Computationally Guided Design of Single-Chain Variable Fragment Improves Specificity of Chimeric Antigen Receptors

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Chimeric antigen receptor (CAR)-T cell-based immunotherapy of malignant disease relies on the specificity and association constant of single-chain variable fragments (scFvs). The latter are synthesized from parent antibodies by fusing their light (V_L) and heavy (V_H)-chain variable domains into a single chain using a flexible linker peptide. The fusion of V_L and V_H domains can distort their relative orientation, thereby compromising specificity and association constant of scFv, and reducing the lytic efficacy of CAR-T cells. Here, we circumvent the complications of domains’ fusion by designing scFv mutants that stabilize interaction between scFv and its target, thereby rescuing scFv efficacy. We employ an iterative approach, based on structural modeling and mutagenesis driven by computational protein design. To demonstrate the power of this approach, we use the scFv derived from an antibody specific to a human leukocyte antigen A2 (HLA-A2)-HER2-derived peptide complex. Whereas the parental antibody is highly specific to its target, the scFv showed reduced specificity. Using our approach, we design mutations into scFvs that restore specificity of the original antibody.

INTRODUCTION

Single-chain variable fragments (scFvs), obtained from antibodies by connecting their light (V_L) and heavy (V_H)-chain domains with a peptide flexible linker, allow harnessing specificity of antibodies in a conceptually simpler single-chain construct. scFvs are generally used in phage display,1 flow cytometry, immunohistochemistry, and more recently, in generating chimeric antigen receptors (CARs) in which the scFv is fused with T cell signaling molecules to redirect the antigen specificity of T lymphocytes.22 Despite successful applications of scFv in CAR-T, the specificity and the association constant of the scFvs do not always match those of the parent antibody, because rewiring the connectivity of V_L and V_H chain domains of the parent antibody can change their dynamics and relative orientation.

To demonstrate this complication in constructing scFv, we selected an antibody that recognizes a peptide processed and presented physiologically into the groove of the human leukocyte antigen A2 (HLA-A2) complex, reasoning that even small distortions of the antibody structure in a scFv format may alter the recognition of the peptide/HLA complex because of the high specificity of the peptide/HLA interaction. We developed an antibody (SF2) specific to the HLA-A2/peptide complex in which the KIFGSLAFL peptide derives from the human HER2 protein. The SF2 antibody shows no binding toward the empty HLA-A2 complex, whereas the binding to the HLA-A2/KIFGSLAFL peptide complex is typically peptide concentration dependent (Figure S1). We then cloned the V_H and V_L chains of the SF2 antibody and generated a scFv by connecting the C terminus of V_L with the N terminus of V_H via a Gly-Ser linker. This scFv was then fused with the hinge and transmembrane domain of CD8α and endodomains of CD28 and CD3ζ to generate the CAR (SF2.CAR) (Figure S2A). Retroviral particles encoding the SF2.CAR were used to generate SF2.CAR-expressing T cells (SF2.CAR-Ts) using previously validated protocols to generate CAR-T cells for clinical use (Figures S2B and S2C). Surprisingly, SF2.CAR-T cells displayed activity not only toward T2 cells loaded with the HER2 KIFGSLAFL peptide, but also against empty T2 cells and T2 cells loaded with an irrelevant peptide as assessed by interferon gamma (IFNγ) release assay (Figure S2D), indicating that the scFv conformation or its assembling in the CAR format substantially modify the specificity of the parental SF2 antibody.

We argued that by mutating key residues in the binding interface between scFv and the HLA-A2/peptide complex, we can restore specificity of scFv toward the target HER2 peptide. This approach requires knowledge of the complex tertiary structure. Traditional methods of tertiary
structure determination such as X-ray crystallography or cryo-electron microscopy are technically challenging and provide only a static snapshot of the structure. NMR can be used to explore structure dynamics, but protein size is a limiting factor for the NMR applicability. Computational modeling provides an alternative to experimental approaches and allows to predict structure and explore its dynamics. However, due to inaccuracies present in modern force fields, computational modeling cannot unambiguously identify the correct structure but can provide a range of alternative models. To validate the model of scFv bound to the HLA-A2/peptide complex, we generated a set of scFv mutants. We developed an empirical scoring function, which allowed us to rank computational models based on comparison between predicted and experimentally measured effects, which these mutants exert on binding between scFv and the HLA-A2/peptide complex. The top-ranked models were selected and evaluated using an additional set of mutants. By following this iterative modeling and redesign procedure, we determined the structure of scFv complexed with HLA-A2 loaded with the HER-2 peptide, and identified mutations that improve the binding specificity of the scFv in the CAR format.

RESULTS
Probing Structure of scFv Bound to the HLA-A2/KIFGSLALF Peptide Complex
The computational methods used for prediction of antibody structure have gradually improved over the last decade as revealed by the Antibody Modeling Assessment, a community-wide competition aimed to evaluate performance of antibody modeling software.5,6 The best models match experimental structures with angstrom accuracy even for the hardest to predict, the third complementarity-determining region (CDR) of the VH variable domain.7 To predict the tertiary structure of the SF2 antibody, we used antibody modeling software.8 The corresponding scFv is obtained by connecting the C terminus of VL to the N terminus of VH by (GGGGS)_3 linker. To explore dynamics of corresponding scFv is obtained by connecting the C terminus of VL to simulations9 these structures, we ran discrete molecular dynamics (DMD) simulations to model HLA-A2/peptide (Materials and Methods). To infer binding pose of scFv to the HLA-A2/peptide complex, we used docking software.12,13 After discarding apparently inconsistent models (Figure 2B), where none of the six complementarity-determining regions (CDRs) of the scFv binds the HER2 peptide, we obtained 270 3D models. We then clustered these models (Figure 2C) and identified eight distinct clusters (Figure S3). For each cluster we chose a centroid structure as a representative model. For each residue within CDRs of representative models, we considered all possible mutations and calculated: (1) change in stability of the scFv/HLA-A2/peptide complex upon mutation; (2) change in the interaction energy between scFv and peptide; and (3) change in the interaction energy between scFv and HLA-A2 (Materials and Methods). We selected a set of six mutations in VH and six mutations in VL domains (named set I) for further experimental probing. The selected mutations stabilize the interaction between scFv and peptide relatively to the interaction between the antibody and HLA-A2 complex in more than one considered model.

We validated these mutations by generating mutant SF2.CAR molecules, constructing CAR-T cells for each specific mutant, and testing their specific activity. For this purpose, we measured IFNγ release by CAR-T cells co-cultured with T2 cells loaded with the specific HER2-P_{369-377} or with the irrelevant MAGEA3-P_{271-279} peptide or not loaded with any peptide (Figure S4). The results showed that the mutations VL-S31Y, VL-G93L, and VH-G55F did not affect the specific binding to HER2-P_{369-377} peptide-loaded HLA-A2 molecules but decreased the binding to irrelevant MAGEA3-P_{271-279} peptide-loaded HLA-A2 molecules and to empty HLA-A2 molecules.

For each computational model we calculated the scoring function in order to assess compatibility of the model and experimental data (Figure 2D) (Materials and Methods). The scoring function is a sum of scores over all experimentally tested mutations and consists of two terms. In the first term the theoretically calculated change in global stability of the HLA-A2/peptide-antibody complex (ΔΔG^{TH}) is compared with the approximate value derived from experimental data (ΔΔG^{EXP}). In the second term the relative stabilization of interaction between scFv and peptide versus interaction between scFv and HLA-A2 is calculated theoretically (ΔE_{p•p-HLA-Ab}^{TH}) and compared with the approximate value derived from experimental data (ΔE_{p•p-HLA-Ab}^{EXP}). The model with minimal score corresponds to the model best matching the experimental data.
CAR-T cells generated from multiple donors and illustrated in Figure 3. They were evaluated using our scoring function. Based on these results, we selected the model with the lowest scoring function (Figure 4A).

**Assessment of the Model Quality**

The selected model correctly describes most of the mutations from sets I and II. Three mutations from set I (V<sub>L</sub> S31Y, V<sub>L</sub> G93L, and V<sub>H</sub> G55F) exhibited decrease in binding affinity to both empty and peptide-loaded HLA-A2 complex (Figure 5A). Based on our model, we predict that upon these mutations, large hydrophobic residues become exposed to the solvent (Figures 4B–4D), thus decreasing overall stability of the complex. Two mutations from set II (V<sub>H</sub> S100V and V<sub>H</sub> S53M) (Figures 4E and 4F) preserved binding affinity of the scFv to peptide-loaded HLA-A2, whereas drastically reducing binding to the empty HLA-A2 complex (Figure 5B). In line with this observation, our model suggested that these residues directly interact with F8 residue of the peptide, which provides an anchor for successful recognition of the peptide by the scFv. These mutations increase contact surface between F8 and the scFv, thus increasing the energy of van der Waals interactions.

The developed structural model also explains differential effects of the A50 mutations, such that A50L completely abrogates the interaction between scFv and the HLA-A2 complex, whereas A50V conserves some residual binding, and A50F preserves the binding but reduces its specificity. According to our model, A50L and A50V mutations result in clashes with the HLA-A2 H1 helix, whereas A50F mutation rests on top of the H1 helix without clashes (Figure 5C). Furthermore, interaction between A50F mutation and H1 helix increases the binding between scFv and the empty HLA-A2 complex, causing reduced specificity for the interaction with the peptide.

We also compared binding pose of the scFv as inferred from the modeling to the orientation of typical T cell receptor (TCR) bound to the HLA-A2/peptide complex (Figure 5D). At least 50 unique TCR-peptide-major histocompatibility complex (MHC) class I complexes with 3D structures have been deposited into the PDB. All of them follow the classical binding pattern of TCR with a few exceptions where TCR orientation is flipped by 180°. A typical TCR binds the MHC complex in the gap between H1 and H2 helices, thus maximizing interaction with a peptide and minimizing non-specific interaction with empty MHC. The interaction with the peptide is mediated by CDR3 loops, whereas germline-encoded CDR1 and CDR2 loops mostly contact MHC H1 and H2 helices. In our model, scFv accommodates a different binding pose. We observed that contact between scFv and peptide is mediated by H2 and H3 CDR loops. V<sub>L</sub> does not bind the peptide but makes extensive contact to MHC walls. This structure explains the high non-specific binding of the wild-type scFv.

**DISCUSSION**

Here, we presented a novel approach for optimization of binding specificity of scFv. It relies on structural modeling and mutagenesis, and does not require prior knowledge of antibody 3D structure. We validated our approach on the scFv derived from an antibody specific to the KIFGSLAFL peptide loaded into the HLA-A2 complex. We built a model of the complex that captures fine structural details, such that it can explain the differences in binding of scFv to the
HLA-A2/peptide complex when the same residue is mutated to different amino acids.

We found that the scFv is more flexible than parental SF2 antibody. We also predicted that binding pose of the scFv on the top of the HLA-A2/peptide complex differs from what is expected based on comparison with TCR binding. These two observations together can explain non-specific binding observed for the wild-type scFv. We established two mutations that reduce non-specific binding, while preserving binding to peptide loaded to the HLA-A2 complex at the same level.

An interesting avenue to explore is possible allosteric effects, which mutations exhibit on binding interface between scFv and the HLA-A2/peptide complex. The phenomenon of allostery, which refers to mutations exhibit on binding interface between scFv and the HLA-A2/peptide complex. The phenomenon of allostery, which refers to the process when perturbation of one site within a macromolecule affects another distant site, attracts broad attention.17 In particular, loop regions are shown to be important for propagation of allosteric effects,20 which suggests potential importance of CDR loops in allosteric modulation of antigen binding. It was previously shown that mutations can trigger allosteric modulations of antibody21 and scFv.22 To reveal allosteric effects within our system, we used the AlloSigMA server23 (Materials and Methods). Interestingly, we found changes in protein dynamics far away from mutation sites (Figure S8), which reveals allosteric modulation of binding between scFv and the HLA-A2/peptide complex.

In the current manuscript we use standard (GGGGS)3 linker to connect VL and VH chain variable domains into scFv. Optimization of the linker length and composition can be used to further improve specificity of scFv. It was previously shown that lengthening of (GGGGS)n linker can increase activity of scFv toward corresponding antigen,24 whereas shortening of the linker can destabilize interaction of VL and VH within a single scFv and leads to multimerization of scFv.25

Although antibodies can be derived to bind peptide inserted into the MHC complex, their sequence is not optimized for minimization of non-specific binding to the MHC complex as it is in the case of TCRs, which evolved complementarity to MHC during millions of years of evolution. Optimizing antibody design using sequence snippets of the α- and β-chains of TCR may improve low non-specific binding affinity of TCRs.

There is a significant demand of finding an efficient way to optimize the affinity of the scFv in CAR molecules with the goal to select and maintain high specificity for antigens expressed at high levels in tumor cells, but also expressed at low levels in some normal tissues. Our proposed computational model can also be adapted to tune the affinity of the scFv to minimize on target but out of tumor activity.

MATERIALS AND METHODS

Structure Modeling and Model Selection Workflow

The 3D structure of the SF2 antibody was built using the Kotai web server.8 In total, 21 models of antibody, which differ in the conformation of the third complementarity-determining regions of a VH variable domain (CDR H3), are considered (Figure 2A). HLA-A2 structure is built based on the template structure (PDB: 1HHI), where we replace the original peptide by the HER2 peptide. For that we kept peptide backbone geometry intact and replaced corresponding side chains. We verified that anchor residues (positions 2, 6, and 9)26 point inward HLA-A2. The initial structure was run through short DMD simulation to release inter-atomic clashes. The simulation was run for 10,000 steps at T = 0.5 kcal/(mol·kB) with heat exchange coefficient = 10. The Andersen thermostat is used.27

DMD simulations for the RMSF plot were performed for 1 million steps at T = 0.55 kcal/(mol kT) with heat exchange coefficient = 0.1. In total, five trajectories for SF2 antibody and five trajectories for the scFv form of the antibody were produced. RMSF is calculated over carbon-alpha atoms of the protein backbones.

Docking was performed with the ClusPro13 web server, resulting in 598 models in total (Figure 2B). The structure used for docking contains VH and VL variable domains without the Gly-Ser linker. After discarding apparently incorrect models, where none of the six complementarity-determining regions (CDRs) of antibodies touch the HER2 peptide, we got 270 3D models. The resulting models were run through DMD simulations [10,000 steps,
atoms of antibodies (Figure 2C), which gave eight distinct clusters by root-mean-square deviation (RMSD) of carbon-alpha backbone. Medusa force field peptide-antibody complex stability upon the mutation (Figure 2D). For that we calculate: (1) change of HLA-A2/peptide interaction energy between antibody and HLA-A2 (ΔE_{HLA-Ab}); and (3) change of interaction energy between antibody and peptide (ΔE_{pep-Ab}). The change of stability is calculated using a software toolkit based on DMD10 and Eris software package in the scanning mode.29,30 The interaction energies are calculated using an empirical energy function:

\[
E_{\text{ Interaction}} = \left( \frac{\text{IFN}_\text{WT}^{\text{HLA-Ab}} - \text{IFN}_\text{MUT}^{\text{HLA-Ab}}}{\text{IFN}_\text{WT}^{\text{HLA-Ab}} - \text{IFN}_\text{WT}^{\text{HLA-Ab}}} \right)
\]

where \( \text{IFN}_\text{WT}^{\text{HLA-Ab}} \) and \( \text{IFN}_\text{MUT}^{\text{HLA-Ab}} \) define interferon gamma response as measured by ELISA assay for the wild-type and mutated antibodies against HLA-A2/peptide complex.

\[
\Delta \Delta G = \left\{ \begin{array}{ll}
-1 & \text{if } x < -1 \\
x & \text{if } -1 < x < 1 \\
1 & \text{if } x > 1
\end{array} \right.
\]

\[
y = \frac{\Delta G}{E_{\text{thresh}}} \text{ and } E_{\text{thresh}} = 4 \text{kcal/mol} \text{ is an empirically derived threshold. The mutations with } \Delta \Delta G \text{ exceeding this threshold severely affect protein stability.}
\]

\[
\Delta \Delta G^\text{Exp} = \left\{ \begin{array}{ll}
-1 & \text{if } y < -1 \\
y & \text{if } y > -1
\end{array} \right.
\]

The first term in Equation 1 evaluates the difference between theoretical and experimentally observed changes in HLA-A2/peptide-antibody complex stability upon mutation, and the second term evaluates the difference between theoretical and experimentally observed ability of mutation to stabilize interactions between antibody and peptide as compared with the interaction between antibody and HLA-A2.

**Evaluation of Allosteric Effects**

To evaluate allosteric effects in the scFv/HLA-A2/peptide system, we employed the AlloSigMA server.23 The effect of three mutations (\( V_\text{V} \_\text{S}31\text{Y} \), \( V_\text{V} \_\text{G}93\text{L} \) and \( V_\text{V} \_\text{G}55\text{F} \)) was calculated using the “DOWN-mutated residue” option. This option defines the loosen local contact network. We chose it to describe local.

\[
\text{SCORE} = \sum \left( \Delta \Delta G^\text{TH} - \Delta \Delta G^\text{Exp} \right)^2
\]

where sum is calculated over all experimentally tested mutations for each model.

\[
\Delta \Delta G^\text{TH} = \left\{ \begin{array}{ll}
-1 & \text{if } x < -1 \\
x & \text{if } -1 < x < 1 \\
1 & \text{if } x > 1
\end{array} \right.
\]

\[
x = \frac{\Delta \Delta G}{E_{\text{thresh}}} \text{ and } E_{\text{thresh}} = 4 \text{kcal/mol} \text{ is an empirically derived threshold. The mutations with } \Delta \Delta G \text{ exceeding this threshold severely affect protein stability.}
\]

\[
\Delta \Delta G^\text{Exp} = \left\{ \begin{array}{ll}
-1 & \text{if } y < -1 \\
y & \text{if } y > -1
\end{array} \right.
\]

\[
y = \left( \frac{\text{IFN}_\text{WT}^{\text{HLA-Ab}} - \text{IFN}_\text{MUT}^{\text{HLA-Ab}}}{\text{IFN}_\text{WT}^{\text{HLA-Ab}}} \right)
\]

\[
\text{IFN}_\text{WT}^{\text{HLA-Ab}} \text{ and } \text{IFN}_\text{MUT}^{\text{HLA-Ab}} \text{ define interferon gamma response as measured by ELISA assay for the wild-type and mutated antibodies against empty HLA-A2.}
\]

We identified CDRs using a previously described set of rules.28 For each structure representing centroid of clusters and for each CDR residue (61 residues in total) we determined the effect of every possible mutation (Figure 2D). For that we calculate: (1) change of HLA-A2/peptide-antibody complex stability upon the mutation (ΔΔG); (2) change of interaction energy between antibody and HLA-A2 (ΔE_{HLA-Ab}); and (3) change of interaction energy between antibody and peptide (ΔE_{pep-Ab}). The change of stability is calculated using Medusa force field package in the scanning mode.29 The interaction energies are calculated using a software toolkit based on DMD10 and Medusa force field.31,32 For experimental testing we selected mutations that stabilize interactions between antibody and peptide as compared with the interaction between antibody and HLA-A2 (ΔΔG < 1.5 and ΔE_{pep-Ab} - ΔE_{HLA-Ab} < -1).

The models are checked for consistency with experimental data. For that we define empirical energy function:
destabilization, which results in decreased binding of scFv to irrelevant MAGEA3-P371-379 peptide-loaded HLA-A2 molecules and to empty HLA-A2 molecules. The effect of mutations V1 H1 S100V and V1 H1 S33M was calculated using the "UP-mutated residue" option. This option defines the stiffen local contact network. It was chosen to describe local stabilization, which results in increased binding of scFv to HER2-P369-377 peptide-loaded HLA-A2 molecules.

Cell Lines
T2 cells were purchased from American Type Culture Collection (ATCC) and cultured in RPMI1640 medium (GIBCO) supplemented with 10% FBS and 2 mM GlutaMAX. All colony lines were mycoplasma free and validated by flow cytometry for surface markers and functional readouts as needed.

Generation and Characterization of mAb SF2
The mAb SF1 is secreted by a hybridoma generated by fusing mouse myeloma cells P3-X63-Ag8.653 with splenocytes from an 8-week-old female BALB/c mouse immunized with HLA-A2-antigen HER2/neu369-377 peptide complexes according to the following schedule. Three days before priming, the 6-week-old female BALB/c mouse was injected intramuscularly with 100 μg (per mouse) of granulocyte-macrophage colony-stimulating factor (GM-CSF) plasmid. Subsequently, the mouse was primed with HLA-A2 antigen HER2/neu369-377 peptide complexes (50 μg) utilizing saponin, which can enhance both B cell and T cell response as an adjuvant. Boosters were given at 2-week intervals for a total of 14 times. Four days following the last booster a mouse was sacrificed. Splenocytes were harvested from the immunized mouse and fused to mouse myeloma cells P3-X63-Ag8.653 at a ratio of 1:1 as previously described. Twenty 96-well, flat-bottom microtiter plates (Corning, Corning, NY, USA) were seeded with 1.5 × 10^5 cells/well. Growth of colonies was observed in all seeded wells. Supernatants were screened in ELISA with HLA-A2 antigen HER2/neu369-377 peptide complexes.

HLA-A2 antigen/MAGE-3P371-379 peptide complexes and HLA-A2/MART1P27-35 peptide complexes were used as specificity controls. In the first screening, about 30 hybridomas displayed higher reactivity with HLA-A2 antigen HER2/neu369-377 peptide complexes than with the other complexes. In additional screenings, only the hybridoma SF1 maintained its selected reactivity with HLA-A2 antigen HER2/neu369-377 peptide complexes. The mAb SF1-stained T2 cells pulsed with the HER2/neu369-377 peptide, but not with peptides derived from other tumor antigens. On the other hand, the mAb SF1 did not stain cell lines that expressed both HLA-A2 and HER2/neu.

Plasmid Construction and Retrovirus Production
The variable regions of the V1 H and V1 L chains of the SF2 mAb were cloned from the SF2 mouse hybridoma. The V1 H amino acid sequence was: 1-DIQMTQSPASLSVSVGTVTITCRPSENLYSLAYQQK QGKSPQLVYAAATHLADGVPSRFSGSGTQYSLKINSLQSEDFT GTYVYQHWGTPYTFGGGTKEIK-107. CDRs as identified using a previously described set of rules are underlined. The corresponding residue ranges are CDR1 (residues 24–34), CDR2 (residues 49–56), and CDR3 (residues 89–97). The V1 L amino acid sequence was: 1-QVQLKQSGPGLLQPSQSLSITCTVSGFSLTSYGVHVRQPS GKGLGLEWLGIVS5GSGTSDYNAAFISLSKDSKQFFKMS LIQDDTAYICYCATGSHWYTFDVWGAAGTVTSS-117. CDRs as identified using a previously described set of rules are underlined. The corresponding residue ranges are CDR1 (residues 23–35), CDR2 (residues 50–58), and CDR3 (residues 96–106). The scFv was created by connecting the C terminus of V1 H with the N terminus of V1 L using 15-residue peptide linker GGGGSGGG GSGGGGS and then cloned into a previously validated CAR cassette that includes the human CD8α hinge and transmembrane domain, CD28 intracellular costimulatory domains, and CD3ζ intracellular signaling domain. The SF2.CAR cassettes were cloned into the retroviral vector SFG. Mutations of the scFv were created by PCR and were listed as follows: set 1: V1 L S31Y (CDR1), N32Y (CDR1), A50L (CDR2), A50F (CDR2), T52Y (CDR2), G93L (CDR3); V1 H S28Y (CDR1), T30E (CDR1), S31L (CDR1), G55F (CDR2), S56Y (CDR2), S100M (CDR3); and set 2: V1 L N32Q (CDR1), A50V (CDR2), G93I (CDR3), G93V (CDR3). HLA-A2 MAGEA3-P271–289 and HER2/neu377 peptide, but not with peptides

Transduction and Expansion of Human T Cells
Buffy coats from healthy donors were obtained through the Gulf Coast Regional Blood Center, Houston, TX, USA. Peripheral blood mononuclear cells (PBMCs) isolated with Lymphoprep density separation (Fresenius Kabi Norge) were activated using 1 μg/mL anti-CD3 (Miltenyi Biotec) and 1 μg/mL anti-CD28 (BD Biosciences) mAb-coated plates. On day 3, T lymphocytes were transduced with retroviral supernatants using retroenectin-coated plates (Takara Bio, Shiga, Japan). After removal from retronectin plates, T cells were expanded in complete medium (45% RPMI 1640 and 45% Cig’s medium [Irvine Scientific], 10% FBS [Hyclone], 2 mM GlutaMAX, 100 U/mL penicillin, and 100 μg/mL streptomycin) with IL-7 (10 ng/mL; PeproTech) and IL-15 (5 ng/mL; PeproTech), changing medium every 2–3 days. On days 12–14, cells were collected for in vitro co-culture experiments.

Co-culture and ELISA
T cells (5 × 10^5) were co-cultured with T2 cells (5 × 10^5) in a 24-well plate without the addition of exogenous cytokines. After 24 h, supernatants were collected, and IFNγ level was measured by using an ELISA kit (R&D Systems) following the manufacturer’s instructions. Each supernatant was measured in duplicate.

Flow Cytometry
The expression of SF2.CAR was detected using anti-Fab antibody (Jackson Immunoresearch laboratories). Samples were analyzed with BD FACSanto II or BD FACSfortessa with the BD Diva software (BD Biosciences); for each sample we
acquired a minimum of 10,000 events, and data were analyzed using FlowJo 10.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2019.08.008.

AUTHOR CONTRIBUTIONS
A.K. and K.P. performed molecular dynamics simulations and interpreted the data. H.D. and K.H. performed and interpreted the molecular and immunologic experiments. T.K., X.W., and S.F. generated and provided the antibodies. G.D. and N.V.D. conceptually designed the project and interpreted the data. A.K., H.D., G.D., and N.V.D. wrote the manuscript with contributions from all of the authors.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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Supplemental Information

Computationally Guided Design of Single-Chain Variable Fragment Improves Specificity of Chimeric Antigen Receptors

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Figure S1. SF2 antibody recognizes the HLA-A2/HER2_{369-377} complex. (A) T2 cells were stained with either mouse IgG isotype or SF2 Ab, secondary goat anti-mouse IgG Ab and analyzed by flow cytometry. (B) T2 cells were loaded with different concentrations of the HER2_{369-377} peptide for 3 hours, stained with the SF2 and secondary Abs and then examined by flow cytometry. The SF2 Ab specifically stains peptide loaded T2 cells at the lowest concentration of the HER2_{369-377} peptide (1µg/mL).
Figure S2. IFNγ release by SF2.CAR-Ts. (A) The scFv derived by the SF2 Ab specific for the HLA-A2/KIFGSLAFL complex was cloned and assembled into a CAR cassette by fusing the scFv with the CD8α hinge and transmembrane domain and the intracytoplasmic CD28 and CD3ζ chain endodomains. The entire cassette was cloned into the SFG gamma-retroviral vector. (B) Expression of the SF2.CAR in transduced T cells (SF2.CAR-Ts) as assessed by flow cytometry. (C) Summary of the SF2.CAR expression in T cells in multiple donors (n=8). (D) SF2.CAR-Ts were cultured for 24 hours with empty T2 cells or T2 cells loaded with the KIFGSLAFL (HER2<sub>369-377</sub>) peptide or an irrelevant peptide (MAGEA3<sub>271-279</sub>). IFNγ released in the supernatant was measured at 24 hours by ELISA.
Figure S3. Structural models of scFv docked on the top of HLA-A2-peptide complex. Each model represents a centroid of corresponding cluster.
Figure S4. IFNγ release by set I scFv mutants expressing SF2.CAR-Ts. T cells were transduced to express the set I scFv mutants of SF2.CAR, then the transduced T cells were normalized to same expression level of CAR and co-cultured with T2 cells without peptide loading, T2 cells loaded with the irrelevant peptide MAGEA3271-279 or T2 cells loaded with low (100 ng/mL) or high (1000 ng/mL) concentration of the HER2369-377 peptide, at 1:1 ratio. Co-culture supernatants were collected after 24 hours to measure IFNγ by ELISA.
Figure S5. IFN$\gamma$ release by set II scFv mutants expressing SF2.CAR-Ts. T cells were transduced to express the set II scFv mutants of SF2.CAR, then the transduced T cells were normalized to same expression level of CAR and co-cultured with T2 cells without peptide loading, T2 cells loaded with the irrelevant peptide MAGEA3_{271-279} or T2 cells loaded with low (100 ng/mL) or high (1000 ng/mL) concentrations of the HER2_{369-377} peptide, at 1:1 ratio. Co-culture supernatants were collected after 24 hours to measure IFN$\gamma$ by ELISA.
Figure S6. Mutants V50L, A50V, A50F
Figure S7. Comparison between structural model of $\text{V}_L/\text{V}_H$ best matching experimental data and canonical binding pose of TCR ($\text{V}_\alpha/\text{V}_\beta$) on top of HLA-peptide complex (PDB ID 2ESV).
Figure S8. Allosteric modulation of scFv/HLA-A2/KIFGSLAFL peptide complex dynamics by mutations in scFv. (A) $V_L$ S31Y (B) $V_L$ G93L (C) $V_H$ G55F (D) $V_H$ S100V (E) $V_H$ S53M. The regions with decreased dynamics (stabilized) are colored red, the regions with increased dynamics (destabilized) are colored blue color, and the regions with unchanged dynamics are colored white.