CRISPR-based gene disruption and integration of high-avidity, WT1-specific T cell receptors improve antitumor T cell function

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T cell receptor (TCR)–based therapy has the potential to induce durable clinical responses in patients with cancer by targeting intracellular tumor antigens with high sensitivity and by promoting T cell survival. However, the need for TCRs specific for shared oncogenic antigens and the need for manufacturing protocols able to redirect T cell specificity while preserving T cell fitness remain limiting factors. By longitudinal monitoring of T cell function and dynamics in 15 healthy donors, we isolated 19 TCRs specific for Wilms’ tumor antigen 1 (WT1), which is overexpressed by several tumor types. TCRs recognized several peptides restricted by common human leukocyte antigen (HLA) alleles and displayed a wide range of functional avidities. We selected five high-avidity HLA-A*02:01–restricted TCRs, three that were specific to the less explored immunodominant WT137–45 and two that were specific to the noncanonical WT178–64 epitopes, both naturally processed by primary acute myeloid leukemia (AML) blasts. With CRISPR-Cas9 genome editing tools, we combined TCR-targeted integration into the TCR α constant (TRAC) locus with TCR β constant (TRBC) knockout, thus avoiding TCRβ mispairing and maximizing TCR expression and function. The engineered lymphocytes were enriched in memory stem T cells. A unique WT137–45-specific TCR showed antigen-specific responses and efficiently killed AML blasts, acute lymphoblastic leukemia blasts, and glioblastoma cells in vitro and in vivo in the absence of off-tumor toxicity. T cells engineered to express this receptor are being advanced into clinical development for AML immunotherapy and represent a candidate therapy for other WT1-expressing tumors.

INTRODUCTION

Adoptive cell therapy (ACT) using transfer of genetically engineered T cells is part of the frontline of cancer immunotherapy and has already produced convincing clinical results, especially against B cell tumors (1–3). In high-risk acute myeloid leukemia (AML), the beneficial impact of T cells has been documented by the graft-versus-leukemia effect observed after allogeneic hematopoietic stem cell transplantation (HSCT) (4) but has been limited by the detrimental effects of alloreactivity (5). Although the infusion of engineered tumor-specific T cells may overcome this hurdle, its use is still limited. First, the expression of leukemic antigens in healthy hematopoietic cells, and the consequent risk of myelosuppression, has so far hampered the use of chimeric antigen receptor (CAR)–T cells in AML (6–8). Second, the genomic instability intrinsic to AML blasts results in an elevated risk of clonal evolution and relapse (9). Third, poor CAR-T cell persistence is linked to loss of immunosurveillance and disease recurrence (6). Thus, the generation of a T cell product should focus on antigens relevant for blast survival, which may be more likely found intracellularly. In contrast to CARs, T cell receptors (TCRs) can target virtually every protein, independent of their subcellular localization, and are sensitive to very low antigen densities (10). Moreover, TCR signaling promotes T cell survival, leading not only to the generation of antitumor effectors but also to the establishment of long-term immunological memory, necessary for counteracting tumor relapse. In this study, we focused on Wilms’ tumor antigen 1 (WT1), a zinc finger transcription factor of proven immunogenicity, which has restricted expression in healthy tissues, a strong correlation with oncogenesis, and is expressed by a wide range of hematological and solid tumors (11, 12). WT1 expression is associated with poor disease outcome and is rarely lost, supporting its use as a pan-leukemic marker for minimal residual disease detection (13, 14). Accordingly, a low risk of tumor escape through antigen loss can be predicted if WT1 is targeted (15). Spontaneous T cell responses against WT1 have been observed in patients with cancer upon HSCT and have been found...
to correlate with disease regression (16–18), validating both the immunogenicity of the antigen and its clinical relevance. As a result, several clinical trials targeting WT1 have been conducted (19–21). TCR-based therapies have highlighted the safety profile of this therapeutical approach, but efficacy data are limited to date, which in part may be attributable to the characteristics of the epitope targeted in these trials. Most published studies relied on high-avidity TCRs specific for the WT1 human leukocyte antigen (HLA)–A*02:01 restricted peptide, an epitope found to require the immunoproteasome for natural processing (22), which may not be operational in all tumors. Hence, the full exploitation of ACT targeting WT1 requires (i) the identification and validation of high-avidity TCRs specific for epitopes naturally processed by the standard proteasome, and thus by most AML blasts, and restricted by different HLA alleles and (ii) the engineering of more potent and durable T cells. To this end, we used healthy donor (HD) T cells to identify 19 TCRs, including receptors with high functional avidity, recognizing multiple WT1-derived epitopes with varying HLA restrictions. Through a funnel-based approach, we selected the lead TCRs from our library to engineer TCR-transgenic (Tg) T lymphocytes using genome editing (23, 24), an emerging technology in cancer immunotherapy (25–27). Through clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9–based targeted integration (TI) of the WT1–TCR genes into the TCR α constant (TRAC) locus, combined with efficient knockout (KO) of the endogenous α and β TCR chain genes, we generated homogeneous T cell products, each expressing a nonmodified, high-avidity TCR specific for the immunodominant and naturally processed HLA-A*02:01–restricted WT1 37–45 epitope. The TCR with the highest avidity showed antigen-specific responses in both CD4+ and CD8+ T cells. WT1–TCR–edited lymphocytes efficiently killed leukemic blasts and glioblastoma cells in vitro and in vivo in the absence of unwanted off-tumor effects.

RESULTS

Repetitive stimulations of HLA-matched HDs’ cells enable the generation of a library of WT1-specific TCRs

To identify WT1-specific T cells and TCRs, we designed a multistep workflow and applied it to 15 HDs (Fig. 1A). T cells from HD1 to HD12 were stimulated with a pool of 141 15-mer peptides that span the entire standard exon 5’ KTS’ WT1 isoform, with the addition of an extra 126–amino acid–long fragment at the N terminus (WT1 pool) (28). T cells from HD13 to HD15 were stimulated with a restricted pool of 11 15-mers (WT1 HLA-A*02:01–restricted pool) previously reported as immunogenic and capable of binding HLA-A*02:01 (29). A peptide length of 15 amino acids was used to elicit both CD4+ and CD8+ responses (30). The activation marker CD137 was used to isolate responding T cells, and their expansion was promoted by serial stimulations with either autologous CD3– peripheral blood mononuclear cells (PBMCs) or immortalized B lymphocytes (31, 32), which provide an unlimited reservoir of autologous antigen-presenting cells (APCs) (fig. S1). WT1-specific T cells were successfully expanded in 14 of 15 HDs (success rate: 93%) with varying kinetics and expansion rates, as shown by longitudinal monitoring of CD107a expression and interferon-γ (IFN-γ) production (Fig. 1, B and C, and fig. S2, A and B, left). In most cases, a clear HLA class I–restricted responding population could be observed after four rounds of in vitro stimulation (11 of 14 HDs, about 70%; Fig. 1D); fewer cultures expanded HLA class II–restricted T cells (about 30%). To identify the recognized epitopes, we cultured responding T cells with irradiated APCs pulsed with individual peptide subpools (SPs), according to a defined mapping grid (HD1 to HD12), or with individual peptides (HD13 to HD15). A skewed T cell response toward SP4, SP5, and SP16 was observed for HD1, HD3, HD6, HD7, and HD10, whereas HD13 and HD14 responded to peptide WT1 37–45. For the remaining donors, a T cell response directed against different portions of the WT1 protein was identified (Fig. 1, B and C, and fig. S2, A and B, right). From two HDs (HD4 and HD7), multiple T cell clones reacting to more than one HLA class I–restricted epitope were detected and further expanded by subculturing T cells with each recognized WT1 SP (fig. S2C). Flow cytometry analyses of the TCR repertoire (Fig. 1E) were performed on 11 responding T cell cultures and confirmed a clear oligoclonal T cell subpopulation in HD1 to HD3, HD5, HD12, and HD14. For HD4, HD6, HD10, and HD13, no dominant Vβ (variable region of TCRβ chain) was detected, probably due to the limitation of the cyt fluorimetric assay, which covers only about 70% of the whole Vβ repertoire, or to the presence of multiple clones. For functional validation of individual TCRs, HLA class I–restricted candidates (13 of 19 clonotypes) were prioritized and classified in three groups: (i) TCRs recognizing WT1 37–45, (ii) TCRs recognizing WT1 78–84 of the WT1 HLA-A*02:01–restricted pool, and (iii) other WT1-specific HLA class I–restricted TCRs.

Five newly identified TCRs specifically recognize the HLA-A*02:01–immunodominant WT1 37–45 peptide

Immune responses observed in HD1, HD3, HD6, HD7, and HD10 (group i) were characterized by IFN-γ secretion and CD107a expression in CD8+ T cells challenged with SP4, SP5, and SP16 (Fig. 2A). By deconvoluting the mapping grid, we predicted peptides 40 and 41 to harbor the key epitope in the 11–amino acid overlap (APVLDFAPPGA), which was confirmed by coculturing T cells with autologous APCs loaded with these two peptides or with the flanking peptides as controls (Fig. 2B). We used NetMHCpan-4.0 algorithm (33) to predict the most favorable HLA- peptide binding, with the results suggesting a strong affinity between the nonamer VDLFAPPGA(WT1 37–45) and the HLA-A*02:01 allele (fig. S3, A to E). Prediction was validated by a degranulation assay (Fig. 2C). WT1 37–45– specific T cells recognized Tg K562 cells (K562-Tg: HLA-A*02:01–transduced and WT1) while sparing the wild-type counterpart (K562: HLA-A*02:01 and WT1), thus indicating that the WT1 37–45 peptide is naturally processed and presented in the context of HLA-A*02:01 (Fig. 2D). To identify WT1 37–45–specific TCRβ clonotypes, we longitudinally monitored the immune repertoire dynamics by high-throughput TCR sequencing (34, 35). Results showed different pattern of fluctuations resulting in the ultimate narrowing of the repertoire overtime for most HDs (Fig. 2E). For HD6, the predominant αβ clonotypes observed at early stimulation rounds (third stimulation) were gradually supplanted by different ones at the sixth stimulation. For HD7, despite the expansion of multiple T cell subpopulations specific for different WT1 epitopes, the WT1 37–45–specific TCRβ pairing was identified. Last, for HD10, because of reduced cell fitness, TCR sequencing was performed only once but the results were sufficient to determine the proper TCRβ pairing. As shown, TCRs with higher functional avidity showed preferential outgrowth in these polyclonal cultures, suggesting that our approach of sequential restimulations selects for T cells with a highly functional TCR.
indicates the percentage of WT1-specific T cells upon sequential stimulations (S), indicated with colors ranging from yellow (S2) to maroon (S8). Rose charts were generated using the Visual Paradigm tool. No WT1-specific T cell expansion was obtained from HD11. Identification of the WT1 SPs (B, right) and peptides (C, right) recognized by expanded T lymphocytes (CD4⁺ T cell responses: open dots; CD8⁺ T cell responses: filled dots) is shown, expressed as the proportion of IFN-γ–secreting CD4⁺ or CD8⁺ T cells of total CD4⁺ or CD8⁺ T lymphocytes. (D) The pie chart indicates the percentage of WT1-specific CD4⁺ and CD8⁺ T cell cultures. (E) Clonal T cell expansion was evaluated by cytofluorimetric quantification of the TCRβ chain variable region (TRBV) families (HD2: CD4⁺-specific T cell response; remaining donors: CD8⁺-specific T cell response).

The TCR hunting procedure identified HLA-A*02:01–restricted TCRs specific for a noncanonical WT1 peptide

HD13 and HD14 (group ii) were characterized by a preferential recognition of WT1−78−64 (Fig. 2F). In silico prediction identified the nonamer LLAA-ILDFL as a high-affinity HLA-A*02:01 binder (Fig. S3, F and G). Although HLA restriction was confirmed by the specific recognition of WT1−78−64 pulsed HLA-A*02:01⁺ APCs (Fig. 2G), natural processing and presentation of the cognate peptide was initially questionable because expanded T cells barely recognized K562-Tg cells (Fig. 2G). WT1−78−64 is located in the N-terminal noncanonical portion of WT1, in a splice variant reported in AML samples (29). We observed that this variant is not efficiently expressed by this cell line and was heterogeneously expressed by leukemic blasts (Fig. S3H). We observed specific and potent killing of primary WT1⁺ AML blasts harvested from three HLA-A*02:01⁺ patients by HD14-expanded T cells, whereas HD13-expanded T cells showed a weak response (Fig. 2H). Clonal tracking of the TCR repertoire overtime revealed WT1-specific TCR genes (Fig. 2I) even in the case of HD13, for which characterization of the TCR clonal expansion by flow cytometry proved unsuccessful. Hence, we identified two TCRs targeting a splice variant–specific naturally processed HLA-A*02:01–restricted WT1 peptide.
Fig. 2. TCRs recognizing two HLA-A*02:01–restricted, pAML-processed epitopes were identified and validated in vitro. (A) Dot plots indicating the percentage of HD1-derived CD8+ T cells responsive to SP4, SP5, and SP16. APC, allophycocyanin; FITC, fluorescein isothiocyanate. (B) P40 and P41 (red), identified by deconvoluting the mapping grid, were validated by assessing T cell degranulation upon challenge with peptide-pulsed APCs. P42 and P43 flanking peptides were used as controls. (C) The prediction for VLDFAPPGA(WT137–45) binding to HLA-A*02:01 was validated by coculturing T cells with WT137–45-pulsed (Target cells) or control peptide–pulsed (Ctr target cells) HLA-A*02:01− WT1+ target cells (T2 cells for HD1, HD3, and HD6 and autologous CD3+ T cells for HD10). Degranulation was measured by CD107a expression. (D) WT137–45 is naturally processed as highlighted by increased CD107a expression (degranulation) on T cells challenged with HLA-A*02:01–transduced K562 cells (K562-Tg, HLA-A*02:01*, and WT1+) compared to control cells (K562 wild type (WT), HLA-A*02:01*, and WT1+). (E) Clonal tracking of the WT1-specific TCRβ (α; red; β, blue; remaining TCRs, gray). Circle size reflects the relative contribution of each clonotype to the TCR repertoire. Percentages of the WT1-specific TCR sequences are reported. (F) Dot plots indicate the percentage of HD13- and HD14-CD8+ T cells responsive to WT1–78–64. (G) HD13 and HD14 T cells efficiently recognized WT1–78–64–Pulsed HLA-A*02:01+ target cells (T2 cells for HD13 and autologous immortalized B cells for HD14) while sparing control cells (control peptide–pulsed T2 or B cells and K562 WT cells) and K562 cells expressing WT1 and HLA-A*02:01 (K562-Tg). Degranulation was measured by CD107a expression. (H) HD14 T lymphocytes specifically eliminated HLA-A*02:01*WT1+ blasts from three patients at different effector-to-target (E:T) ratios. WT1 expression is reported in parentheses. Induction of apoptosis in target cells was measured by percentage of caspase 3 (Cas3+) in living cells. Primary acute myeloid leukemia control (pAML ctr) (HLA-A*02:01* WT1+). (I) Clonal tracking of the WT1-specific TCRβ chains is shown as in (E). (J) The pie chart shows the contribution of each CD8+ T cell culture specific for HLA class I–restricted epitopes (predicted, not validated nonamers are shown in italics). HLA-A*02:01–specific T cell cultures are highlighted in red.

Generation of a library of TCRs specific for WT1 epitopes restricted by multiple HLAs
Cultures from HD5 (fig. S4, A to E), HD12 (fig. S4, F to J), and HD15 (fig. S4, K to M) generated clear responses toward defined epitopes restricted by HLA-B*35:01 (HD5), HLA-C*07:02 (HD12), and HLA-B*18:01 (HD15), based on strong prediction scores. HD4-derived cultures were more enigmatic. These T cells responded to several SPs (fig. S5A); thus, the culture was split in two: A portion of cells was stimulated with SP14 (stronger response, HD4A; fig. S5B, left), and the remaining portion was stimulated with the combination of
SP18 and SP21 (weaker response, HD4B; fig. S5B, right). By challenging T cells with autologous APCs pulsed with individual peptides, we identified a response to 15-mers 17 and 18 for T cells stimulated with SP14 (fig. S5C, left) and to 15-mers 99 and 100 for T cells stimulated with SP18 and SP21 (fig. S5C, right). These peptides were then used to specifically expand T cells in both subpopulations. In silico prediction failed in identifying a peptide from 15-mers 17 and 18 with strong HLA binding (fig. S5D, left). Thus, as done for HD5 and HD12, we cocultured T cells with a panel of Epstein-Barr virus (EBV)–B lymphoblastoid cell lines sharing at least one HLA allele with HD4 (fig. S5E, left). T cells responding to 15-mers 17 and 18 demonstrated restriction for HLA-B*35:02 (fig. S5E, left), whereas T cells responding to 15-mers 99 and 100 were predicted to be restricted for HLA-B*40:01 (fig. S5D, right). Specific and selective killing of HLA-B*35:02− and WT1+, but not of HLA-B*35:02− and WT1+, primary AML blasts confirmed the natural processing and presentation of the target peptide by leukemic cells (fig. S5F). TCR immunoprofiling performed over time accurately recapitulated our observations in the cell culture, with a major and faster selection of a defined TCRβ chain in cultures responding to 15-mers 17 and 18 (fig. S5G, left) and a weaker expansion of defined TCRs in samples belonging to cultures reactive to 15-mers 99 and 100 (fig. S5G, right). For HD7-derived T cells, in addition to the dominant response directed toward the WT137–45 epitope, a minor immune reaction was observed specific for SP7, SP8, and SP20 (fig. S6, A and B), leading to the prediction of peptide NQMNLAGTL as a strong binder of the HLA-A*02:05 allele (fig. S6, C and D). The cognate TCRβ pairing was identified by high-throughput sequencing (fig. S6E).

Our TCR identification efforts were culminated in a library of 19 TCRβ chain clonotypes, specific for 14 WT1 peptides and encompassing different HLA restrictions (fig. 2). Out of these, seven TCRs were specific for two epitopes (five for the WT137–45 peptide and two for WT178–64), naturally processed by leukemic blasts and presented on HLA-A*02:01, the most common allele in the Caucasian population (36). Overall, five (HD1, HD3, HD7, HD13, and HD14) HLA-A*02:01–restricted TCRs, for which a clear enrichment of the dominant TCR sequences could be tracked in the T cell cultures, were selected for further validation.

High-avidity WT1–TCR CRISPR-Cas9 genome-edited T cells efficiently kill primary leukemias

To redirect T cell specificity, we modified our previously reported TCR gene editing protocol (23, 24), leveraging on the ability of CRISPR-Cas9 genome editing technology to precisely and efficiently disrupt multiple genes simultaneously in one manipulation step. We deleted the endogenous TCR genes, thus abrogating the risks of TCR mispairing and achieving maximal surface expression of our WT1-specific TCRs (fig. 3A). To KO the endogenous TCRγ chain, we identified a highly active guide RNA (gRNA) targeting exon 1 of the TRAC locus (TRAC023; fig. S7A). In addition, to account for the evolutionary duplication of the TCRβ chain, gRNAs were designed, which target both TCR β constant 1 (TRBC1) and TRBC2 loci, and selected gRNA TRBC019 (fig. S7B). Two days after activation, cells were electroporated with preassembled ribonucleoprotein (RNP) complexes. KO efficiencies at each locus, assessed on the protein abundance using CD3 surface expression, were >99% for the α chain and >90% for the β chain, with an overall disruption efficiency of 98 ± 0.6% (means ± SEM) when concomitant targeting of both loci was performed (fig. 3B). On day 3, we redirected T cells with WT137–45-specific receptors upon transduction with bidirectional lentiviral vectors (LVs) (fig. S7C) (37) encoding for HD1-, HD3-, or HD7-derived TCRs. Transduction efficiency, as evaluated by determining the rescue of CD3 surface expression, was about 50% (HD1-TCR: 41.0 ± 3%; HD3-TCR: 51.5 ± 3.5%; HD7-TCR: 58.5 ± 3.5%; means ± SEM) (fig. 3B) for the three WT137–45-specific TCRs. Engineered cells displayed a stem memory T cell (%TSCM) phenotype (fig. 3C and fig. S7D) and a CD4+ to CD8+ T cell ratio similar to unedited lymphocytes, indicating that gene editing does not affect T cell subsets or differentiation. Homogeneous and high expression of the three different TCRs was further confirmed by high WT137–45 dextramer binding on CD8+ cells (fig. S7E). HD1 LV TCR–edited T cells showed a superior degranulation activity and cytokine production compared to HD3 LV and HD7 LV TCRs when challenged with T2 cells pulsed with decreasing concentrations (1 µM to 1 pM) of WT137–45 peptide (figs. 3D and fig. S7F). HD7-TCR required saturating peptide concentrations (100 µM) for recognition, highlighting an extremely low functional avidity (fig. S7G). The half-maximal effective concentration (EC50) indicates the superior performance of HD1-TCR, followed by HD3- and HD7-TCR (fig. S7H). Adoptive T cell therapy benefits from combined activity of CD4+ and CD8+ T cells (38–40); however, the activation of CD4+ cells by a HLA class I–restricted TCR requires high avidity, as described for some virus-specific lymphocytes (41). Of interest, HD1-TCR was able to activate both T cell subsets, although with an about 100-fold higher peptide threshold for CD4+ cells. This characteristic was not observed with HD3-TCR (fig. 3E). Both HD1- and HD3-TCR–engineered T cells specifically killed blasts harvested from three HLA-A*02:01+ patients with AML, with an efficiency of up to 75% at an effector-to-target ratio of 5:1 (fig. 3F). Overall, HD1-TCR–edited T cells exhibited superior cytotoxic ability, supporting an enhanced fitness of this cellular product.

To further highlight the relevance of the manipulation protocol implemented here, peripheral blood T cells harvested from patients with AML were cultured and engineered with the same protocol used for HDs. Robust gene editing efficiency and preferential expansion of TSCM cells were observed (fig. S8, A to C). CD4+ and CD8+–edited HD1-TCR–expressing patients’ T cells recognized T2 cells pulsed with the WT137–45 peptide (fig. S8D) and killed HLA-matched primary leukemic blasts (fig. S8E). These results highlight the feasibility of our protocol in generating highly functional early memory TCR-edited T cells.

HD1-TCR–engineered T cells are superior in eliminating primary blasts compared to other WT1-specific HLA-A*02:01–restricted TCRs

In addition to the WT137–45-specific TCRs, we also evaluated the two receptors specific for the noncanonical WT178–64 epitope (HD13- and HD14-TCR–engineered T cells). Experimental validation of HLA-peptide prediction (fig. S3, F and G) confirmed the predicted HLA-A*02:01 restriction of HD13- and HD14-TCRs, whereas no recognition of cells expressing the predicted HLA-C*03:03 allele was observed (fig. S9A). Data revealed recognition of two nonamers (WT178–70 and WT177–69) by HD13, one of which (WT177–69), not predicted in silico, was also recognized by HD14 (fig. S9B). WT178–70 elicited wider and larger polyfunctionality than WT177–69 in HD13-TCR–edited T cells and overall a superior immune response than the HD14-TCR counterpart (fig. S9C). When challenged in a 24-hour coculture with primary leukemic blasts, each engineered T cell
Fig. 3. WT137–45-specific TCRs enable engineered T lymphocytes to efficiently recognize target cells. (A) TCR gene editing strategy. (B) Efficiency of TCR gene disruption using gRNAs targeting TRAC, TRBC, or both (n = 3). Untransduced stimulated T lymphocytes (mock) were included as a control. Edited T lymphocytes were transduced (Td) with HD1-, HD3-, or HD7-derived WT1-TCRs. TCR KO and transduction efficiency were assessed by measuring CD3 surface expression (n = 3). Mock T cells were included as a control. (C) TCR-editing strategy. (D) Functional avidity of WT137–45-edited T cells. Polyfunctionality of CD8+ T cells (measured as CD107a expression, IFN-γ, IL-2, and tumor necrosis factor–α (TNF-α) secretion) after a 6-hour coculture with T2 cells loaded with decreasing concentrations of WT137–45 is shown (n = 3). The results of a peptide titration assay show the ability of the natural HD1-TCR, and not of HD3-TCR, to activate both CD4+ and CD8+ T cells as detected by flow cytometry (n = 2, target cells: OCI-AML3 cells). (F) HD1- and HD3-edited T cells efficiently recognized HLA-A*02:01+WT1+ leukemic blasts harvested from three patients with AML at different E:T ratios (n = 3). Total WT1 expression is reported in parentheses. pAML ctr (HLA-A*02:01+WT1+). (G) HD1-edited T cells showed superior and consistent elimination of primary leukemic blasts compared to HD13- and HD14-TCR T cells. pAML ctr (HLA-A*02:01+WT1+). Black stars refer to the statistics versus both HD13- and HD14-TCRs; gray versus HD13-TCR; magenta versus HD14-TCR. Expression of the WT1 isoform exon 5’ KTS + in pAML1, pAML5, and pAML11 can be found in fig. S3H. Functional studies have been performed with T cells generated from distinct donors. Data are presented as means ± SEM. **P < 0.01, ***P < 0.001, and ****P < 0.0001 by two-way analysis of variance (ANOVA) with Sidak’s (E) or Tukey’s (F and G) multiple comparisons test. CM, central memory; EM, effector memory; EMRA, terminally differentiated effector memory.

product (HD1 LV, HD13 LV, and HD14 LV TCR) was able to eliminate target cells. HD1-TCR–expressing cells were superior to the other cellular products in mediating a consistent and potent killing of leukemic blasts (Fig. 3G), thus further strengthening the selection of this receptor, specific for a canonical portion of WT1, as the lead candidate for immunotherapy of AMLs.

TI of HD1-TCR in the TRAC locus, combined with TRBC disruption, generates an efficient and specific cell product

To reduce the manipulation steps, minimize insertional mutagenesis risk, and further standardize the cellular products, we explored TI of the Tg-TCR into the TRAC locus by combining CRISPR-Cas9 with an adeno-associated virus (AAV) carrying the TCR α and β genes
separated by a 2A peptide and flanked by homology arms complementary to sequences present on both sides of the nuclease cut site (Fig. 4A). We started from gRNAs selected and validated to promote specific disruption of TRAC and TRBC1/2 loci, with an efficiency greater than 90% (fig. S7, A and B). The TRAC guides were further evaluated for insertion efficiency of the Tg-TCR, because high editing efficiency is not predictive for high insertion rates (fig. S10A). All lead guides were carefully assessed for off-target editing properties by a combination of bioinformatic (CasOFFinder) (42) and biochemical (SITE-Seq) (43) approaches (fig. S10B). Each potential site was validated in T cells using targeted off-target sequencing (fig. S10C). In particular, gRNA TRBC004 showed no detectable off-target activity and the gRNA TRAC002 showed a high degree of insertion and KO and also displayed no validated off-target events. To evaluate whether KO of both endogenous TCR genes is necessary, cellular products obtained upon insertion of the Tg-TCR at the TRAC locus were compared with (TI TCR) or without (TRAC-TI TCR) concomitant TRBC1/2 gene disruption. We observed a high degree of TCR insertion in the TRAC locus (60 to 70% CD3+ cells; Fig. 4B, left, and fig. S11A) in both experimental settings. The addition of TRBC-KO to TRAC-TI increased the expression of the Tg-TCRs [median fluorescence intensity (MFI) of V8β: about 12,500 in TRAC-TI TCR versus about 40,000 in TI TCR; Fig. 4B, right] and nearly completely abrogated TCR mispairing (Fig. 4B, left, and fig. S11A, top versus bottom). Accordingly, TI TCR cells displayed a significantly increased functional avidity (P < 0.0001; Fig. 4C and fig. S11B) and reduced cross-reactivity in a mixed lymphocyte assay (fig. S11C), thus corroborating the notion that TCR mispairing can lead to new, unknown reactivities (44, 45). The generation of an additional disulfide bond between TCR chains (46, 47) had no effect on reducing TCR mispairing and unexpectedly reduced the expression of our HD1-TCR (fig. S11, A and D). TI of a CAR gene into the TRAC locus, under the control of the TCRα promoter, has been reported to improve CAR-T cell function by averting tonic signaling (25). We thus explored the effects of the endogenous TCRα promoter (promoterless) versus a strong exogenous promoter [full EF1α (elongation factor 1 alpha) promoter] in driving HD1-TCR expression. EF1α drove stronger, more homogenous Tg-TCR expression (fig. S11E) to a degree comparable to natural TCRs, resulting in increased functionality (Fig. 4D and fig. S11F). Treatment of cells with AAV in the absence of CRISPR editing did not result in any episomal WT1-TCR expression, confirming that expression is due to TI in the TRAC locus (fig. S11E). Furthermore, we confirmed HD1 insertion at the genomic level using droplet digital polymerase chain reaction (ddPCR) with primers designed to flank the insert junction region (fig. S11G). The functional avidity of HD1-TCR proved about two logs higher than that of HD3-TCR, even when tested in TI TCR T cells, built with natural TCR constant chains driven by the EF1α promoter (fig. S11H). HD1 T1 TCR T cells efficiently killed WT1* HLA-A*02:01* K562 cells (Fig. 4E) and exhibited a very potent and efficient killing of primary leukemic cells at different effector-to-target ratios (Fig. 4F) to a comparable degree observed with LV-edited T lymphocytes.

**HD1-TCR–edited lymphocytes display an optimal safety profile**

A thorough characterization of the TCR specificity necessitates special consideration given the unexpected toxicities observed in clinical trials with affinity-enhanced TCRs (48–50). Although our TCR is natural and isolated from an HLA-matched HD and cells are engineered to minimize TCR mispairing, we sought to assess potential risks of off-target reactivity. We interrogated the residues within the WT1[37–45] epitope that are critical for HD1-TCR recognition by sequentially substituting each amino acid with an alanine (alanine scanning). All peptides but peptide VADFAPPGLA (peptide 2) were predicted to bind HLA-A*02:01 with similar affinity to the wild-type peptide. No activity was detected with peptides 1 to 3 and 5, and minimal activity was detected with peptide 4. In contrast, peptides 6 and 7 exhibited activities comparable to the wild-type peptide. These data indicate that amino acids V1, D3, and P6 are critical for TCR binding, and F4 is a strong contributor. Amino acids L2 (predicted and experimentally confirmed) and A9 (predicted) were determined critical for HLA binding, whereas P7 (peptide 6) and G8 (peptide 7) likely did not contribute to HLA or TCR binding (Fig. 4G). The alanine-substituted peptide at position 4 induced T cell reactivity only at high concentrations (500 nM); thus, the phenylalanine in position 4 was considered essential for high-avidity interactions. Positions A5 and A9 were not evaluated in this study because they are natural alanine positions. These preliminary data suggest that the minimal functional motif for the WT1[37–45]–specific HD1-TCR is VLDFApXxA (where A is a natural alanine that was not investigated, and x is not critical for binding). In silico analysis using ScanProsite (https://prosite.expasy.org/scanprosite/) and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify protein sequences that contained the minimum binding motif VLDFxPxx with a predicted affinity for HLA-A*02:01 of at least 1000 nM revealed one other expressed protein in humans outside of WT1. Testing of cell lines expressing high abundance of this protein (PIF1) did not reveal any reactivity with the HD1-TCR T cells, suggesting high specificity of this TCR to WT1. The same analysis was performed on our second-in-rank HD13- and HD14-TCRs. Although the latter proved highly selective for the cognate epitope, HD13-TCR, despite impressive functional avidity, displayed a promiscuous recognition motif (fig. S12), thus limiting its translational potential.

Reactivity of HD1-TCR T cells to normal cells was also evaluated. WT1 is expressed at 100- to 1000-fold lower concentrations, compared to AML, in normal tissues including stromal cells within the reproductive organs, podocytes, and CD34+ cells (GTEx, Tissue Atlas) (11, 51, 52). Because of the relevance of potential bone marrow toxicity and the accessibility of this normal tissue, HD1-redirected T cells were tested against HLA-A*02:01 and HLA-A*02:01− CD34+ cells and did not induce relevant cytotoxicity (Fig. 4H). The data indicate that the WT1[37–45] peptide concentrations naturally presented by CD34+ cells are not sufficient to trigger HD1-TCR T cell–mediated recognition. Overall, results of this validation support the safety of HD1-TCR T cells and further strengthen the selection of this receptor as a candidate for clinical implementation.

**EF1α-driven expression of the TCR generates T cells with an optimal activation profile in vivo**

We investigated the effects of the different engineering processes for their effect on T cell activity against AML in vivo. To this aim, WT1-expressing primary leukemic blasts harvested from an HLA-A*02:01+ patient (pAML1) were infused into immunodeficient NOD (non-obese diabetic) scid (severe combined immunodeficient) gamma (NSG) mice. TCR expression and phenotype of infused T cells was evaluated (fig. S13A). Leukemia-bearing mice were treated with two doses of HD1 LV TCR, EF1α HD1 TI TCR, or
**Fig. 4. TI of WT1-TCR in the TRAC locus coupled to TRBC disruption generates T cell products endowed with enhanced functionality.** (A) TI of the WT1-TCR into the TRAC locus alone (HD1 TRAC-TI TCR) (blue) or in combination with endogenous TRBC disruption (HD1 TI TCR) (red) (n = 2). PE, phycoerythrin. (C) Increased functional avidity and enhanced TCR pairing were detected in HD1 TI TCR compared to HD1 TRAC-TI TCR cells by measuring degranulation activity in peptide titration assays (n = 2, target cells: T2 cells). (D) Promoterless (PL) and elongation factor 1 alpha (EF1a) WT1 T cells were assessed for their functionality by measuring cytokine release when cocultured with WT137–45-pulsed OCI-AML3 cells (n = 4). (E) Cytotoxicity of HD1-engineered T lymphocytes against hematologic tumors was shown by measuring loss of luciferase signal upon coculture with luciferase-expressing HLA-A*02:01-transduced (K562-A2.1-luc), but not control (K562-luc), WT1+ chronic myelogenous leukemia (CML) (K562) cells (n = 2). Unedited T cells were used as a control. Statistics refer to the condition with unedited T cells. (F) TI of TCR-expressing cells showed a comparable killing activity to LV-engineered T lymphocytes as evaluated by measuring caspase 3 expression upon coculture with HLA-A*02:01 WT1+ pAML blasts (total WT1 expression in parentheses) harvested from two patients at different E:T ratios (n = 2). Statistics refer to the condition with control T cells (engineered lymphocytes expressing an unrelated TCR). (G) The specificity of TCR-edited T cells was assessed by determining the critical amino acid residues involved in TCR-HLA binding. Caspase 3 and 7 induction was measured in T2 cells pulsed with the WT137–45 wild-type peptide or with the epitopes generated by mutating each individual non-alanine amino acid with alanine (left) upon coculture with HD1-TCR T cells (n = 2). Critical amino acid residues involved in the TCR binding are highlighted with blue circles (right). The phenylalanine in position 4 (dashed circle) was considered essential for high-avidity TCR-ligand interactions. (H) HD1-engineered T cells do not show on-target/off-tumor toxicities against CD34+ stem or progenitor cells, as measured by caspase 3/7 spots per image at different peptide concentrations (n = 2).
promoterless HD1 TI TCR T cells \((1 \times 10^7 \text{ TCR}^+ \text{ T cells per dose})\) starting 3 days after blast infusion (Fig. 5A). As control, mice were either left untreated or treated with melanoma-associated antigen recognized by T cells 1 (MART1) TI TCR T cells (recognizing an HLA-A*02:01–restricted MART1 epitope). All HD1-based cellular products significantly controlled leukemic outgrowth, compared to controls \((P < 0.0001; \text{Fig. 5B})\). Results highlighted a greater efficacy for HD1 TI TCR T cells than HD1 LV TCR T cells \((P < 0.0001)\) in killing blasts in vivo, independently from the promoter used to drive TCR expression. A thorough phenotypic analysis showed that, once harvested ex vivo, LV-engineered T lymphocytes, which proved enriched in highly differentiated TEMRA \((\text{terminally differentiated effector memory T})\) cells at the time of infusion (fig. S13A), were phenotypically different and displayed a reduced expression of the HLA-DR activation marker and an increased expression of TIM3 and PD-1 exhaustion markers compared to TI-based cellular products (fig. S13, B and C). Comparative analysis of EF1\(\alpha\)- versus promoterless-based products revealed a higher MFI for surface CD3 expression (Fig. 5C), an increased proportion of activated CD4\(^+\) and CD8\(^+\) T cells (Fig. 5D), and an increased percentage of HLA-DR\(^+\)TIM3\(^-\)PD-1\(^-\) T cells when the tumor-specific TCR expression was driven by the EF1\(\alpha\) promoter (fig. S13C). On the basis of the aggregate results, the EF1\(\alpha\)-driven T cell product was selected for further validation. The funnel diagram summarizes the filters applied to our pipeline of TCR discovery and T cell engineering to select a candidate (HD1 TI TCR) for clinical validation (fig. S13D).

**HD1-TCR–engineered T cells efficiently control human leukemia and glioblastoma outgrowth in vivo**

To further evaluate the efficacy of this HD1 TI TCR T cell product in vivo, we tested engineered cells in three separate immunodeficient murine models. TCR expression and phenotype of infused T cells were evaluated (fig. S14A). In the AML model described above (pAML1, Fig. 6A), we compared the efficacy of one versus two lymphocyte infusions. At day 20, a time point associated with a nearly complete disappearance of circulating infused T cells (Fig. 6B, left), half of the mice belonging to the MART1- and HD1-TCR groups received an additional dose of engineered cells. After initial fluctuations in their frequencies, circulating blasts started to increase rapidly in control mice, but not in HD1-TCR–treated animals.
Fig. 6. HD1-engineered T lymphocytes mediate specific antitumor activity in acute leukemia models and in glioblastoma-bearing mice. 

(A) Timeline of the AML in vivo model. (B) T cell kinetic (left) in vivo model with pAML1 and leukemic blast (pAML1 and pAML5) outgrowth (middle and right) for mice treated with engineered HD1 TI TCR T cells. Mice only infused with leukemic blasts or treated with MART1-TCR T cell infusions were included as controls. (C) Cytotoxicity of HD1-engineered T cells was measured in vitro upon coculture with luciferase-expressing HLA-matched WT1+ ALL cell line (n = 2). Unedited T cells were used as a control. (D) Timeline of the ALL in vivo model (left) and noninvasive bioluminescence monitoring of ALL growth in treated and untreated tumor-bearing hIL-15 NOG mice (right). (E) Cytotoxicity of HD1-engineered T cells against the HLA-A*0201 WT1+ glioblastoma U87MG cell line at an E:T ratio of 2:1 (n = 2). The image area covered by red (target cells) and green (caspase 3/7-positive cells) signals is shown as the G+R area. (F) Timeline of the glioblastoma mouse model (left) and outcomes (right) of the three treatment groups (U87MG only, MART1-TCR, and HD1-TCR). The Kaplan-Meier curve indicates the overall survival (in %) of untreated (U87MG only) mice and animals injected with an unrelated MART1-TCR or with HD1-TCR T cells. Animals were euthanized and censored when one tumor diameter reached >10 mm or when tumor ulceration was present. (G and H) Increased activation status of engaged CD4+ and CD8+ T cells was observed in HD1-engineered lymphocytes compared to the MART1-TCR counterpart. The first two time points after the first and second T cell infusion in the glioblastoma settings (G) and in the in vivo model with pAML1 (H) are shown. In vitro functional studies were performed with T cells generated from distinct donors. Data are presented as means ± SEM [in (E), means ± SD]. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 by two-way ANOVA with Sidak’s (C, G, and H) or Tukey’s (B and D) multiple comparisons test. In (F), the log-rank Mantel-Cox test was used. ALL, acute lymphoblastic leukemia; HBSS, Hank’s balanced salt solution.

(P < 0.0001). At day 45, we observed the complete absence of circulating blasts in animals treated with two doses of HD1-TCR T cells (Fig. 6B, middle). Longitudinal profiling of circulating T cells showed a preferential accumulation of activated effector memory T cells in the HD1 TI TCR–treated animals (fig. S14, B and C). Results were confirmed in an independent experiment with blasts (pAML5) from a different HLA-A*02:01 patient (Fig. 6B, right). At euthanasia, evaluation of the tumor burden in the bone marrow and
in the spleen confirmed the efficiency of HD1-treated cells in controlling the leukemic outgrowth (fig. S14D).

Because WT1 is overexpressed in several hematological and solid tumors (12), we reasoned to challenge HD1 TI TCR T cells in two additional models. Upon in vitro assessment of T cell–mediated cytotoxicity against an aggressive acute lymphoblastic leukemia (ALL) cell line (ALL 697) (Fig. 6C), HD1-Ti–engineered T cells were infused in tumor-bearing human interleukin-15 (hIL-15) NOG (NOD/Shi-scid/IL-2Rnull) mice. In this context, HD1 T1 TCR–engineered T cells proved highly effective in controlling leukemic cells compared to control groups (P < 0.0001) (Fig. 6D).

Last, as a model of solid tumor expressing WT1 (53), we selected glioblastoma, which represents a major unmet clinical need. At first, we verified the ability of HD1 TI TCR T cells to effectively kill HLA-A*02:01+, WT1-expressing U87MG glioblastoma cells in vitro (Fig. 6E). Using an in vivo heterotopic glioblastoma NSG model, we found that HD1 TI TCR T cells infused in mice with a measurable tumor burden (≥5 × 10^6 total flux in photons per second) (fig. S14E) efficiently controlled tumor outgrowth, leading to an improved survival at day 24 (P < 0.01 versus mice infected only with the tumor and P < 0.001 versus mice treated with MART1-TCR T cells) (Fig. 6F).

Longitudinal profiling of engineered T cells circulating in mice confirmed our in vitro observations and showed a preferential enrichment of CD4+ and CD8+ activated T lymphocytes in the HD1 TI TCR group (Fig. 6, G and H, and fig. S14F) in each in vivo model tested. No substantial body weight loss (fig. S14G), signs of graft-versus-host disease (GVHD), or other treatment-related toxicities were observed in any experimental setting. Overall, these results further underline the potential of HD1-Ti–engineered T cells as a safe and potent controller of tumor outgrowth in vivo and support advancement of this product toward clinical testing.

**DISCUSSION**

Endowing T cells with new TCRs can effectively redirect some of the most potent players of our immune system against virtually any tumor antigen. Because of the low frequency of high-avidity cancer-specific T cells, identifying the most potent and safe tumor-specific TCRs remains a major challenge for the broad use of TCR-based therapies in clinical practice. In this study, we coupled the discovery of optimal antitumor TCRs with genome editing to generate safe and highly efficient living drugs with the potential to persist long term and patrol for tumor recurrence. We focused on WT1, a transcription factor widely overexpressed on a variety of hematological and solid tumors and collectively recognized as a high-priority antigen (11). Tumor-specific T cells in patients are rare, often exhausted (54, 55), and difficult to expand. Thus, although more time consuming, we chose to stimulate T cells with autologous APCs because of reported issues of promiscuity in peptide binding observed with high-avidity TCRs generated from mismatched HLA cultures (56). Being selected from the natural immune repertoire of HDs, the newly identified high-avidity TCRs are expected to be intrinsically safer than affinity-enhanced TCRs (57, 58), having gone through negative selection in the thymus.

Self-HLA–restricted T cells specific for tumor-associated antigens have been isolated from the peripheral blood of HDs (59, 60). Here, upon repeated stimulations of HDs’ PBMCs with tumor antigen and by monitoring fluctuations of the TCR repertoire over time, we could select an array of clinically relevant, high-avidity TCRs in the absence of the time-consuming procedure of T cell cloning. We isolated 19 TCRs recognizing 14 WT1-derived epitopes encompassing various HLA alleles. Of interest, 7 of 19 WT1-specific αβ clonotypes are specific for two epitopes (WT137–45 and WT1–178–64) presented by HLA-A*02:01, an allele expressed in about 40% of the Caucasian population (36). In particular, a shared potent response was observed in T cells originating from five HDs toward epitope WT137–45 and toward WT1–178–64 in two HDs.

A TCR targeting the WT1126–134, HLA-A*02:01–restricted epitope has been safely tested in clinical trials, but its efficacy has been limited, in part, by its requirement of processing by the immunoproteasome (22, 61). In contrast, WT137–45 and WT1–178–64 are naturally processed and presented on the surface of primary blasts harvested from patients with leukemia, as shown by the ability of newly isolated TCRs to efficiently eliminate these targets. Despite these promising data, given the localization of WT1–178–64 in a WT1 isoform that is not uniformly expressed in all AMLs and has limited data on its relevance in tumor biology, further investigation in different tumor types is warranted before targeting it therapeutically. In our study, although the known WT1126–134 epitope was included in the peptide pool, T cells specific for this epitope were not retrieved, indicating that other epitopes can be more immunodominant, as also shown by vaccination studies. T cells specific for WT137–45 could be detected in 60% of patients with cancer upon vaccination, whereas only 20% were specific for WT1126–134 epitope (62). Overall, these data indicate the presence of an endogenous immunity toward this epitope (62, 63); however, identification of high-avidity T cells has not been reported to date.

We selected the most potent TCRs recognizing the WT137–45, WT1–178–64, and WT1–126–134 peptide (HD1-, HD3-, and HD7-TCRs) as well as the noncanonical WT1–178–64 (HD13- and HD14-TCRs) and genetically engineered these receptors into T cells for further characterization, leveraging the CRISPR-Cas9 editing platform. Despite recognizing the same peptide, the three TCRs specific to WT137–45 showed a wide range of functional avidities, with HD1-TCR outperforming the rest. HD1-TCR also showed a superior and more consistent killing of primary leukemic samples when compared to HD13- and HD14-TCRs. To generate a more homogeneous product and to further boost functionality and specificity, we exploited T1 of the TCR genes into the TRAC locus (silencing the endogenous TCRα chain) while also removing the endogenous TCRβ chain. T1 into TRAC has already been shown beneficial for improving CAR-T cell fitness (25). In case of TCR insertion, this strategy may be particularly useful, generating a cellular product with only one TCR pair and thus avoiding mispairing issues and the need to generate modified TCR constant regions that can be immunogenic (64). This engineering approach resulted in improved TCR expression, as the transferred TCR α and β chains no longer compete with the endogenous ones for other TCR complex components. This led to increased functionality and specificity, as highlighted by the comparison of T cells engineered with and without TRBC KO. Unlike what has previously been shown for CAR-T cells, our in vivo data showed that a more efficacious cellular product, with an optimal in vivo phenotypic profile (superior engagement of activated CD4+ and CD8+ T cells, enhanced TCR surface expression, higher percentage of the HLA-DR activation marker, and reduced expression of exhaustion receptors), was obtained when TCR expression was driven by the EF1α promoter, rather than by the endogenous TCRα.
promoter. The results reported by Eyquem et al. (25) compared random integration of a CAR using a promoter-driven LV construct to T1 into the TRAC locus without an exogenous promoter and found improved in vivo activity with the latter, attributing the effect to the TCRs versus EF1α promoter and the genomic context. The data reported here show a clear benefit for the T1, regardless of the promoter; however, activity was further improved with the strong exogenous promoter in our setting, which could be due to the length of the transcript, here encoding for two TCR chains instead of one CAR, or the need for two 2A self-cleaving peptides in the promoterless construct. T1 of the EF1α construct generated more potent T cell activation than the construct driven by the endogenous TCRβ promoter while also resulting in less exhausted T cells. Recently, Müller and colleagues (65) showed that the orthotopic integration of viral specific TCR genes leads to homogeneous TCR expression and more predictable in vivo activity of engineered cells, when compared to retrovirally transduced, edited T cells. Our data suggest that optimal activation of the TCR is critical and dependent on genomic context as well as the right promoter strength. Accordingly, edited T cells, generated from HDs' peripheral blood and from culturing T cells isolated from patients with AML, displayed an early differentiated phenotype, a characteristic that has been associated with long-term T cell persistence in multiple clinical trials (66–68).

HD1-TCR–edited T cells showed high functional avidity in the low nanomolar range. They also displayed potent recognition of target cells, including primary AML blasts. HD1-TCR–edited T cells were also endowed with the unique ability to activate both CD4+ and CD8+ T cells, a characteristic not yet reported for TCRs specific for this epitope (69), which could be crucial for effective and long-lasting clinical effects.

Because a genome-edited product may contain unwanted off-target changes, and because WT1 is expressed at lower concentrations in normal tissues, we carefully evaluated its safety profile. In this regard, no off-target editing sites were observed for the gRNAs used in the genome editing process. Furthermore, the TCR recognition motif proved to be highly specific for the WT1 37–45 epitope. In addition, no killing of HSCs that express physiological concentrations of the WT1 protein was observed. Although on-target off-tumor toxicity cannot be assessed in the mouse because of the lack of HLA-A*02:01 expression, no cross-reactivity or GVHD was observed, and the infusion of HD1-TCR–edited T cells effectively controlled tumor outgrowth in different tumor settings, mirroring hematological (primary AML and ALL models) and solid (glioblastoma) tumors. T cell engraftment, activation, and persistence for about 21 days were observed in these mice, as expected in an in vivo setting without human cytokine support. No uncontrolled T cell outgrowth was detected, supporting the specificity of the TCR and the lack of chromosomal alterations due to the genome editing process, as further confirmed by detailed genomic analyses of the product being developed for clinical investigation.

Our study has some limitations. In TCR-based gene therapy studies, the TCR needs to be matched to patient HLAs, thus poten- tially limiting its wide-range exploitation in the clinical arena. However, to overcome this hurdle, we have focused on the in-depth profiling of a TCR restricted by the HLAs A*02:01 allele, present in 40% of the Caucasian population. In addition, our pipeline has led to the generation of a library of TCRs encompassing diverse HLA restrictions, an aspect that may further enlarge the cohorts of patients who may benefit from a WT1-TCR therapy. Furthermore, our study has focused on the identification of TCRs targeting a single antigen, with the consequence that antigen loss may potentially be envisaged as an immune evasion mechanism. To limit this potential drawback, we have selected WT1, an antigen highly relevant for tumorigenesis and leukemogenesis.

As our study highlights, although a library of TCRs specific for a selected antigen can be established, the application of a systematic comparative multistep analysis of their characteristics allows for prioritization of receptors and selects for those endowed with all the relevant features necessary for its implementation in clinical trials. Hence, we here show an innovative and robust pipeline for TCR discovery able to lead to the generation of T cell products for the treatment of hematological malignancies and possibly solid tumors. These results support the development of this cellular product for clinical investigation.

MATERIALS AND METHODS

Study design

The objective of this study was to identify tumor-specific TCRs to be used in TCR gene editing with the final aim of generating cellular products for the treatment of hematological and solid malignancies. For expansion of WT1-specific T cells from HDs, cell cultures were functionally tested at different time points and analyzed by TCR sequencing. Newly identified TCRs were further tested and validated in TCR-engineered T cells. For the in vitro evaluation of TCR-engineered T cells, we used as effector cells lymphocytes harvested from different healthy individuals or AML patients and engineered to redirect their TCR specificity. The detailed number of biological replicates is reported in each figure legend. As target cells, we used either leukemic blasts harvested from different AML patients or tumor cell lines. Experiments were performed at increasing effector-to-target ratios, and several parameters were evaluated (killing ability, CD107a expression, and cytokine secretion). Peripheral blood was obtained from HDs at San Raffaele Hospital (Ospedale San Raffaele Scientific Institute) upon informed consent, in agreement with the Declaration of Helsinki. PBMCs were isolated using Ficoll-Hypaque (Fresenius) density gradient centrifugation.

For in vivo studies, the number of animals was selected on the basis of variability observed in pilot experiments and on availability of primary blasts. Growth kinetics of the infused leukemias and of U87MG cells was assessed before treatment. Animals were randomized to groups (6 to 10 mice per group), and no mice were excluded from the experiment. Mice were treated by an operator who was blinded to treatment groups. All in vitro and in vivo experiments were replicated by different investigators, and all replicates were successful. Analysis of in vitro and in vivo data was based on objectively measurable data (cell counts and MFI).

Stimulation, isolation, and expansion of WT1-specific T cells

Freshly isolated PBMCs were resuspended in X-VIVO 15 medium (Lonza/Euroclone) supplemented with 5% human AB serum (Lonza/Euroclone), 1% penicillin-streptomycin (Lonza/Euroclone), 2 mM glutamine (Lonza/Euroclone) [culture medium (CM)], and anti-CD28 monoclonal antibody (1 μg/ml) (BD Biosciences); seeded at a density of 10^5 cells/ml and stimulated with the WT1 overlapping peptide pool or with the WT1 HLA-A*02:01–restricted pool (table S1). Only for the first stimulation of T lymphocytes with the WT1 peptide pool, antigen-specific T cells were isolated after 26 to 30 hours by CD137 expression using magnetic sorting (Miltenyi Biotec). The
CD137− fraction was depleted of the CD3 cells, γ-irradiated at 30 Gy, and used as peptide-loaded APCs in coculture with the CD137− fraction at ratios from 20:1 to 100:1. Cells were seeded at a final density of 5 × 10^6 cells/ml. CM supplemented with IL-7 (5 ng/ml) (PeproTech), IL-15 (5 ng/ml) (PeproTech), and IL-21 (10 ng/ml) (Milenyi Biotec) was used and replaced every 2 to 3 days. Cells were restimulated every 7 to 14 days with WT1-pulsed autologous γ-irradiated (30 Gy) APCs and seeded at a final density of 1.5 × 10^6 to 2 × 10^6 cells/ml. To identify the immunogenic peptides, T cells stimulated with the WT1 pool were seeded in different wells and cocultured with autologous APCs loaded either with individual WT1 SPs or with individual peptides.

**Editing of T lymphocytes**

PBMCs from HDs or from AML patients were activated and sorted using magnetic beads conjugated to antibodies to CD3 and CD28 (ClinExVivo CD3/CD28, Invitrogen) and seeded at a concentration using magnetic beads conjugated to antibodies to CD3 and CD28 directed to WT1 SPs or with individual peptides. Stimulated with the WT1 pool were seeded in different wells and cocultured with autologous APCs loaded either with individual peptides to seeded T cells directly after the electroporation step.

(i) the redirection of T cell specificity was achieved by adding AAV vectors to seeded T cells

(ii) the redirection of T cell specificity was achieved by adding AAV vectors to seeded T cells

Statistical analysis

Raw, individual-level data are presented in data file S1. Statistical analyses were performed with Prism software (GraphPad, version 8). Student’s t test, one-way analysis of variance (ANOVA), and two-way ANOVA with Tukey’s and Sidak’s multiple comparisons tests were performed for the analysis of the set of data throughout the study. Log-rank ( Mantel-Cox) test was performed for the survival analysis. **EC50** values were calculated using a nonlinear regression model (least squares fit) by using the dose-response equation of the GraphPad Prism software. A P value of <0.05 was set as a threshold for significance.

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designed and supervised the study and wrote the manuscript. Competing interests: C.B. is an inventor on a patent application submitted by Ospedale San Raffaele on the T cell manufacturing protocol used in the manuscript (Use of common g-chain cytokines for the visualization, isolation and genetic modification of memory T lymphocytes; patent family PCT/IT2006/000600) and on a patent application submitted by Sangamo Bioscience and Ospedale San Raffaele on the exploitation of zinc finger nucleases for the TCR gene editing (TCR targeted disruption of T cell receptor genes using engineered zinc finger protein nucleases; U.S. Patent 12/927,292). C.B., S.M., and Z.I.M. are co-inventors on a patent application submitted by Sangamo Bioscience and Ospedale San Raffaele on the TCR gene editing approach (Targeted disruption of T cell receptor genes using engineered zinc finger protein nucleases; PCT/US2014/031360). C.B., E.R., F.C., L.V., and Z.I.M. are co-inventors on a patent application submitted by Ospedale San Raffaele and Fondazione Centro San Raffaele on the discovery of new WT1-TCRs (WT1-TCRs; patent family PCT/EP2018/060477, WO2018197492A1). C.B., E.R., Z.I.M., F.C., and E.C. are co-inventors on a patent application submitted by Ospedale San Raffaele on the WT1-TCRs (WT1-TCRs; PCT/EP2019/079916). S.A.Y. and B.S. are co-inventors on a patent application submitted by Intellia Therapeutics on compositions and methods for engineered T cell therapies using CRISPR-Cas9 (Compositions and methods for immunotherapy; PCT/US2019/056399, WO2020/081613A1). A. Prodeus, I.D., M.M., M.S.A., D.L., I.B., and B.S. are employees and shareholders of Intellia Therapeutics. S.A.Y. and D.O. were employees of Intellia Therapeutics at the time this work was conducted and are shareholders of Intellia Therapeutics. C.B. has been a member of the advisory boards and a consultant for Molmed, Intellia, TxCell, Novartis, GSK, Allogene, Kite/Gilead, Miltenyi, and Kiadis and received research support from Intellia Therapeutics. L.V. received research support from GenDx and Moderna Therapeutics. The other authors declare that they have no competing interests.

Data and materials availability: All data associated with this study are present in the paper or the Supplementary Materials. After publication, all reasonable requests for materials, data, and code will be fulfilled after completion of a material transfer agreement between Intellia Therapeutics and the University or Institution. TCR sequencing data are deposited on Gene Expression Omnibus under the accession number GSE189518.