Bispecific antibodies targeting mutant RAS neoantigens

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Mutations in the RAS oncogenes occur in multiple cancers, and ways to target these mutations has been the subject of intense research for decades. Most of these efforts are focused on conventional small-molecule drugs rather than antibody-based therapies because the RAS proteins are intracellular. Peptides derived from recurrent RAS mutations, G12V and Q61H/L/R, are presented on cancer cells in the context of two common human leukocyte antigen (HLA) alleles, HLA-A3 and HLA-A1, respectively. Using phage display, we isolated single-chain variable fragments (scFvs) specific for each of these mutant peptide–HLA complexes. The scFvs did not recognize the peptides derived from the wild-type form of RAS proteins or other related peptides. We then sought to develop an immunotherapeutic agent that was capable of killing cells presenting very low levels of these RAS-derived peptide–HLA complexes. Among many variations of bispecific antibodies tested, one particular format, the single-chain diabody (scDb), exhibited superior reactivity to cells expressing low levels of neoantigens. We converted the scFvs to this scDb format and demonstrated that they were capable of inducing T cell activation and killing of target cancer cells expressing endogenous levels of the mutant RAS proteins and cognate HLA alleles. CRISPR-mediated alterations of the HLA and RAS genes provided strong genetic evidence for the specificity of the scDbs. Thus, this approach could be applied to other common oncogenic mutations that are difficult to target by conventional means, allowing for more specific anticancer therapeutics.

INTRODUCTION

Selective killing of cancer cells is challenging because cancer cells and their normal counterparts are so similar. At the genetic level, the most important differences between cancer cells and normal cells are driver gene mutations, most often single–base pair substitutions (1). Targeted therapies with small-molecule anticancer drugs, such as epidermal growth factor receptor tyrosine kinase inhibitors, exploit these subtle differences by selectively inhibiting the function of the proteins encoded by the mutant genes (2). However, the vast majority of mutations in cancer driver genes cannot yet be targeted.

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One prime example of this challenge is KRAS; despite the high frequency of mutations of this gene in multiple tumor types, drugs that could selectively target these mutations have only recently shown promising clinical activity (3–5).

Antibodies in current clinical use target extracellular proteins, such as ERBB2, which are overexpressed in cancers compared with normal tissues (6). Although these antibodies have led to unprecedented clinical successes, they are not truly cancer specific, because their target proteins are present at lower amounts on normal cells. However, antibodies can also be generated that specifically react with peptides or proteins that differ in only a single amino acid or even by a posttranslational modification of an amino acid (7). Thus, antibodies are an ideal agent for specifically targeting subtly altered proteins in cancer cells. However, antibodies generally cannot penetrate the cell membrane, and the great majority of proteins encoded by commonly mutated oncogenes are intracellular and therefore inaccessible to antibodies (1).

Although the proteins encoded by mutant cancer driver genes are intracellular, peptides derived through proteolytic processing within the cell can be presented on the cell surface by human leukocyte antigen (HLA) molecules as a peptide–HLA (pHLA) complex (8). Such peptides are called mutation-associated neoantigens (MANAs). To take advantage of this truly tumor-specific cell surface antigen, our group has developed a class of T cell receptor (TCR)–mimic antibodies called MANA-directed antibodies (MANABodies) (9, 10). Although MANABodies are potentially powerful weapons against cancer, most MANAs are presented at levels too low to be recognized by MANABodies in a way that leads to cell killing. Established antibody-based therapies, such as bispecific antibodies, antibody drug conjugates, and chimeric antigen receptor (CAR) T cells, typically
require on the order of a few hundred to thousands of antigens on a tumor cell for recognition and cytolysis (11–13). Here, we show that MANAbodies, when grafted into an optimized T cell–engaging bispecific antibody format, are capable of specifically recognizing and killing cancer cell lines bearing extremely low levels of their cognate antigens.

RESULTS

MANAs derived from clinically relevant RAS gene mutations

Mutations in the RAS family of genes (KRAS, HRAS, and NRAS) are found in about 25% of all patients with cancer (14). The G12V mutation is one of the most frequent KRAS mutations and is also present in HRAS and NRAS (15), which are identical to KRAS at the first 86 residues (14). In an attempt to target this mutation (informal in silico binding predictions), we had identified a MANAbody specific to a G12V peptide (KLVVVGAVGVK) derived from codons 5 to 14 and bound to HLA-A*02:01 (henceforth referred to as HLA-A2) (9). However, this MANAbody could not recognize the G12V peptide naturally presented in HLA-A2, raising the possibility that the in silico algorithm may be inaccurate. Subsequently, we developed a highly sensitive mass spectrometry (MS)–based approach (MANA-SRM) to analyze HLA-bound peptides (16). We found that even in an overexpression model using transfected SV40 virus–immortalized monkey kidney COS-7 cells, the G12V peptide was not detected by MANA-SRM (table S1).

Thus, we turned our attention to two peptides, a 9-mer (VVGA VGVGK) from codons 8 to 16 (“G12V[8-16]”) and a 10-mer (VVVGVAVGVK) from codons 7 to 16 (“G12V[7-16]”), containing the G12V mutation that were predicted via NetMHC v4.0 to bind HLA-A*03:01 (henceforth referred to as HLA-A3), one of the most common HLA-A alleles (17, 18). Using MANA-SRM, we detected the G12V[7-16] peptide at 102 copies per cell and the G12V[8-16] peptide at 24 copies per cell in COS-7 cotransfected with HLA-A3 and KRAS G12V (fig. S1A and table S1). When COS-7 cells were transfected with HLA-A2 and KRAS G12V, neither of these peptides was detected (table S1). When we performed MANA-SRM on two human cancer cell lines, NCI-H441 (lung adenocarcinoma) and CFPAC-1 (pancreatic ductal adenocarcinoma), which both express endogenous levels of HLA-A3 and harbor the KRAS G12V mutation (19). The CFPAC-1 and NCI-H441 lines presented on average three and nine copies per cell, respectively, of the G12V[7-16] peptide (fig. S1, B and C, and table S1), and the G12V[8-16] peptide was not detected. Given the higher abundance of the 10-mer G12V[7-16] (henceforth referred to as “G12V”) peptide, we focused our efforts on targeting this MANA.

Another frequently mutated residue in RAS genes is the glutamine at codon 61 (15). Using NetMHC v4.0, we predicted that a 10-mer RAS peptide from codons 55 to 64 (ILDTAGQEEY) would bind HLA-A*01:01 (henceforth referred to as HLA-A1), another common HLA-A allele (17, 18). Using MANA-SRM, we previously evaluated cell surface presentation of 10-mer peptides from codons 55 to 64 containing the Q61H, Q61L, and Q61R mutations (ILD TAGHEEY, ILDTAGLEEY, and ILDTAGREEY, respectively) (16). In the COS-7 overexpression system, an average of 583, 512, and 127 copies of the Q61H, Q61L, and Q61R peptides per cell were found (16). These peptides were also presented on cell lines expressing endogenous levels of Q61 mutant RAS proteins, with four copies of the Q61L peptide per cell found for the acute promyelocytic leukemia cell line HL-60 (table S1) (16). Together, these data showed that G12V and Q61 mutant RAS proteins could be processed into peptides that were presented on the surface of cancer cells, albeit at extremely low antigen densities below what is considered the minimum required for recognition by conventional antibody-based immunotherapeutic agents (11–13).

Phage library design and construction

The phage library was designed on the basis of the principles from the construction of our first library (9) but with modifications (10). The library DNA was synthesized using trinucleotide mutagenesis (TRIM) technology, permitting fine tuning of the amino acid composition at particular codons considered most critical for antigen binding (20). We introduced diversity in five of the six complementarity-determining regions (CDRs), with the most amino acid diversity and length diversity incorporated into the third CDR of the heavy chain (CDR-H3) (fig. S2, A and B). The completed library was estimated to contain ~3.6 × 10^10 unique clones. As a quality control, a portion of the library was subjected to massively parallel sequencing of the heavy-chain CDR3 region, demonstrating that the expected and actual amino acid diversity were in excellent alignment (fig. S2C). Highlighting the utility of the more diverse library, all the candidate clones identified for the G12V pHLA-A3 and Q61 pHLA-A1 targets using the second library included amino acids not available in the first library.

Identification of scFv-expressing phage clones targeting HLA-restricted RAS mutant peptides

We screened the phage display library for phage specific to the recombinant G12V pHLA-A3 complex with negative selection against controls including the KRAS WT[7-16] (G12WT) pHLA-A3 (table S2) (9, 10). After the screening process, individual phage clones were subjected to enzyme-linked immunosorbent assay (ELISA) to assess their specificity (fig. S3A). Although multiple clones appeared promising on ELISAs with plate-bound pHLA, one clone (V2) stood out upon subsequent flow cytometry assays on cells displaying these complexes (fig. S3, B and C, and table S3). The V2 phage had substantially greater binding to G12V pHLA-A3 compared with other pHLA-A3, including those formed with G12WT peptides and other mutant RAS peptides (fig. S3D and table S2).

The recombinant V2 single-chain variable fragment (scFv) retained the same binding profile as its phage counterpart (fig. S3E), with only minimal binding to the G12WT pHLA complex at the highest concentration tested (fig. 1A). TAP-deficient T2 cells modified to express HLA-A3 (T2A3) (9) were pulsed with the G12V peptide or control peptides, and the cells were assessed for binding to V2 scFv or an HLA-A3–specific antibody. Flow cytometric analysis showed specific binding of V2 scFv to G12V peptide–pulsed T2A3 cells compared with cells pulsed with the G12WT, G12C, or G12D RAS peptides (Fig. 1B and fig. S3, F and G). As in the ELISA, the V2 scFv was unable to detectably bind to cells pulsed with the 9-mer [8-16] RAS peptides. Surface plasmon resonance (SPR) binding analysis of the V2 scFv demonstrated an equilibrium dissociation constant (K_d) value of 0.28 nM for G12V pHLA-A3, with no appreciable binding to G12WT pHLA-A3 (fig. S3H and table S4). The interaction between V2 scFv and G12V pHLA-A3 was best modeled with a two-state binding model, rather than a one-to-one fit, suggesting that the interaction involves a conformational change.

A similar screening procedure was used for the RAS Q61H, Q61L, and Q61R pHLA-A1 MANAs. Using phage staining and T cell–based
assays, we identified one phage clone displaying high specificity for each of the three targets, clones H1, L2, and R6, respectively (fig. S4, A to F, and table S3). These clones bound to their cognate mutant pHLA with no detectable binding to the RAS Q61WT pHLA-A1 (Fig. 1, C to E). To further assess binding to pHLA on the cell surface, the HLA-A1+ acute myeloid leukemia line SigM5 was pulsed (Fig. 1, C to F). To further assess binding to pHLA on the cell surface, we incubated the wells at 1 μg/ml before detection with protein L.

**Fig. 1.** Characterization of RAS MANA scFvs. (A and C to E) Biotinylated G12V or G12WT pHLA-A3 (A) or Q61WT, Q61H, Q61L, or Q61R pHLA-A1 (C to E) was coated on a streptavidin plate at the specified concentrations. Recombinant RAS G12V clone V2 (A), Q61H clone H1 (C), Q61L clone L2 (D), or Q61R clone R6 (E) scFv was incubated in the wells at 1 μg/ml before detection with protein L. ELISAs were performed in duplicate. OD450, optical density at 450 nm. (B and F) T2A3 or SigM5 cells were pulsed with the specified peptides at 50 μM, followed by flow cytometric analysis. (B) T2A3 cells were stained with V2 scFv preconjugated to anti-FLAG-PE, with mean fluorescence intensities (MFIs) plotted. (F) SigM5 cells were stained with clone H1, L2, or R6 phage.

**T cell–engaging bispecific antibodies could recognize mutant RAS-derived pHLA complexes**

A variety of T cell–engaging bispecific antibody formats have been developed for targeting T cells to specific ligands (21). It is unknown whether any of these formats can recognize targets when they are presented on the cell surface at very low densities. To elucidate this, we evaluated six bispecific formats: diabodies, single-chain diabodies (scDb), bispecific T cell engagers (BiTEs), dual affinity targeting molecules (DARTs), bivalent scFv-Fcs, and trivalent scFv-Fcs (fig. S5A) (21). The scFv-Fcs were heterodimerized via the knob-into-hole method (21). The V2 scFv was used as the pHLA-targeting moiety, and different anti-CD3 clones were used for engaging T cells (tables S3 and S5). In several of the formats, we tested different configurations of the heavy (“H”) and light (“L”) chains of the V2 and anti-CD3 scFvs (fig. S5B).

First, we tested 32 recombinant proteins to identify the most effective format and configuration (fig. S5B and table S5). Binding to G12V pHLA-A3 and recombinant CD3ε/δ protein (fig. S5C) was assessed by ELISAs. Although a few formats exhibited weak binding to one or both antigens, most had similar performance on ELISA (fig. S5C). To further compare the formats, T2A3 cells were pulsed with two concentrations of the G12V peptide and cocultured with T cells and each of the bispecific proteins at two concentrations, followed by measurement of interferon-γ (IFN-γ) to assess T cell activation (fig. S5D). Despite their similar performance on ELISA, the ability of the bispecific formats to recognize the G12V peptide at low antigen densities on cells was highly variable. scDb generally performed better than other formats. Switching the order of the heavy and light variable domains in the analogous scDb proteins (“LHLH” to “HLHL”) abolished their ability to activate T cells, despite these formats showing similar functionality on ELISA (fig. S5). In addition, although the bivalent and trivalent scFv-Fcs performed particularly well on ELISA, they consistently performed poorly in the coculture with peptide-pulsed cells.

In the initial tests of formats, we used five different anti-CD3 scFvs. On the basis of the results indicating that the scDb performed best, we tested seven additional anti-CD3 clones (tables S3 and S5). A total of 12 V2 scDb were expressed (fig. S6A) and tested with ELISA for their ability to bind CD3ε/δ (fig. S6B). Two target cell lines were used to further assess these V2 scDb. The first was KRAS G12V and HLA-A3 cotransfected COS-7, and the second was the lung cancer cell line NCI-H441, which expressed endogenous HLA-A3 and mutant KRAS G12V. In a coculture experiment with the COS-7 overexpression system, 10 of the scDBs activated T cells (fig. S6C). However, with NCI-H441 cells, only the scDb containing the UCHT1 (“U”) and UCHT1.v9 (“U2”) anti-CD3 clones activated T cells (fig. S6D), with the UCHT1-based scDb (Fig. 2A) performing...
Fig. 2. Schematic and characterization of MANA scDbs. (A) Schematic showing the optimal bispecific format, a scDb with variable light (VL or L), and variable heavy (VH or H) domains arranged in the following order: VLV2-VHUCHT1-VLUCHT1-VH2. SL, short linker (GGGGS); LL, long linker (GGGGS). (B and D to F) Anti-MANA/anti-CD3 scDbs were characterized via ELISA. Biotinylated pHLA-A3, pHLA-A1, or CD3e/δ was coated on a streptavidin plate. V2-U (B), H1-U (D), L2-U (E), or R6-U (F) scDb was incubated at the specified concentrations then detected with protein L. All ELISAs were performed in triplicate. (C and G) V2-U and L2-U scDb binding was evaluated by single-cycle kinetics using SPR. CMV, cytomegalovirus.

particularly well. Both scDbs (hereafter referred to as V2-U and V2-U2, respectively) (tables S3 and S5) retained remarkable specificity of V2 phage toward G12V pHLA-A3, as they failed to interact with pHLA-A3 folded with the other RAS peptides or an unrelated CT-NNB peptide in ELISAs (Fig. 2B and fig. S7A). They also bound to CD3e/δ (Fig. 2B and fig. S7A) and could simultaneously interact with G12V pHLA-A3 and CD3e/δ (fig. S7B), suggesting that the proteins were folded and functioned properly. Given these data, we chose to focus on the V2-U scDb (N terminus-LV2-H2-LU2-HV2-C terminus) (Fig. 2A) for further studies, although we also used V2-U2 for select assays.

We performed further biophysical characterization of the V2-U scDb, which exhibited stability in human serum at 37° and 40°C for at least 168 hours as assessed by CD3e/δ binding (fig. S7C). Thermal stability of the V2-U scDb as measured by differential scanning fluorimetry (DSF) showed a single melting temperature (Tm) at 68°C, suggesting that the protein was stable (fig. S7D). SPR analysis of the V2-U scDb revealed a KD value of 24 nM for G12V pHLA-A3 and no appreciable binding to G12WT pHLA-A3 (Fig. 2C and table S4). As with the V2 scFv, the V2-U scDb G12V pHLA-A3 interaction was best modeled with the two-state binding model.

On the basis of the V2 data, the Q61 scFv was performed in triplicate. (Fig. 2, D to F; fig. S7, E to G; and table S2). Like the V2-U scDb, the L2-U scDb was stable in human serum for at least 168 hours (fig. S7C) and had a single Tm at 70.7°C as measured by DSF (fig. S7H). SPR analysis of the L2-U scDb revealed a KD value of 65 nM for Q61L pHLA-A1 and no appreciable binding to Q61WT pHLA-A1 (Fig. 2G and table S4). These results showed that the format chosen on the basis of the RAS G12V scFv data was generalizable and applicable to three other scFvs with independent targets.

scDbs recognized cells pulsed with exogenous peptides at low nanomolar concentrations

To assess the minimal concentration of target antigen required for activating T cells, we pulsed T2A3 cells with G12V or G12WT peptides and cocultured them with T cells in the presence of the V2-U scDb. T cells were activated with G12V peptide concentrations as low as 1 nM (Fig. 3A and fig. S8A). Even at low peptide concentrations, the scDb mediated antigen–dependent lysis of the peptide-pulsed T2A3 cells (Fig. 3B). There was no appreciable cytokine secretion or cell killing with the G12WT peptide. Similarly, specific activation was seen with the V2-U2 scDb (Fig. 3, A and B).

To determine the approximate antigen density on peptide-pulsed T2A3 cells, the cells were stained with the V2 scFv bound to an anti-FLAG antibody (2:1 ratio of scFv:antibody) and then assessed by flow cytometry. Bound antibodies were quantified using QIFIKIT and Quantibrite Beads (fig. S8, B and C). We found that cells pulsed with 320 nM G12V peptide presented about 300 to 800 antibody sites per cell, each representing one or two G12V pHLA-A3. This method did not have the sensitivity required to quantify antibodies on cells pulsed with <320 nM G12V peptide. However, T2A3 cells pulsed with 300-fold lower concentrations of G12V peptide could activate T cells (Fig. 3, A and B). Although there may not be a linear relationship between the concentration of the pulsed peptide and the number of pHLA on the cell surface, these results suggested that the V2-U scDb was capable of inducing T cell activation in the presence of target cells bearing far fewer than 300 pHLA. We performed
cells were combined with 5 × 10^4 human T cells (effector:target ratio or E:T, eabd5515 (2021) 1 March 2021) and an analogous peptide-pulsing experiment with HLA-A3 + monocyte-derived immature dendritic cells (iDCs) to present the pHLAs. To assess whether scDbs recognize pHLAs formed through endogenous antigen processing, COS-7 cells were cotransfected with plasmids encoding HLA-A1 and full-length WT or mutant HRAS, KRAS, or NRAS to assess whether each scDb was capable of recognizing the cognate mutant pHLAs derived from each of the RAS genes. Each scDb, at subnanomolar concentrations, elicited T cell responses highly specific for the COS-7 cells expressing the RAS gene with the cognate Q61 mutation, regardless of the RAS gene assessed (Fig. 4, B to D). Moreover, no activation of T cells was observed when cocultured with COS-7 cells expressing HRAS, KRAS, or NRAS genes that were WT or contained the noncognate Q61 mutations (Fig. 4, B to D).

**Fig. 3. MANA concentration-dependent T cell activation.** (A and B) T2A3 cells were pulsed with either the G12V or G12WT peptides at the specified concentrations. (C and D) SigM5 cells were pulsed with either the Q61L or Q61WT peptides at the specified concentrations. T2A3 (5 × 10^4) or SigM5 (2.5 × 10^4) peptide-pulsed cells were combined with 5 × 10^4 human T cells (effector:target ratio or E:T, eabd5515 (2021) 1 March 2021) and an analogous peptide-pulsing experiment with HLA-A3 + monocyte-derived immature dendritic cells (iDCs) to present the pHLAs. To assess whether scDbs recognize pHLAs formed through endogenous antigen processing, COS-7 cells were cotransfected with plasmids encoding HLA-A1 and full-length WT or mutant HRAS, KRAS, or NRAS to assess whether each scDb was capable of recognizing the cognate mutant pHLAs derived from each of the RAS genes. Each scDb, at subnanomolar concentrations, elicited T cell responses highly specific for the COS-7 cells expressing the RAS gene with the cognate Q61 mutation, regardless of the RAS gene assessed (Fig. 4, B to D). Moreover, no activation of T cells was observed when cocultured with COS-7 cells expressing HRAS, KRAS, or NRAS genes that were WT or contained the noncognate Q61 mutations (Fig. 4, B to D).

**scDbs activated T cells when exposed to cancer cells harboring endogenous mutant RAS genes**

As noted above, the NCI-H441 cancer cell line presents only ~9 copies of KRAS G12V pHLA complexes per cell (table S1). Despite this extremely low level of the target MANA, T cells could be activated by NCI-H441 cells in the presence of the V2-U scDb (Fig. 5, A and B). The potency of V2-U was high, with a median effective concentration (EC_{50}) of 140 and 76 pM for IFN-γ secretion and cytotoxicity, respectively (Fig. 5, A and B). To rigorously assess the specificity of the V2-U scDb, the HLA-A3 allele was disrupted in NCI-H441 cells using CRISPR, and the knockout (KO) was confirmed (fig. S10). KO of the HLA-A3 allele eliminated the ability of the V2-U scDb to elicit T cell activation upon exposure to the NCI-H441 cells (Fig. 5, C and D). We then used CRISPR to replace the "knock-in" (KI) KRAS G12V with KRAS G13D to maintain the viability of the cells that require mutant RAS genes (22) in parental NCI-H441 cells containing the HLA-A3 allele (fig. S11). Two independent NCI-H441 clones with the KRAS G13D substitution were tested, and both induced substantially abrogated T cell activation through V2-U scDb (Fig. 5, C and D). Similarly, specific activation of T cells cocultured with NCI-H441 cells was observed with the V2-U scDb, but as expected, the potency of the V2-U scDb was not as great as the V2-U scDb (fig. S12). In all the experiments with endogenous levels of HLA-A3 and KRAS G12V alleles (Fig. 5, A and C, and fig. S12A), T cell activation was considerably lower than T cells activated by the transiently COS-7 cells (Fig. 4A and fig. S9C), consistent with greater numbers of pHLA on COS-7 cells. Even so, the scDb was able to induce efficient NCI-H441 cell lysis in a KRAS G12V and HLA-A3-dependent fashion (Fig. 5, B and D, and fig. S12B). Moreover, other markers of T cell activation [tumor necrosis factor-α (TNFα), interleukin-2 (IL-2), granzyme B, and perforin] were released in a dose-dependent manner (fig. S13), demonstrating that the V2-U scDb induced a polyfunctional T cell response against cells expressing very low levels of antigen.

To further assess the specificity of the V2-U scDb, we used a second cell line, NCI-H358. This lung adenocarcinoma line contains the HLA-A3 allele and a KRAS G12C mutation (19). Using CRISPR, we introduced the G12V mutation in the KRAS locus in three independent clones (fig. S11). Applying the MANA-SRM technique to the NCI-H358 cell lines, we detected the HLA-presented G12V[7-16] peptide at between 6 and 16 copies per cell in the three G12V-expressing lines, with no copies detected in the parental NCI-H358 cells (fig. S14 and table S1). All three G12V clones were able to induce T cell activation in the presence of the subnanomolar concentrations of V2-U scDb, whereas parental cells or a parental clone was not (Fig. 5, E and F). Similarly, T cell activation was observed in the
Fig. 4. T cell activation mediated by scDb recognizing pHLAs formed through endogenous processing. COS-7 cells were transfected with plasmids encoding HLA-A3 ("A3") or HLA-A1 ("A1") and RAS variants or other negative controls. Twenty-four hours later, 1 × 10⁴ COS-7 cells were combined with 5 × 10⁴ T cells (E:T = 5:1) and V2-U (A), H1-U (B), L2-U (C), or R6-U (D) scDb at specific concentrations. Supernatants were assayed for IFN-γ at 24 hours. All experiments were performed in triplicate. GFP, green fluorescent protein.
presence of V2-U2 scDb (fig. S15). All NCI-H358 variants expressed about the same level of HLA-A3 (fig. S10).

We also assessed T cell activation in cocultures with several other HLA-A3+ cancer cell lines without the KRAS G12V mutation. These lines included A-427 (lung adenocarcinoma), COLO 741 (melanoma), Hs 578T (breast invasive ductal carcinoma), Jurkat (acute T cell leukemia), SK-MES-1 (lung squamous cell carcinoma), and SW780 (bladder transitional cell carcinoma) (19). We also assessed CFPAC-1, the KRAS G12V and HLA-A3+ pancreatic adenocarcinoma cell line that presents an average of only ~3 copies of the G12V peptide per cell (table S1). All these cell lines expressed HLA-A3 (fig. S10). Neither V2-U nor V2-U2 scDb resulted in appreciable T cell activation cocultured with cell lines without the KRAS G12V mutation (fig. S16). V2-U scDb induced a low but significantly higher level of IFN-γ release with CFPAC-1 cells compared with the cell lines without the KRAS G12V mutation (fig. S16A); however, there were no consistently significant differences when V2-U2 was used (fig. S16B).

Likewise, to study the ability of the L2-U scDb to induce T cell activation, coculture with a panel of cell lines that differed in RAS mutation status and HLA-A1 expression were used (fig. 6A and fig. S17). Substantial dose-dependent T cell activation was only observed with the one cell line (HL-60) containing both HLA-A1 and RAS Q61L alleles (fig. 6A). As noted above, HL-60 presents an average of four Q61L pHLA per cell. In a titration experiment, the L2-U scDb induced IFN-γ release from T cells with an EC50 value of 60 pM (Fig. 6B) and HL-60 cell death with an EC50 value of 34 pM (fig. 6C), despite the very low level of Q61L pHLA per cell. Other markers of T cell activation were similarly released in a dose-dependent manner, showing that the L2-U scDb, like the V2-U scDb, was capable of inducing a polyfunctional T cell response (fig. S18). To rigorously assess the antigens on HL-60 cells recognized by the L2-U scDb, we generated NRAS Q61H-KI, Q61R-KI, and HLA-A1-KO variants of the HL-60 cell line (fig. S11). Coculture experiments with these variants confirmed that T cell activation (Fig. 6, D and E) were dependent on the copresence of NRAS Q61L and HLA-A1 genes.

Assessing potential cross-reactivity to other putative HLA-A1- and HLA-A3–binding peptides
To investigate whether the V2 scFv could bind similar peptides derived from other proteins, we performed a protein BLAST (BLASTp) search of the human RefSeq proteome (23) with the amino acid sequences of the G12V peptide. Thirty-two proteins containing similar peptides were identified. Of these, NetMHC v4.0 predicted that 17 peptides could bind HLA-A3 (table S6). Each of these 17 peptides was pulsed on T2A3 cells. Although most bound to HLA-A3, as assessed by anti–HLA-A3 antibody staining (fig. S19), 9 peptides only recognized the peptide IIVGAIGVVK ("Blast2") derived from the protein Rab-7b (fig. S19A and table S6).

Rab-7b is expressed in monocytic cells and keratinocytes (24, 25). To investigate whether this peptide could cause V2-U2 scDb off-target toxicity, we first assessed Rab-7b expression in HLA-A3+ peripheral blood mononuclear cells (PBMCs), monocytes, iDCs, and mature dendritic cells (fig. S19B) and then cocultured these cells with T cells. None of these cells was able to significantly activate
T cells with V2-U scDb unless pulsed with the G12V peptide (fig. S19C). Next, we tested V2-U scDb against a skin-derived cell line, Hs 695T, that highly expresses Rab-7b (fig. S19B) but lacks the HLA-A3 allele. Hs 695T was transfected with either HLA-A3 or HLA-A2 and cocultured with T cells. Despite high levels of Rab-7b expression, Hs 695T cells did not activate T cells in the presence of V2-U scDb, although they were capable of inducing T cell activation when pulsed with the G12V peptide (fig. S19D). As a final assessment of the potential Rab-7b cross-reactivity, plasmids expressing full-length Rab-7b, KRAS WT, or KRAS G12V, in combination with HLA-A3, were transfected into COS-7. Cells overexpressing the mutant KRAS induced robust T cell activation in the presence of V2-U scDb (fig. S19E), whereas COS-7 cells expressing KRAS WT or Rab-7b showed only marginal, non–dose-dependent T cell activation (fig. S19, B and E). We repeated this experiment in HCT 116 cells and again found that T cells were activated by KRAS in a mutant–dependent fashion, but no activation was observed in Rab-7b–expressing cells (fig. S19, B and F).

To further assay for potential cross-reactivity of both the V2-U and L2-U scDbs, we evaluated their binding to libraries of positional scanning variant peptides (26). The library of variant peptides was generated by systematically substituting each amino acid of the original peptides with the other 19 common amino acids. This resulted in 190 variants for each of the G12V and Q61L peptides. T2A3 cells (for V2-U scDb) or SigM5 cells (for L2-U scDb) were pulsed with the individual variant peptides and then cocultured with T cells to access recognition via IFN-γ release (Fig. 7, A and B, and fig. S20, A and B). For both scDbs, amino acid positions in the peptides’ C-terminal half, where the mutant residues resided, demonstrated greater specificity. Most changes at these positions abolished scDb recognition. On the other hand, amino acids at the N terminus could, in many cases, be substituted without substantially changing interaction with the scDbs. These recognition patterns are also illustrated as Seq2Logo graphs (Fig. 7, C and D) (27). Next, using a 20% cognate peptide reactivity as a cutoff for permissive scDb recognition, a search of these motifs in the UniProtKB human protein database yielded 162 hits (including the Rab-7b peptide). Using ScanProsite (28) and a 20% cognate peptide reactivity as a cutoff for permissive scDb recognition, a search of these motifs in the UniProtKB human protein database yielded 162 hits (including the Rab-7b peptide). Next, using a 20% cognate peptide reactivity as a cutoff for permissive scDb recognition, a search of these motifs in the UniProtKB human protein database using ScanProsite (28) yielded 162 (including the Rab-7b peptide IIIVGAQVGK) and 232 peptides that could potentially bind to V2 and L2, respectively (tables S7 and S8). Comparing these peptides with an extensive database of peptides actually presented by HLA as assessed by MS (29), we found that none of the 162 peptides and only 1 of the 232 peptides was known to be presented by the cognate HLA. However, when SigM5 cells were pulsed with this one peptide [DTTELQGMNEY, from chromodomain helicase DNA binding protein 4 (CHD4)] and then cocultured with T cells and L2-U scDb, they did not elicit IFN-γ release (fig. S20C), demonstrating that L2-U scDb did not bind the CHD4 peptide.
Fig. 7. Peptide scanning mutagenesis to assess potential V2-U and L2-U scDb cross-reactivity. Each amino acid of the G12V and Q61L peptides was systematically substituted with the other common 19 amino acids, thereby generating libraries of variant peptides each differing from the original peptide by a single amino acid. T2A3 cells were pulsed with 10 μM of individual peptides from the G12V peptide library (A and C) or SigM5 cell cells were pulsed with 10 μM of individual peptides from the Q61L peptide library (B and D). Peptide-pulsed target cells (2.5 × 10^4) were combined with 5 × 10^4 human T cells (E:T = 2:1) and either the V2-U (A and C) or L2-U scDb (B and D) at 1 nM. (A and B) Supernatant was assayed for IFN-γ at 24 hours, with the mean of three technical replicates plotted as a heatmap. Black boxes indicate amino acids in the parental peptides. (C and D) Illustration of the binding patterns of V2-U and L2-U scDbs as Seq2Logo graphs, calculated by dividing the IFN-γ value by 10^3 and using the PSSM-Logo algorithm.

Evaluation of scDbs in mouse models

To determine whether the V2-U scDb could affect KRAS G12V HLA-A3+ tumors in vivo, we assessed intrasplenic xenografts of NCI-H358 (KRAS G12C/WT) cells and their G12V-KI variant in NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mice. At the time of tumor injection, an infusion pump containing either V2-U scDb or an isotype control scDb was placed in the peritoneum. Once the mice were confirmed to have tumors through bioluminescence imaging, human T cells were administered intravenously. Although not statistically significant, the V2-U scDb resulted in a trend toward slowed tumor growth of the G12V cells (fig. S21A) but not of isogenic G12C cells (fig. S21B). Given that the pump only secreted scDb for 14 days, therapeutic effects at later time points were not assessed.

Similarly, to determine whether the L2-U scDb could control tumor growth in vivo, we intravenously injected HL-60 leukemia cells (NRAS Q61L/WT) and T cells into NSG mice to establish widespread leukemic infiltrates. As controls, NRAS Q61H/Q61H HL-60 cells were used in separate mice. Bioluminescence imaging established tumor uptake, and mice were randomized to receive the L2-U scDb or a control scDb through intraperitoneal 14-day infusion pumps. The L2-U scDb slowed the growth of the Q61L HL-60 leukemic cells (fig. S21C) but not the Q61H cells (fig. S21D). Although the tumor growth retardation was significant (P < 0.05, multiple t tests with Bonferroni-Dunn correction), the effect size was modest. Therapeutic effects were not assessed at later time points because of the lifetime of the pumps. No substantial changes in body weight were noted in any of the V2-U or L2-U scDb–treated mice (fig. S22).

DISCUSSION

This study shows that bispecific antibodies can mediate mutant gene product–specific killing of cancer cells containing only a few pHLA complexes per cell. The ability to induce target cell death at such low antigen densities was previously documented for T cells bearing TCRs but was thought to be well out of the range of antibody-based reagents (11–13). The keys to the success of these bispecific antibodies were (i) the development of high-affinity scFvs through multiple rounds of positive and negative selection and (ii) experimentation to identify the optimal format of bispecific antibodies that would have activity against very low levels of antigen presentation.

In addition to the discovery of scFvs that specifically recognize the pHLA, the particular anti-CD3 scFv component that activates the T cells was also critical for the success of the bispecific antibodies.

The reported affinities of the tested scFvs for CD3 varied widely, from at least 1 to 263 nM (30–38), but there was no obvious correlation with performance here. This suggests that other factors, such as the precise way in which the scFv binds CD3 or the juxtaposition of the T cell and target cell that is mediated by the bispecific antibodies, play a role. Given that the TCR α and β chains of the TCR complex appear to function as a mechanoreceptor (39), subtle differences in orientations of the antibodies or cells could translate to large differences in T cell activity.

Several groups have performed bispecific antibody format optimizations, particularly with regard to the orientation of the variable light and heavy chains (40, 41). A few groups have compared BiTEs with the more compact scDb, diabody, or DART formats, with contradictory results (36, 40–42). In addition, a number of other antibody-based therapeutic formats exist, including full-length antibodies, antibody drug conjugates, and CAR T cells (11–13). All of these are reported to require relatively high antigen densities for effective target cell killing. Our experiments were designed to compare formats that could be used in the realm of extremely low antigen densities, which characterize the vast majority of pHLA derived from mutated
driver genes. In our experiments with the V2 clone, although the scDb format did result in reduced affinity for the G12V pHLA-A3 relative to that of the V2 scFv, the scDb format outperformed other formats for targeting low–antigen density targets while maintaining specificity and potency.

Although many bispecific antibodies are currently under investigation, the only U.S. Food and Drug Administration–approved T cell–engaging bispecific is the anti-CD19–targeting blinatumomab, based on the BiTE format (21). Blinatumomab is used to treat B cell leukemia cells that typically express thousands of times more antigen (CD19) molecules per cell than is the case with the cells used in our study. The BiTE format has also been used for the TCR-mimic antibody ESK1, which targets a pHLA derived from the tumor-associated antigen WT1 (43). An ESK1-based BiTE did demonstrate effective tumor control in vivo (43); however, the WT1 peptide is presented at higher levels, on the order of 100s to 1000s per cell (44), than the peptides targeted in the present study. The BiTE format is of a similar size to the scDb but contains a single flexible linker between the scFvs. The scDb, on the other hand, may have limited flexibility between the antigen target and CD3, and it is conceivable that the more compact scDb structure may result in a tighter immunological synapse, resulting in more effective T cell activation (36, 45). However, the best bispecific antibody format for a given scFv and target antigen pair likely requires independent optimization.

Bispecific antibodies containing Fc domains are attractive because of the much longer predicted in vivo half-life. The scFv-Fcs initially looked promising; in particular, trispecific variants that were bivalent for the V2 scFv exhibited a high level of G12V pHLA-A3 binding, expected given the effects of bivalency on functional affinity (46). However, the scFv-Fcs performed poorly in coculture experiments. Others have observed that close spatial proximity between the target and T cell membranes results in more efficient T cell activation, likely due to a tighter immunological synapse (47, 48). Thus, we hypothesize that the added size of the scFv-Fcs results in a weaker synapse, either due to steric hindrance or reduced membrane proximity.

Alternatively, mutant pHLA can be targeted by TCR-based therapeutics, including ex vivo expanded tumor-specific T cells, TCR transgene-expressing T cells, and TCR-based bispecific antibodies (5, 49–51). Like scDbs, TCR-based therapeutics can elicit T cell reactivity when incubated with target cells pulsed with single-digit nanomolar peptide concentrations (5). Furthermore, MANA-targeting TCRs have shown promise in human clinical trials, including those directed against mutant KRAS-derived peptides (5, 51). Although TCR-based T cell therapies have advantages, including lower risk of peptide cross-reactivity and the possibility of targeting nonrecurrent passenger mutations, most of these formats require time and resource-intensive ex vivo T cell manipulation (52). TCR-based bispecific T cell–engaging proteins are another promising approach, because they, like bispecific antibodies, can be used as “off-the-shelf” reagents. However, TCRs typically have weaker affinities (100 nM to 10 μM) and require affinity maturation to reach an affinity range comparable to that of an antibody (49).

One major concern about any immunotherapeutic agent is specificity. The specificity for the scDbs here was demonstrated for both the HLA and peptide components through genetic KO and KI experiments. However, such experiments do not exclude the possibility of cross-reactivity to other peptides, which we assessed in several ways. First, we demonstrated the lack of reactivity against panels of HLA-matched cell lines and normal hematopoietic-derived cells. Second, we used BLAST tools to identify normal human peptides containing amino acid sequences related to the G12V peptide. Last, we systemically substituted amino acids at each position of the targeted peptide with each of the remaining 19 amino acids. Although these experiments provide good evidence supporting the absence of significant cross-reactivity, absence of evidence is not the same as evidence of absence, and further testing is necessary to assess for potential reactivity to other human peptides.

Although the V2 scDbs were able to activate T cells when cocultured with KRAS G12V mutant cell lines NCI-H441 and NCI-H358, they were not potent when cocultured with CFPAC-1. This lower potency may be the result of the threefold lower peptide density on the surface of CFPAC-1 versus NCI-H441 (an average of three versus nine peptides per cell). Evidence from the literature suggests that, in the regime of very low antigen density, small differences in antigen density can markedly change whether a T cell will be activated and that, whereas as few as three peptide HLA complexes can induce cytotoxicity, a fully functional mature synapse requires 10 peptide HLAs (53, 54). However, it is also possible that there are other variables such as target cell characteristics, including proteins that inhibit T cell function or inhibit cell death processes. Determining the nature of such variables is likely to be important in the further development of all T cell therapies designed to target low-density antigens.

Another limitation of the bispecific antibodies described here was in vivo potency. Although both the V2-U and L2-U scDbs had high potency in cell culture systems (EC50 value at subnanomolar concentrations), they were not potent in mouse models. The basis for this discrepancy is not clear, and many explanations are possible. For example, the models that we used, using exogenously introduced human T cells and antibodies, may not be optimal. In addition, we were not able to continue treatment for longer than 2 weeks because of the necessity to deliver scDbs through osmotic pumps. We found that replacement of these intraperitoneal pumps was not well tolerated by the mice. Although similar in vivo experiments with other bispecific antibodies have demonstrated more impressive results in mouse models (36, 43), few have used target cells with very low antigen densities. Thus, it is not clear that the suboptimal in vivo results represent a problem with the models, the bispecific antibodies, or the method of therapeutic delivery.

In sum, this work shows that it is possible to generate bispecific antibodies that are highly specific for pHLA derived from common mutations and that are capable of inducing target cell killing at very low antigen densities. We believe that the format of the bispecific antibodies developed here is generally applicable, as we have used it to create scDbs against four genetically altered protein products in this study and other targets in cancer cells (55, 56). Further work is required to determine whether the MANAbody-based scDb approach is a viable therapy, particularly clinical trials to assess efficacy and toxicity in patients with cancer.

**MATERIALS AND METHODS**

In areas where protocols are described in brief, additional details are available in Supplementary Materials and Methods.

**Study design**

The objective of this study was to generate therapeutic agents that target common mutations in RAS genes. Phage display was used to
identify scFvs specific to MANAs that had been confirmed to be presented via MS. These scFvs were grafted into an optimized bispecific antibody format, the scDb. The scDBs mediated MANA-specific T cell activation and target cell cytotoxicity in overexpression and endogenous-level expression model systems. All data presented are representative of data collected during this study. All experiments were performed in triplicate with three technical replicates (unless otherwise noted) and performed in a way to minimize confounding variables, such as plate layout effects.

Plasmids
Plasmids for eukaryotic cell line transfections encoding KRAS (isoform B), HRAS (isoform 1), and NRAS variants (WT and mutant) and HLA class I alleles A*01:01 and A*03:01 were synthesized and cloned into pcDNA3.1 by GeneArt (Thermo Fisher Scientific) or synthesized into gBlocks (IDT) and assembled into pcDNA3 using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs).

Cell lines and primary cells
All cells were grown at 37°C under 5% CO2. In brief, peripheral blood cells were obtained from healthy volunteer donors under the Johns Hopkins Institutional Review Board approval or purchased as leukopheresis samples (STEMCELL Technologies). PMBCs were purified by density gradient centrifugation. T cells were expanded from PBMCs using anti-human CD3 antibody (clone OKT3, BioLegend) or Human T-Activator CD3/CD28 Dynabeads (Thermo Fisher Scientific). Monocytes were negatively isolated from PBMCs and differentiated into dendritic cells.

Phage display library construction
Oligonucleotides were synthesized by GeneArt (Thermo Fisher Scientific) using TRIM technology. The oligonucleotides were incorporated into the pADL-10b phagemid (Antibody Design Labs) (fig. S23). Library construction was performed following previously published protocols (9). In brief, electrocompetent SS320 cells (Lucigen) were electroporated with library DNA. Bacteria were grown on plates containing agar with 2xYT medium (Sigma-Aldrich) supplemented with carbenicillin. After harvesting the bacteria, phages were produced by infecting mid-log phase bacteria with M13K07 helper phage (Antibody Design Labs); resuspending in 2xYT medium with carbenicillin, kanamycin, and isopropyl-β-D-thiogalactopyranoside (Thermo Fisher Scientific); and growing overnight at 30°C. Phages were precipitated on ice followed by centrifugation and resuspension. Library DNA was subjected to next-generation sequencing as previously described (57).

Peptides and pHAs
Peptides (tables S2 and S6) were synthesized at a purity of >90% (Peptide 2.0 or ELIM Biopharm), with the exception of the crude peptides used for the positional scanning library. Peptides were re-suspended in N,N'-dimethylformamide at 10 mg/ml and stored at −80°C. HLA-A1 or HLA-A3 was refolded with peptide and β2-microglobulin, purified by gel filtration, and biotinylated (Fred Hutchinson Immune Monitoring Lab, Seattle, WA or Baylor MHC Tetramer Production Lab, Houston, TX). These pHAs were confirmed to be folded before selection via ELISA using the W6/32 antibody (BioLegend). Cognate peptide reactivity search of the UniProtKB human protein database using ScanProsite was performed using binding motifs with a 20% parental peptide IFN-γ value as a cutoff (28). The V2 motif was [FWDY]-[ILMVT]-[RE]-[ILV]-x-[ILV]-[GNST]-[VP]-[AG]-[HKY]. The L2 motif was x-{PWRHD-EY}-[APRDEQSC]-[DE]-[AMFPGDHNDQSTYC]-[AG]-[ILM]-[AIMGRDENQ]-[DE]-[AY].

Selection of phage clones
Phage selection was performed following previously published protocols (9, 10). In brief, phages were negatively selected against streptavidin, heat-denatured HLA, unrelated pHLA, and/or WT pHLA, followed by positive selection for the mutant pHLA, followed by amplification of eluted phage for up to six rounds. A fraction of the previous round’s phage was used as input for the current round. Some rounds included a “competition” step, where WT pHLA and mutant pHLA were coincubated with phage, with selective retrieval of mutant pHLA. After selection, monoclonal phages were obtained by producing phage from bacteria transduced at limiting dilutions.

Enzyme-linked immunosorbent assays
In brief, ELISAs were performed in streptavidin-coated, 96-well plates (R&D Systems), incubated with biotinylated pHLA or biotinylated recombinant heterodimeric CD3ε/δ (Abcam). Plates were washed using a BioTek 405 TS plate washer. Plate-bound phage, scFv, and bispecific antibodies were detected as outlined in the figure legends and Supplementary Materials and Methods.

Flow cytometry
In brief, cells were peptide-pulsed by incubating with peptide and human β2-microglobulin (ProSpec) in serum-free media for 4 hours. Cell-bound phage or scFv was detected as outlined in the figure legends. HLA staining was performed using anti–HLA-A3 clone GAP. A3-PE (phycoerythrin) (eBioscience, Thermo Fisher Scientific) or anti–HLA-A1/A11/A26 clone 8.L.101 (Abcam), followed by anti-mouse-PE (BioLegend). Stained cells were analyzed using an LSRII flow cytometer (Becton Dickinson) or an Intellicyt iQue3 flow cytometer (Sartorius).

Recombinant scFv and bispecific antibody production
In brief, recombinant scFv was produced in Escherichia coli with periplasmic expression followed by nickel chromatography purification by AxioMx Inc. (Abcam). Bispecific antibody constructs were obtained by subcloning gBlocks (IDT) with an IL-2 signal sequence and C-terminal 6xHis tag into the pcDNA3.4 vector (Thermo Fisher Scientific). Bispecific antibodies were produced by secretion from either human embryonic kidney 293F by the Eukaryotic Tissue Culture Core Facility at Johns Hopkins University, followed by nickel resin purification, or alternatively produced in Expi293s, followed by purification with a HisTrap column and size exclusion chromatography by GeneArt. Proteins were quantified via miniPROTEAN TGX stain-free gels (Bio-Rad) and bicinechonic acid protein assay (Pierce, Thermo Fisher Scientific).

SPR measurements
SPR binding experiments were performed using a Biacore T200 SPR instrument (GE Healthcare). In brief, biotinylated pHLA was captured by a streptavidin chip, followed by injection with increasing concentrations of scFv or scDb. Binding responses for kinetic analysis were both blank and reference subtracted. Binding curves were fitted using Biacore Insight evaluation software.
pHLA immunoprecipitation and MS

pHLA immunoprecipitation and MS were performed as previously described (16), with more details provided in Supplementary Materials and Methods.

CRISPR on cell lines

In brief, the Alt-R CRISPR system (IDT) was used to modify the HLA and RAS alleles. KOs were obtained by duplexing Alt-R CRISPR-Cas9 CRISPR RNAs (crRNAs) (IDT) and Alt-R CRISPR-Cas9 trans-activating CRISPR RNA (tracrRNA) (IDT) before combining with Cas9 nuclease (IDT). This Cas9 ribonucleaseprotein (Cas9 RNP) mixture was combined with cells and electroporated. KIs were generated by electroporating cells with Cas9 RNPs and single-strand DNA homology–directed repair templates (58, 59), obtained as Ulramer Oligos from IDT. Modified polyclonal cell pools were plated at 0.5 to 2 cells per well and grown for 3 weeks before screening using flow cytometry (for HLA-A3 KOs) or genomic DNA Sanger and targeted next-generation sequencing (for RAS KIs). Sequences are provided in Supplementary Materials and Methods.

Cocultures

In brief, coculture was assembled by combining the target cells, T cells, bispecific antibody, and IL-2 in a 96-well plate, followed by incubation for 24 hours. The supernatant was assayed for cytokines using the Human IFN-γ Quantikine and Human TNFα Quantikine ELISA Kits (both R&D Systems Bio-Techne). Cell viability was assayed by CellTiter-Glo Luminescent Cell Viability Assay (Promega). Cytotoxicity was calculated by taking the luciferase signal of a given cell population divided by the signal of an untreated control and multiplying by 100. Cell viability was assessed using a Human IFN-α Quantikine ELISA Kit (R&D Systems Bio-Techne). Cytotoxicity was calculated by taking the signal of a given cell population divided by the signal of an untreated control and multiplying by 100.

Mouse xenograft model

NOD.Cg-Pkd1+/-cd41+/-Ii2+/-+129SvJ (NSG) mice were acquired from the Johns Hopkins Sidney Kimmel Comprehensive Cancer Center Animal Resources Core and treated in compliance with a research protocol approved by the Johns Hopkins University Animal Care and Use Committee. Littermate controls were used for all experiments. Further details are provided in the figure legends and Supplementary Material and Methods.

Statistics

Statistical analyses were performed with Prism 8 (GraphPad software). Unless otherwise indicated, error bars represent the SD of three technical replicates that were independently assembled. Error bars smaller than the symbols used to represent the mean of these replicates are not shown. Percent cytotoxicity of target cells for in vitro experiments was calculated as described above. For in vivo experiments, statistical significance was performed with an unpaired, two-tailed t test with Bonferroni-Dunn correction for multiple comparisons.

SUPPLEMENTARY MATERIALS

immunology.sciencemag.org/cgi/content/full/6/5/eaabd5515/DC1
Fig. S1. KRAS neoantigen transitions detected through MANA-SRM.
Fig. S2. Design and sequencing of CDR-H3 of the phage library.
Fig. S3. Characterization of the V2 scFv.
Fig. S4. Characterization of the RAS G61W, G61L, and Q61R scFvs.
Fig. S5. Bispecific antibody format comparison.

Fig. S6. V2 scDBs made with various anti-CD3 clones.
Fig. S7. Specificity and stability of scDBs.
Fig. S8. Additional peptide-pulsing data.
Fig. S9. Western blots and cocultures with transfected COS-7 cells.
Fig. S10. HLA-A3 expression in target cell lines.
Fig. S11. Sanger sequencing of RAS alleles in CRISPR-modified cell lines.
Fig. S12. V2-U2 scDB–mediated T cell activation in cocultures with NCI-H441 isogenic cell lines.
Fig. S13. Polyfunctional T cell response elicited by L2-U2 scDB.
Fig. S14. KRAS neoantigen transitions detected through MANA-SRM for NCI-H358 variants.
Fig. S15. V2-U2 scDB–mediated T cell activation in cocultures with NCI-H358 isogenic cell lines.
Fig. S16. Effects of V2 scDBs on IFN-γ secretion from T cells in cocultures with HLA-A3+ cell lines.
Fig. S17. HLA-A1 expression in target cell lines.
Fig. S18. Polyfunctional T cell response elicited by L2-U2 scDB.
Fig. S19. Testing potential cross-reactive peptides.
Fig. S20. Positional scanning of target peptides that could potentially react with V2 or L2 scDB.
Fig. S21. V2-U2 and L2-U2 effects on tumor growth in mouse model systems.
Fig. S22. Body weights of NSG mice treated with scDBs.
Fig. S23. Diagram of scFv phage library phagemid.
Table S1. MANA-SRM quantification.
Table S2. Peptides for pHLA complexes.
Table S3. scFv sequences.
Table S4. Binding kinetics of clones V2 and L2 to their respective MANA pHLA.
Table S5. V2 bispecific antibody sequences.
Table S6. KRAS G12 BLAST peptides.
Table S7. Human peptides matching the V2 or L2 scDb.
Table S8. Additional peptide-binding data.
Table S9. Western blotting and cocultures with transfected COS-7 cells.
Table S10. Specificity and stability of scDBs.
Table S11. Additional peptide-pulsing data.
Table S12. Western blotting and cocultures with transfected COS-7 cells.
Table S13. Polyfunctional T cell response elicited by L2-U2 scDB.
Table S14. Testing potential cross-reactive peptides.
Table S15. Positional scanning of target peptides that could potentially react with V2 or L2 scDB.
Table S16. V2-U2 and L2-U2 effects on tumor growth in mouse model systems.
Table S17. Human peptides matching the L2-U2 binding motif.
Table S18. Raw data table.
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View/request a protocol for this paper from Bio-protocol.

REFERENCES AND NOTES

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Directed Cas9 by inhibition induced DNA breaks.

CD3 antibody


Author contributions: Conceptualization: J.D., E.H.-C., M.S.H., Q.W., A.D.S., B.V., S.Z., N.P., K.W.K., and C.B. Methodology: J.D., E.H.-C., Q.W., M.S.H., B.J.M., A.H.P., S.R.D., M.S.M., K.M.W., and A.D.S. Investigation: J.D., E.H.-C., Q.W., B.J.M., S.R.D., A.H.P., M.S.H., S.P., A.S., M.S.M., K.M.W., P.A.A., M.B.M., M.D.M., Q.L., E.W., and Y.L. Analysis and interpretation of data: J.D., E.H.-C., Q.W., M.S.H., B.J.M., A.H.P., S.R.D., M.F.K., K.W.K., N.P., K.W.K., S.B.G., B.V., and S.Z. Writing—original draft: S.Z., J.D., and E.H.-C. Writing—review and editing: S.Z., S.B.G., and B.V. Supervision: C.B., N.P., K.W.K., S.B.G., B.V., and S.Z. Competing interests: The Johns Hopkins University has filed patent applications related to technologies described in this paper, on which J.D., E.H.-C., K.W.M., Q.W., A.D.S., M.S.H., B.J.M., A.H.P., N.P., K.W.K., S.B.G., B.V., and S.Z. are listed as inventors: HLA-restricted epitopes encoded by somatically mutated genes (US20180086832A1), MANAbodies and methods of using (US20200079854A1), MANAbodies targeting tumor antigens and methods of using (PCT/US2020/065617). B.V., K.W.K., and N.P. are founders of Thrive Earlier Detection. K.W.K. and N.P. are consultants to and were on the Board of Directors of Thrive Earlier Detection. B.V., K.W.K., N.P. and S.Z. own equity in Exact Sciences. B.V., K.W.K., N.P., and C.B. are founders of Neophere. N.P. is an advisor to and holds equity in CAGE Pharma. C.B. is a consultant to DeCyte-Synthes and Bionaut Labs. The companies named above, as well as other companies, have licensed previously described technologies related to this paper from Johns Hopkins University. B.V., K.W.K., S.Z., N.P., and C.B. are inventors on some of these technologies. Licenses to these technologies are or will be associated with equity or royalty payments to the inventors as well as to Johns Hopkins University. The terms of all these arrangements are being managed by Johns Hopkins University in accordance with its conflict of interest policies. Q.W. is the founder and CEO of Complete Omics Inc. M.F.K. received personal fees from Bristol-Myers Squibb and Celldyn. M.P. reports grant and patent royalties through institution from BMS, grant from Compugen, stock from Triaza Therapeutics and Dracen Pharmaceuticals, and founder equity from Potenza; being a consultant for Aduro Biotech, Agenon, AstraZeneca (Medimmune/Ampimmune), Bayer, DANAtrix, Dynavax Technologies Corporation, Enveva, FLX Bio, Rock Springs Capital, Janssen, Merck, Tizona, and Immunometric-Therapeutics; being on the scientific advisory board of Five Prime Therapeutics, Camden Nexus II, and WindMil and being on the board of directors for Dracen Pharmaceuticals. S.B.G. is a founder and holds equity in Advanced Molecular Sciences LLC. Data and materials availability: The MS data reported in this article have been deposited via ProteomeXchange and can be accessed through identifier PASS01603. All other data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. All materials will be made available to the scientific community through a material transfer agreement from Johns Hopkins University.

Submitted 29 June 2020
Accepted 1 February 2021
Published 1 March 2021
10.1126/sciimmunol.abb5515


Acknowledgments: The expression of antibodies and scDBs was carried out at the Eukaryotic Tissue Culture Facility of The Johns Hopkins University School of Medicine. We thank S. Sur, N. Wyhs, A. Cook, R. L. Blasser, A. Lee, J. Cao, S. Sengupta, R. Siliciano, and A. Timmons for assistance with this study. Heatmaps for peptide positional scoring were generated by Display. Funding: This work was supported by The Virginia and D.K. Ludwig Fund for Cancer Research, Lustgarten Foundation for Pancreatic Cancer Research, The Commonwealth Fund, The Bloomberg–Kimmel Institute for Cancer Immunotherapy, Bloomberg Philanthropies, The Mark Foundation for Cancer Research, NIH Cancer Center Support Grant P30 CA06973, and NCI R37 grant CA230400. J.D., B.J.M., A.H.P., and S.R.D. were supported by NIH T32 grant GM73009. M.F.K. was supported by the National Institute of Arthritis and Musculoskeletal and
Bispecific antibodies targeting mutant RAS neoantigens

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Sci. Immunol. 6, eabd5515. DOI: 10.1126/sciimmunol.abd5515

Diabodies see the unseeable

RAS oncogene mutations are common in various cancers, controlling their growth and survival. Targeting mutant RAS proteins with antibodies has been unsuccessful due to low surface expression, even when targeting mutant RAS peptides presented via HLA on the surface of cancer cells. Douglass et al. used phage display to generate single-chain variable fragments (scFv) specific for mutant RAS peptide-HLA complexes. The authors tested various bispecific, T cell-engaging antibody formulations, finding that single-chain diabodies (scDbs) combining the aforementioned scFv with an anti-CD3 scFv were able to induce T cell activation and subsequent killing of tumor cells expressing mutant RAS peptide-HLA complexes. This scDb approach opens the door for antibody-based therapies against mutant neoantigens expressed at very low levels on the surface of cancer cells.