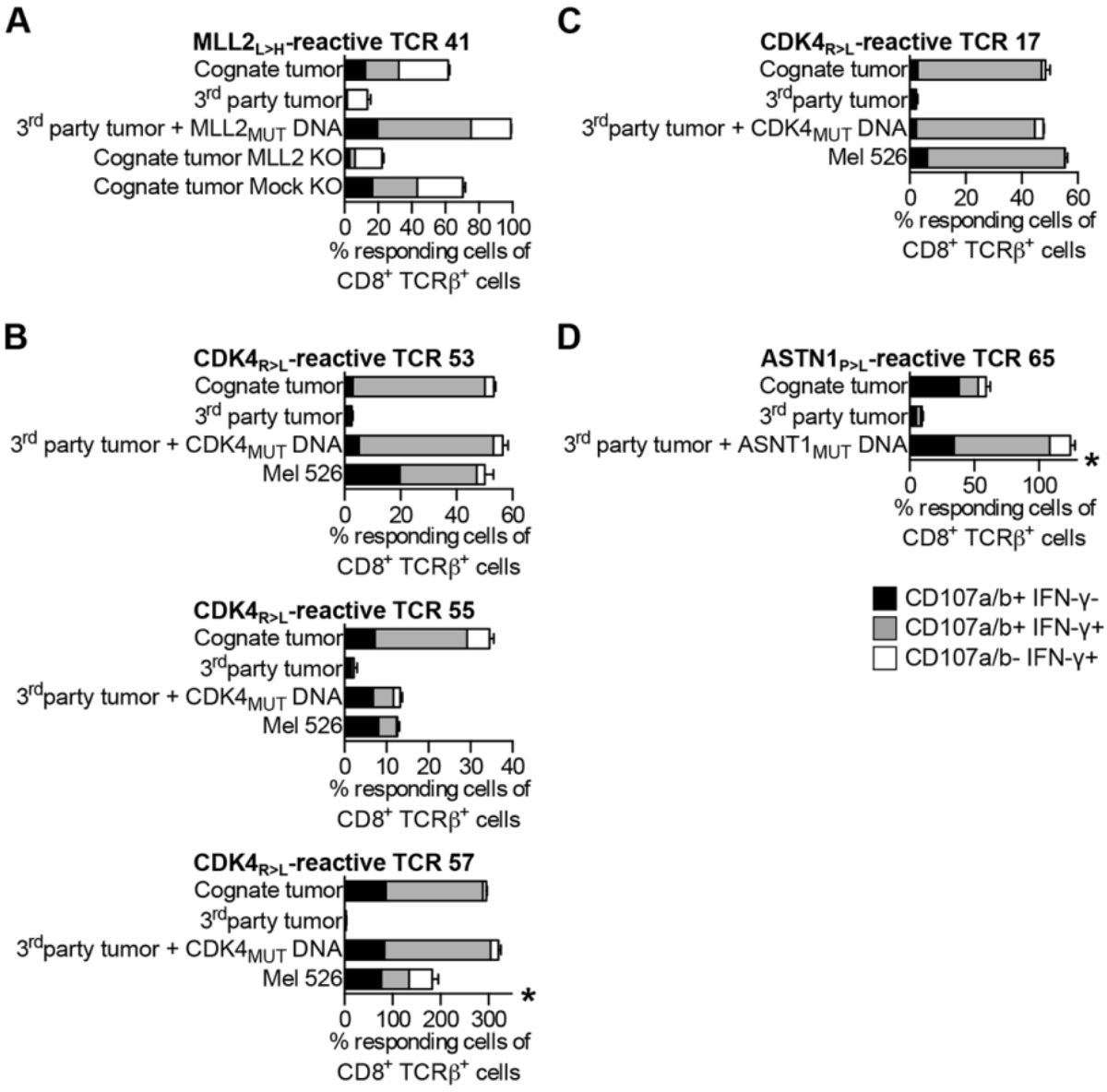

Fig. 3. Tumor recognition by genetic transfer of donor-derived neoantigen specific TCRs. Healthy donor peripheral blood T cells transduced with indicated donor-derived TCRs [(A), (B), (D)] or a patient-derived TCR (C) were incubated with either cognate melanoma line (Cognate tumor), an HLA-A*02:01^{pos} third party melanoma line (3rd party tumor), or melanoma cells modified as indicated. Percentage degranulating CD8 cells was analyzed as described in Fig. 2. (**A**) Melanoma cells in which the mutant MLL2 gene was knocked out (Cognate tumor MLL2 KO), mock-treated cognate melanoma cells (Cognate tumor Mock KO), or third party melanoma cells stably transfected with DNA encoding the relevant mutant neoantigen (3rd party tumor + MLL2$_{MUT}$ DNA) were used as target cells. (**B** and **C**) Third party melanoma cells stably transfected with DNA encoding the relevant neoantigen (3rd party tumor + CDK4$_{MUT}$ DNA), or the melanoma cell line Mel 526 were used as target cells. (**D**) Third party melanoma cells stably transfected with DNA encoding the relevant neoantigen (3rd party tumor + ASTN1$_{MUT}$ DNA) were used as target cells. Data for each TCR are representative of 2-3 independent experiments using T cells from different healthy donors. Graphs depict mean of duplicate samples, error bars indicate SD. Values were corrected for transduction efficiency, measured as percentage of CD8^{pos} cells staining positively with anti-mouse TCRβ chain antibody. Asterisk (*) indicates TCRs for which the fraction of TCR-expressing T cells is underestimated by staining with anti-mouse TCRβ chain constant domain antibody (fig. S6).

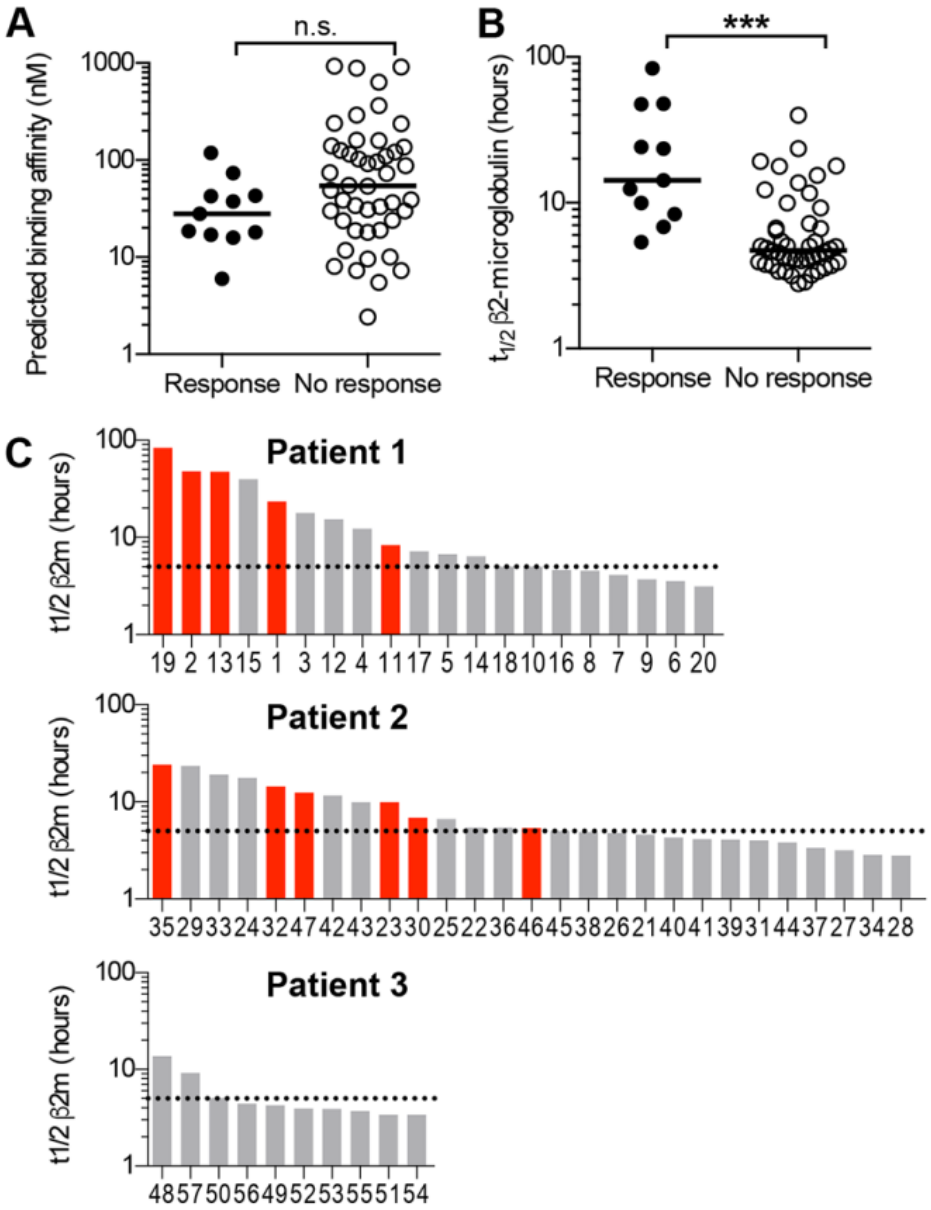

Fig. 4. pMHC stability predicts neoantigen immunogenicity. Predicted binding affinity to HLA-A*02:01 (**A**) and experimentally determined half-life of peptide-HLA-A*02:01 complexes, as measured by dissociation of β2-microglobulin (**B**) for the 57 predicted neoantigens from patients 1-3 that do, or do not, induce a T cell response. Peptide sequences and predicted affinities are listed in tables S1 to S3. (**C**) Red bars represent predicted neoantigens that were shown to be immunogenic, grey bars represent predicted neoantigens for which no T cell response could be detected. Dotted line represents suggested cut-off value of $t_{1/2}$ = 5 hours. Values in (B) and (C) represent means of triplicates. ***; $p < 0.0001$, n.s.; not significant, Mann-Whitney U.

Targeting of cancer neoantigens with donor-derived T cell receptor repertoires

Erlend Strønen, Mireille Toebes, Sander Kelderman, Marit M. van Buuren, Weiwen Yang, Nienke van Rooij, Marco Donia, Maxi-Lu Böschen, Fridtjof Lund-Johansen, Johanna Olweus and Ton N. Schumacher

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