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Editing T cell specificity towards leukemia by zinc-finger nucleases and lentiviral gene transfer

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Abstract

The transfer of high-avidity T-cell receptor (TCR) genes isolated from rare tumor-specific lymphocytes into polyclonal T cells is an attractive cancer immunotherapy strategy. However, TCR gene transfer results in competition for surface expression and inappropriate pairing between the exogenous and endogenous TCR chains, resulting in suboptimal activity and potentially

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Author Contributions

EP and PG designed experiments, performed research, analyzed data and wrote the manuscript. EP and PG equally contributed to this work. AL designed research and analyzed data. ZM performed research and analyzed data. PQL, AR and VC designed and were responsible for assembly of the *TRBC*-ZFN. DP and LZ designed and were responsible for assembly of the *TRAC*-ZFN. JK optimized WT1-TCR genes. AB setup the protocols of T cell culture and assisted with experimental design. MP supervised histological analyses. GC, FC, CBordignon, PDGreenberg, MCH, PDGregory assisted with experimental design and revised the paper. LN designed the research, analyzed the data and wrote the manuscript. C. Bonini designed the research, analyzed the data, wrote the manuscript and acted as senior author of the study.

harmful unpredicted specificities. We designed zinc-finger nucleases (ZFNs) promoting the disruption of endogenous TCR β and α chain genes. ZFN-treated lymphocytes lacked CD3/TCR surface expression and expanded with IL-7 and IL-15. Upon lentiviral transfer of a TCR for the WT1 tumor antigen, these TCR-edited cells expressed the new TCR at high levels, were easily expanded to near-purity, and proved superior in specific antigen recognition to matched TCR-transferred cells. In contrast to TCR-transferred cells, TCR edited lymphocytes did not mediate off-target reactivity while maintaining anti-tumor activity *in vivo*, thus demonstrating that complete editing of T-cell specificity generate tumor-specific lymphocytes with improved biosafety profile.

Introduction

Adoptive T cell immunotherapy has demonstrated promise in humans, as shown by the persistent and complete clinical responses observed in leukemia patients who underwent allogeneic hematopoietic stem cell transplantation followed by the adoptive transfer of donor T lymphocytes[1], and in solid cancer patients following adoptive transfer of tumor specific cytotoxic T lymphocytes (CTLs)[2,3]. However, several hurdles remain before adoptive immunotherapy can become a broadly applicable therapeutic approach. Tumor-associated antigens are often self-antigens, and thus lymphocytes with the potential to recognize cancer cells with sufficiently high-avidity are usually deleted or anergized to prevent detrimental autoimmunity. If rare high-avidity tumor specific lymphocytes can be isolated from a cancer patient, expansion to numbers sufficient for therapy generally requires long-term culture, that may result in lymphocyte terminal differentiation, and that is often incompatible with the treatment of rapidly growing tumors.

To overcome these difficulties, the α and β chain genes from high-avidity T cell receptors (TCR) have been cloned from tumor-specific CTLs and genetically inserted into large numbers of human T lymphocytes to impart specificity for autologous tumor cells[4]. Initial clinical studies demonstrated the feasibility and safety of TCR gene transfer, but the therapeutic results have been disappointing compared to infusion of naturally isolated and expanded tumor-specific T cells[5-7]. This likely reflects limitations of current gene transfer technologies, which often fail to establish stable high levels of expression of the exogenous TCR chains and cannot fully re-direct T cell specificity. In fact, the tumor-specific α and β chains are added to mature lymphocytes that already express an endogenous TCR. Thus, the transduced cells express four different TCR chains that compete for binding to components of the CD3 complex -the rate-limiting factor controlling translocation of paired TCR chains to the cell surface- leading to reduced assembly of the tumor-specific TCR at the plasma membrane. Such dual-specific T cells exhibit impaired function, with lower avidity for targets expressing either the tumor antigen or the ligand for the endogenous TCR. This problem is further exacerbated by the potential for inappropriate pairing of exogenous and endogenous TCR chains which (i) further limits the assembly of proper tumor-specific TCRs; and (ii) leads to the assembly of novel TCRs that have not been subjected to tolerance mechanisms and thus have unpredictable specificities including potential autoreactivity[8,9]. Indeed, the risk of severe autoimmune toxicity resulting from the formation of self-reactive

TCR from mispairing of endogenous and exogenous chains has been recently highlighted in a murine model closely mimicking the clinical setting[10].

Current strategies addressing these hurdles aim at promoting preferential assembly/surface expression[11,12] of the exogenous α and β chains, for example by using murine instead of human TCR constant regions, which can enhance homologous pairing but proved immunogenic[13]; by incorporating additional cysteines into the exogenous chains to promote homologous pairing and enhance the stability of the exogenous TCR; and/or by codon usage optimization of the exogenous TCR genes[14]. These strategies increase exogenous TCR expression but fall short of ensuring the high expression levels required for high-avidity tumor-specific effectors and, most importantly, do not eliminate mispairing with the endogenously expressed TCR[15].

To overcome these limitations, we report a novel strategy based on zinc-finger nucleases (ZFNs) to edit T cell specificity at the DNA level by knocking out the endogenous TCR. ZFNs are engineered endonucleases composed of a tandem array of C_2H_2 zinc-finger DNA binding domains, which can be designed to selectively bind a DNA sequence of choice, coupled to the catalytic domain from the Type IIS restriction enzyme FokI[16], to introduce a DNA double strand break (DSB) at a predetermined genomic site. DSBs are predominantly repaired by non-homologous end joining (NHEJ), an error-prone process that often introduces nucleotide deletions or insertions (indels) at the site of the break and, consequently, can lead to gene disruption if the ZFN induced DSB is targeted to a protein coding sequence[17-20]. Our approach combines the permanent and heritable somatic knockout of the endogenous TCR genes by transient exposure to the ZFNs with the transfer of tumor-specific TCR by state-of-the-art lentiviral vectors. Such TCR gene editing was evaluated in central memory T (T_{CM}) lymphocytes, which are highly clonogenic, exhibit self-renewal capacity and are thus less prone to exhaustion, and demonstrate enhanced anti-tumor potential[21-24]. TCR-edited cells showed enhanced tumor killing activity with sharply reduced non-specific alloreactivity as compared to matched cells undergoing conventional TCR gene transfer.

Results

Efficient ZFN mediated disruption of *TRBC* genes in human T lymphocytes

To edit T cell specificity, we first designed a pair of ZFNs (**Supplementary Table 1**) targeting a sequence found within the TCR β chain conserved between the β1 and β2 constant region genes (*TRBC1* and *TRBC2*; **Fig. 1a**). The binding specificity of each ZFN was determined by SELEX (Systematic Evolution of Ligands by Exponential Enrichment; **Supplementary Table 2**). Integrase-defective lentiviral vectors (IDLV) were employed to transiently express the *TRBC*-targeting ZFNs in lymphocytes (*TRBC*-ZFN IDLV; **Fig. 1b**)[18,19]. In Jurkat cells, we observed a vector dose-dependent abrogation of cell surface expression of the TCR/CD3 complex reaching up to 20% of treated cells (**Fig. 1c**). A mismatch-selective endonuclease assay (Cel1 assay) confirmed these results at the genetic level, with up to 26% of the *TRBC* alleles (18% *TRBC1* + 8% *TRBC2*) disrupted at the ZFN target site in treated cells (**Fig. 1d**). To extend this observation, peripheral blood T cells from healthy donors were activated with CD3 and CD28 antibody-conjugated beads (baCD3/

CD28), exposed to increasing doses of the *TRBC*-ZFN IDLVs after 48 hrs, and cultured in the presence of IL-7 and IL-15 (5ng ml^{-1}). Abrogation of cell surface expression of the TCR/CD3 complex was observed in up to 7% of treated cells (**Fig. 1e**). Cel1 analysis of the DNA from bulk treated cells showed that up to 6% of the *TRBC1* and 7% of the *TRBC2* alleles carried mutations at the ZFN target site, consistent with the disruption of either allele and the observed loss of CD3 surface expression. In cells sorted for loss of CD3 expression, the frequency of *TRBC1* and *TRBC2* disruption at the DNA level was >30% and >40% respectively, more than the minimal expected level of 25% each, considering that only one out of four alleles is expressed in each cell and that disruption of that allele is sufficient to abrogate CD3 expression (**Fig. 1f**). Amplification and sequencing of the *TRBC1* and *TRBC2* DNA from treated lymphocytes confirmed the occurrence of indels at the ZFN target site resulting in the disruption of the targeted genes (**Fig. 1g**).

Expansion and purification of *TRBC*-disrupted T lymphocytes

Since memory T lymphocytes are less dependent upon TCR signals for homeostatic proliferation than naive T cells[25], we investigated whether homeostatic cytokines could promote survival and growth of previously activated cells, in the absence of TCR expression. Remarkably, the *TRBC*-ZFNs treated cells expanded by supplementation with low dose IL-7 and IL-15 (**Fig. 2a**), with the proportion of $CD3^{neg}$ cells remaining stable for more than 50 days in the absence of TCR triggering. In the absence of cytokines, both $CD3^{neg}$ and $CD3^{pos}$ cells died within 7 days (not shown), indicating that homeostatic cytokines are required for T cell survival. Thus ZFN exposure was well-tolerated in primary lymphocytes and resulted in the stable disruption of the targeted *TRBC* gene. As expected, $CD3^{neg}$ cells failed to proliferate in response to the TCR-dependent mitogen PHA, and were rapidly overgrown by the responding $CD3^{pos}$ lymphocytes (**Fig. 2b**). No phenotypic differences were observed in $CD3^{pos}$ and $CD3^{neg}$ lymphocytes, which displayed a similar CD4/CD8 ratio and appeared to acquire in culture a T_{CM} surface phenotype, as evidenced by high expression of CD62L, CD27, CD28 and IL-7Rα (**Fig. 2c**). Sorted $CD3^{neg}$ cells (**Fig. 2d**) expanded with IL-7 and IL-15 for more than three weeks with growth rates similar to $CD3^{pos}$ cells (**Fig. 2e**), demonstrating that homeostatic cytokines do not require TCR signaling to promote survival/proliferation of previously activated cells. These data demonstrate the successful generation of a novel population of CD8 lymphocytes with phenotypic characteristics of T_{CM} but with TCR expression permanently disrupted. We assessed if these cells could be further manipulated to generate tumor-specific T cells.

TCR β-gene editing results in high avidity tumor-specific lymphocytes

We selected the Wilm's Tumor Antigen 1 (WT1), a therapeutically relevant antigen that contributes to oncogenesis and is broadly overexpressed by leukemias and many solid tumors. We used codon-optimized, HLA-A2 restricted TCR α and β chain cDNAs specific for the $WT1_{126\text{-}134}$ peptide (WT1-TCR), modified to incorporate matched cysteines in the constant regions to facilitate homologous pairing and high expression[26]. To promote robust and coordinate expression of the α and β TCR chains in transduced lymphocytes we generated integrating lentiviral vectors (LVs) encoding both chains under a bidirectional promoter derived from the human phosphoglycerate kinase gene (PGK, **Fig. 3a**)[27].

T lymphocytes were first treated with *TRBC*-ZFN IDLVs to disrupt the endogenous *TRBC* gene. Resulting $CD3^{neg}$ cells were sorted and transduced (49.5±30% mean±SD transduction efficiency, *n=4*) with WT1-TCR LV, indicating that these cells were permissive to further genetic manipulation. Expression of the transferred WT1-TCR rescued surface translocation of CD3, which was co-expressed in balanced proportion with the WT1-TCR Vβ21 chain in these TCR-β-edited cells (**Fig. 3b**, top row). In contrast to matched unedited TCR-transferred lymphocytes (**Fig. 3b**, middle row), TCR-β-edited cells expressing the WT1-TCR could be enriched to >90% purity by polyclonal stimulation, indicating that surface expression of the transferred TCR/CD3 complex was necessary and sufficient to promote TCR-mediated expansion (compare to **Fig. 2b** above). The exogenous Vβ21 chain was expressed in TCR-β-edited lymphocytes at up to two-fold higher mean levels than in unedited TCR-transferred cells and reached similar expression as the endogenous Vβ21 chain of control T cells (**Fig. 3c**, top). Vβ21 expression was stably maintained in culture (**Fig. 3d**). Accordingly, after transduction with the same dose of WT1-TCR LV, up to 22% of TCR-β-edited lymphocytes bound the $WT1_{126\text{-}134}$ pentamer as compared to only 2.6% of unedited cells (**Fig. 3c**, bottom). Thus, in the absence of competition from the endogenous TCR β chain, surface expression of the transgenic TCR β chain reaches physiological levels. TCR-β-edited cells killed $HLA\text{-}A2^{pos}$ targets pulsed with $WT1_{126\text{-}134}$ peptide more effectively than unedited cells (**Fig. 3e**; EC50: TCR-β-edited cells: 90.45 nM, with 95%CI: 58.47-139.9; TCR-transferred cells: 312.3 nM, with 95%CI: 235-415), likely reflecting the higher frequency and expression level of the transgenic WT1-TCR in the former samples, and demonstrating the functional advantage offered by expression of a tumor specific exogenous TCR in a host CTL with abrogated endogenous TCR β chain expression.

Theoretically, surface re-expression of the endogenous TCR α chain may still occur in TCR-β-edited cells, following TCR gene transfer. To directly assess the potential for residual mispairing in TCR β chain disrupted lymphocytes, $CD3^{neg}$ cells were transduced by a LV encoding only the WT1-specific TCR β chain gene and the ΔLNGFR marker (WT1-TCRβ/ΔLNGFR-LV). Despite the absence of WT1-specific α chain, Vβ21 expression was detected in up to 84% of $\Delta LNGFR^{pos}$ *TRBC*-disrupted cells (**Fig. 3f**), demonstrating that even a cysteine-modified TCR β chain, when expressed in a cell with endogenous *TRBC* disruption, can efficiently mispair with the endogenous TCR α chain.

Full TCR a/β-gene editing results in high avidity tumor-specific lymphocytes with sharply reduced alloreactivity

To eliminate the potential for TCR chain mispairing we therefore designed another pair of ZFN targeting the constant region of the TCR α chain (*TRAC*) gene (**Fig. 4a**) and obtained TCR-α-edited T lymphocytes, following the same protocol described above for TCR-β-editing (**Supplementary Fig. 1**). We then developed a strategy for complete α/β TCR editing that permits rapid isolation of the engineered cells at each sequential step of TCR chain disruption/replacement, making use of LV to stably express either the α or β WT1-specific TCR chain, and IDLV or adenoviral vectors (AdV) to transiently express *TRBC*- or *TRAC*-targeting ZFNs in lymphocytes. Whereas $CD3^{neg}$ cells were efficiently generated with both ZFN-expressing vector tested (**Supplementary Fig. 2**), AdV proved more efficient and were selected for the purpose of complete TCR editing (**Fig. 4b**). After AdV

transduction, ZFN activity was transient in the treated cells, detectable only for the first 2-3 days after infection and peaking at 24 hrs (**Supplementary Fig. 3**). For TCR α/β editing, cells harvested from healthy donors were first exposed to *TRAC*-ZFN-AdV post-activation with baCD3/CD28, cultured in the presence of IL-7 and IL-15, and the resulting $CD3^{neg}$ cells (36±13% mean±SD) were sorted 5-8 days after ZFN transduction, when surface protein turnover allows discriminating $CD3^{neg}$ cells and ZFN activity is no more detectable because of vector dilution during cell proliferation. $CD3^{neg}$ cells were transduced (65±31% mean±SD) with a LV encoding the WT1-TCR α chain under the PGK promoter (WT1-TCR-α LV). Cells with rescued CD3 expression were then sorted, stimulated with baCD3/CD28 and exposed to *TRBC*-ZFN-AdV. The second round of ZFN exposure yielded 19%±8 newly $CD3^{neg}$ cells, indicating that primary T lymphocytes are permissive to multiple rounds of ZFN treatment. The $CD3^{neg}$ cells were sorted and transduced (25±15% mean±SD, *n=4*) with a WT1-TCR β chain LV. Expression of the transferred WT1 β chain again rescued surface translocation of CD3, which was now co-expressed in balanced proportion with the WT1-TCR β chain in TCR-edited cells (**Fig. 4b**). TCR-α/β-edited cells could be enriched to near purity by polyclonal stimulation following TCR gene transfer (**Fig. 4b** right plot), and homogenously expressed the high levels of the WT1-specific TCR required to bind the $WT1_{126\text{-}134}$ pentamer (**Fig. 4c**). These results indicate that surface expression of the transferred TCR/CD3 complex in TCR-α/β-edited cells is necessary and sufficient to promote expansion of the cells with the desired antigen specificity. The concomitant disruption of α and β endogenous TCR chains was confirmed in TCR-α/β-edited cells by Cel1 assay (**Supplementary Fig. 4**). The consensus DNA binding site experimentally determined by SELEX for *TRBC* and *TRAC*-ZFNs was used for bioinformatic prediction of the 15 most likely off-target sites in the human genome. Cel1 assays performed on α-edited, β-edited and α/β-edited T lymphocytes revealed no detectable signs of NHEJ (1% limit of detection) at any of these 30 genomic sites, suggesting a high rate of target-specificity for both ZFNs sets. (**Supplementary Figs. 5** and **6**). No phenotypic differences were observed in TCR-transferred and TCR-α/β-edited lymphocytes, which displayed a T_{CM} surface phenotype (**Supplementary Fig. 7a**) and, upon stimulation in the absence of co-stimulation, expanded and differentiated with a similar kinetic (**Fig. 4d** and **Supplementary Fig. 7b**). To verify their function and avidity, TCR-α/β-edited and TCR-β-edited cells were compared to unedited cells, which had been transduced with the WT1-TCR LV and selected for high transgene expression, for the ability to recognize $HLA\text{-}A2^{pos}$ and $HLA\text{-}A2^{neg}WT1^{pos}$ targets. Similar to TCR-β-edited cells, TCR-α/β-edited lymphocytes were superior to TCR-transferred lymphocytes in recognizing specific $WT1^{pos}HLA\text{-}A2^{pos}$ targets (**Fig. 4e**). Full editing was required to completely abrogate off-target reactivity (**Fig. 4f**). These results were confirmed on clones that could be isolated and expanded with similar efficiency from TCR-α/β-edited and TCR-transferred cells, indicating that the editing process does not impair expansion/responsiveness of T lymphocytes (**Supplementary Fig. 8**). The high specificity and lytic capacity of TCR-α/β-edited cells was confirmed by challenge with $HLA\text{-}A2^{pos}$ and $HLA\text{-}A2^{neg}$ AML blasts, harvested from leukemic patients (**Fig. 4g**). To confirm that the α/β-editing process abrogated the off-target reactivity of gene-modified cells, we infused donor matched TCR-transferred, TCR-α/β-edited, and unmanipulated cells to 10-12 weeks old immunodeficient NSG mice that had been conditioned by 175 cGy irradiation. Six of six mice receiving unmanipulated T cells and four of five infused with TCR-transferred

lymphocytes developed lethal graft-versus-host disease (GvHD) within two weeks, while no mice receiving TCR-α/β-edited cells (*n=7*) developed GvHD in four weeks follow-up (**Fig. 5a, b, c**), despite substantial and similar engraftment rates in all experimental groups (**Fig. 5d**). Interestingly, despite gene modified cells homogenously expressed high levels of the WT1-specific TCR before infusion, expression of the transgenic TCR was significantly lower in TCR-transferred cells than in TCR-α/β-edited lymphocytes harvested from mice (**Fig. 5e**), suggesting a selective *in vivo* expansion of T cells expressing alloreactive TCR pairs in TCR-transferred cells, a condition that was prevented by abrogating endogenous TCR expression by the editing process. Finally, for a comprehensive assessment of safety and anti-tumor efficacy of TCR-α/β-edited cells, we infused matched TCR-transferred, TCR-α/β-edited, and unmanipulated cells to 10-12 weeks old immunodeficient NSG mice conditioned by 175 cGy irradiation and infused with $WT1^{pos}$ leukemic blasts harvested from two $HLA\text{-}A2^{pos}$ patients. In this model, mice are expected to die either from GvHD or leukemia. Event-free survival, defined as survival in the absence of GvHD and leukemia, was superior in the cohort of mice receiving TCR-α/β-edited cells than in mice infused with TCR-transferred ($p<0.05$) or unmanipulated lymphocytes ($p<0.01$; **Fig. 5f**). Note that anti-tumor activity was dependent on WT1-TCR expression and substantial in mice treated with TCR-transferred ($p<0.05$) and TCR-α/β-edited cells ($p<0.05$) when compared to untreated mice and mice treated with untransduced lymphocytes. As also observed in **Fig. 5a**, however, mice infused with TCR-transferred cells suffered rapid-onset and lethal GvHD. Indeed, the observed mortality in mice receiving TCR-transferred cells was entirely due to GvHD, while 50% and 100% of deaths in mice that received unmanipulated lymphocytes or PBS, respectively, were due to leukemia growth. These results indicate the advantage of TCR-α/β-edited cells, in comparison to TCR-transferred cells, in mediating a safe anti-leukemia response *in vivo*.

Discussion

Suboptimal activity towards the specific target and the generation of potentially harmful unpredicted specificities are common drawbacks of TCR gene transfer in human T cells[9]. Our results demonstrate that combining ZFN-driven disruption of the endogenous TCR α and β chain genes with lentiviral delivery of a tumor-specific TCR results in the successful and complete editing of primary $CD8^{pos}$ T cell specificity. Because both disruption and replacement are performed at the genetic level, edited TCR specificity is stable and transmitted to cell progeny. The absence of the endogenous TCR chains on TCR-edited cells allows for more robust and homogenous expression of the introduced tumor-specific TCR resulting in higher avidity for the specific target and completely overcomes the safety issues raised by TCR mispairing. The advantage of these edited cells is best exemplified by the much higher fraction of pentamer-binding cells compared to unedited TCR-transferred lymphocytes and by higher activity against relevant targets, including blasts harvested from patients affected by acute myeloid leukemia. Most importantly, the TCR-α/β-edited cells showed sharply reduced activity against non specific targets, demonstrating the utility of preventing the formation of mispaired TCR and suggesting that such TCR-edited cells possess improved safety with respect to the risk of inducing autoimmune diseases. In a murine preclinical model, mispairing of endogenous and exogenous TCR chains was clearly

linked to severe toxicity[10]. While molecular modifications of the exogenous TCR genes can reduce the extent of mispairing, strategies able to abrogate this risk have not been previously reported. Recently, the expression of small interfering RNA (siRNA) has been proposed to specifically down-regulate endogenous TCR expression. This approach could only reduce, but not abrogate the expression of the endogenous TCR[12]. Here, we showed that even cysteine modification of the TCR chains, one of the most promising approaches to address mispairing[26,28], is not sufficient to abrogate this unwanted effect. The described strategy allows the rapid generation of large numbers of tumor specific T cells expressing high functional levels of only the desired TCR, thus abrogating the risk of TCR mispairing. In a humanized GvHD model, we showed that no GvHD is observed following infusion of TCR-α/β-edited lymphocytes, whereas TCR-transferred lymphocytes induce lethal GvHD within two weeks in the majority of infused mice, demonstrating *in vivo* that forced expression in human T cells of an exogenous TCR *per se* does not prevent alloreactivity even when TCR chains include cysteine modifications. Several sequential steps of genetic manipulation ensure complete α/β TCR editing in the final cellular product. In the current work disruption of each TCR chain gene by ZFN is accomplished separately because: i) the simultaneous expression of four individual ZFNs (two sets) targeting distinct genomic sites has the potential to lead to unwanted ZFN dimer pairings and therefore increased off-target activity at least with some ZFNs[29]; ii) identification of the gene-targeted cells takes advantage of the dependence of the surface expression of CD3 on expression of both TCR chains. Lack/rescue of surface CD3 expression thus provides a convenient surrogate marker to rapidly select viable cells with the desired gene modification at each disruption/replacement step of the protocol, respectively. This approach allows the whole procedure to be completed within a culture time shorter than that reported in some settings to isolate and expand viral-specific T cells[30], and to generate a pure population of antigen-specific T cells, in the absence of antigenic stimulation. To our knowledge, this is the first time that a double genetic disruption efficiently followed by gene replacement has been achieved in primary human cells. Importantly, gene editing did not detectably affect the phenotype, function and proliferative potential of engineered cells. TCR-edited cells engrafted in NSG mice with similar kinetics to that observed following infusion of unmanipulated lymphocytes. Interestingly, TCR-edited lymphocytes circulating four weeks after infusion continued to express high levels of the tumor-specific TCR, suggesting potential long-term stability of such cells *in vivo*. Indeed, when challenged *in vivo* with primary human leukemias, TCR-edited cells displayed substantial anti-tumor efficacy and, being devoid of off-target reactivity, proved superior to TCR-transferred lymphocytes in promoting event-free survival. Further advances in humanization of murine models, possibly including *in vivo* production of human cytokines, will likely allow to assess the TCR-editing protocol for long-term T cell viability and to stringently and separately evaluate the impact of culture time, each genetic manipulation step and cytokines in determining the long-term fate of edited cells. Clinical trials based on ZFN-mediated disruption of the *CCR5* (NCT 00842634 and NCT 01044654) and glucocorticoid receptor (NCT 01082926) genes in T lymphocytes are ongoing, indicating the feasibility of pursuing a ZFN-based therapy for clinical application[17]. Clinical development of our strategy could thus proceed in currently well-established protocols for producing genetically engineered T cells for use in humans, and the application of this new

approach to modify distinct T cell subpopulations may substantially enhance the therapeutic potential and reduce the risks of toxicity of immunotherapy in a variety of cancers.

Online Methods section

We designed and assembled *TRBC*- and *TRAC*-ZFNs as previously described[16]. The recognition helices are shown in Supplementary Table 1. We generated lentiviral vectors encoding *TRBC*- and *TRAC*-ZFNs from the HIV-derived self-inactivating transfer construct pCCLsin.cPPT.SFFV.eGFP.Wpre, packaged by an integrase-defective third generation packaging construct carrying the D64V mutation in the HIV integrase, and pseudotyped by the VSV envelope as described[31]. The Ad5/F35 adenoviral vectors were generated on a E1/E3 deleted backbone. We linked the ZFNs targeting either the *TRBC* or *TRAC* gene via a 2A peptide sequence and cloned them into the pAdEasy-1/F35 vector under control of the CMV TetO promoter, and the Ad5/F35 virus for each construct was generated using TREx 293T cells as described[32]. We generated lentiviral vectors encoding both WT1-specific TCR chains and single α21 or β21 WT1-specific TCR chains from the bidirectional self-inactivating transfer vector pCCLsin.cPPT.ΔLNGFR.minCMV.hPGK.eGFP.Wpre[27], packaged by an integrase-competent third generation construct, and pseudotyped by the VSV envelope as described[31]. The codon-optimized, cysteine-modified WT1-TCR α and β chain genes were previously described[26]. We cultured T2 and Jurkat cell lines in RPMI 1640 (GIBCO-BRL) 10% FBS (BioWhittaker). We obtained human T lymphocytes and fresh leukemia samples from patients with acute myeloid leukemia upon informed consent according to a protocol approved by the San Raffaele Bioethical Committee. For LV transduction with ZFN-IDLV, ZFN-AdV or WT1-TCR LV, we activated T lymphocytes by anti-CD3/CD28-conjugated magnetic beads (ClinExVivo CD3/CD28; Invitrogen) and grew them in IMDM (GIBCO-BRL) 10% FBS, supplemented with 5ng ml^{-1} of IL-7 and IL-15 (PeproTech) as previously described[23]. We performed transductions with the indicated doses of IDLV or LV vectors, measured as HIV Gag p24 equivalents, 48 hours after activation. Vector infectivity ranged from 1 to 5 $\times 10^4$ Transducing Unit ng^{-1} p24 by vector DNA titration on 293T cells. In the complete TCR editing procedure, the second hit of ZFN treatment was performed at 30°C according to a cold shock protocol previously described[33], to increase efficiency. We sorted $CD3^{neg/pos}$ cells, $V\beta21^{pos}$ cells, and $WT1_{126\text{-}134}$ pentamer binding cells using LD/MS columns with anti-CD3 MACS MicroBeads and MS columns with anti-FITC or -PE MACS MicroBeads respectively (Miltenyi Biotec), following manufacturer instructions. To measure the mutations consequent to NHEJ at the ZFN target site we used the Mismatch selective Cel1 nuclease assay[18] using the following primers: for *TRBC1*, 5'-CTGAACAAGGTGTTCCCACCC-3' and 5'-GTGTGCGCTGGTTCCTTTCTT-3'; for *TRBC2*, 5'-CCTGGCCACAGGCTTCTACC-3' and 5'-CCACCTTGTCCACTCTGGCTT-3'; and for *TRAC*, 5'-GGGCAAAGAGGGAAATGAGA-3' and 5'-CAATGGATAAGGCCGAGACC-3'. For TCR editing, ZFN-IDLV or ZFN-AdV transduced lymphocytes, sorted as $CD3^{neg}$ cells, were subsequently transduced with the LV encoding 1 or both WT1-TCR chains, as described in the text, and expanded by polyclonal stimulation[34]. We evaluated the functional activity of T cells three weeks after stimulation, in a cytotoxicity assay or in a γIFN-ELISpot assay as described[35]. Briefly, we plated cells at the concentration of 2×10^4 cells/well for 18 hrs at a

stimulator/responder ratio of 1. We quantified γIFN using KS-Elispot Reader (Zeiss). We tested T cells for recognition of T2 cells pulsed with $WT1_{126\text{-}134}$ or $pp65_{495\text{-}503}$ peptides (Proimmune), unrelated PBMCs or γIFN cultured (200UI ml^{-1}) human primary leukemic blasts. For in vivo studies, we obtained 10-12 weeks old NSG mice from Jackson lab. The experimental protocol was approved by the internal committee for animal studies of our Institution (IACUC). We re-suspended cells in 250μl IMDM medium with 5% FBS and infused them i.v. We monitored mice for clinical GvHD three times per week as described[36,37]. The following signs were included in the clinical index: weight loss (0 for weight loss <5%, 1 for 5-10%, 2 for >10%), hunching (0-2), activity (0-2), fur texture (0-2), and skin integrity (0-2) yielding a maximum index score of 10. We killed moribund mice for ethical reasons. We determined human chimerism every 3 days for the first week by flow-cytometry after bleeding from the tail vein. Human chimerism was calculated as follows: human chimerism (%) = $[huCD45^+/(huCD45^+ + mCD45^+)] \times 100$. Expression of WT1-specific TCR was quantified on gated ($huCD45^+$) human cells by flow cytometry. For in vivo assessment of anti-tumor efficacy, primary AML blasts harvested after informed consent from 2 HLA-$A2^{pos}$ AML patients, were infused i.v. at the dose of $5\text{-}10\times10^6$ cells/mice in mice preconditioned with 175 cGy irradiation. After T cell infusion (day 0-2), mice were monitored 3 times per week for GvHD or leukemia occurrence, defined by the appearance of circulating blasts (>1%).

Statistical analyses on functional data were performed by Mann-Whitney's U test for non-parametric data and by 2way ANOVA test. EC50 was calculated by nonlinear regression analysis of ^{51}Chromium release data by using the sigmoidal dose-response equation of the GraphPad Prism Software. Log-rank Mantel cox test, and chi-square were used to analyze in vivo experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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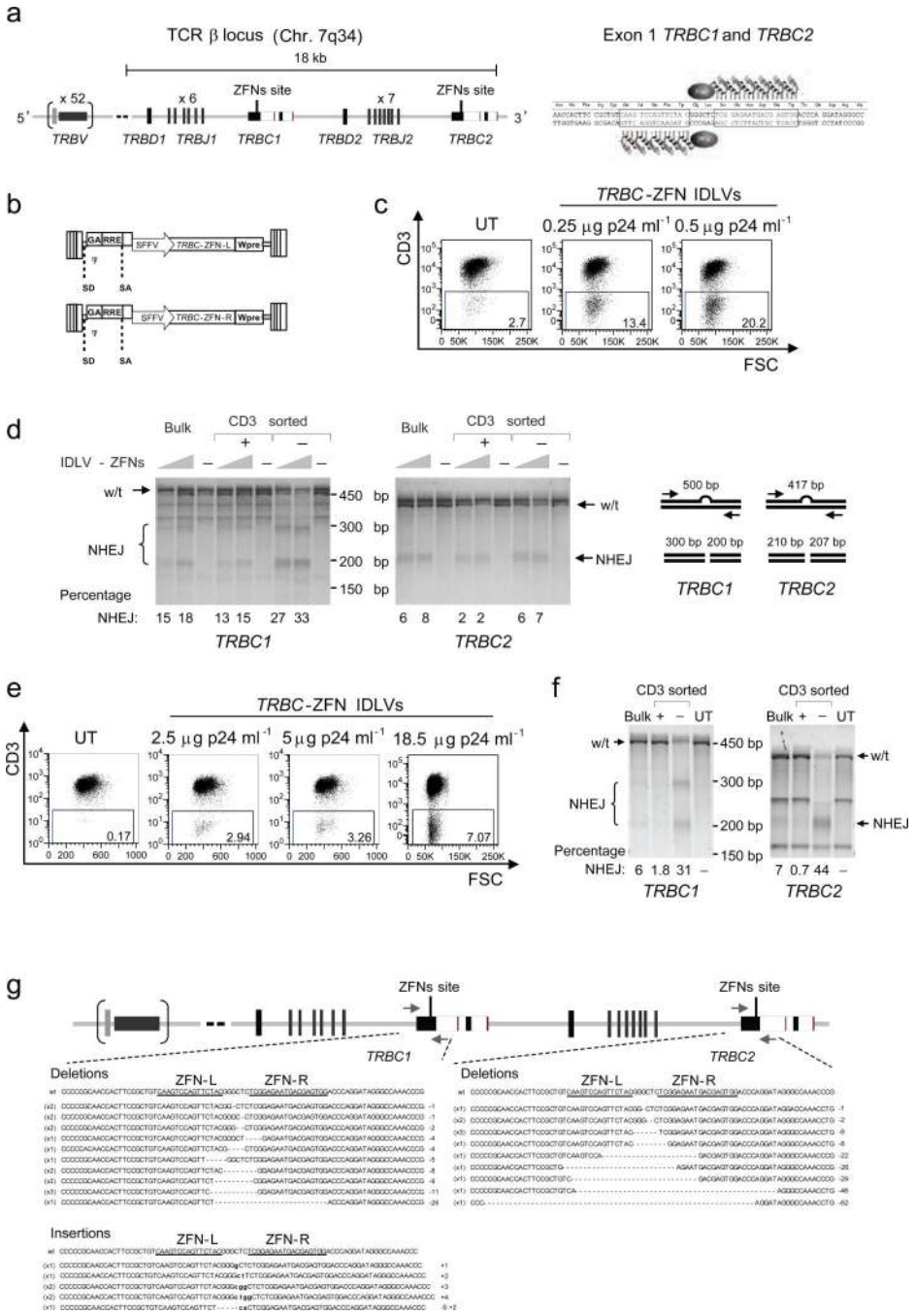

Figure 1. ZFN mediated disruption of *TRBC* genes in human T lymphocytes

a. Left: diagram of the human locus encoding for the TCR β chain; *TRBV*, variable region genes, *TRBD*, diversity region genes, *TRBJ*, junction region genes, *TRBC*, constant region genes. Right: the genomic DNA sequences in *TRBC1* and *TRBC2* bound by each *TRBC*-ZFN. **b.** Schematic representation of lentiviral vectors expressing the ZFN pair targeting the T cell receptor β chain (*TRBC*) genes and packaged by an integrase-defective system. SD and SA, splice donor and acceptor sites; Ψ encapsidation signal, including the 5'portion of the gag (GA) gene; RRE, Rev-responsive element; Wpre, woodchuck hepatitis virus post-

transcription regulatory element. SFFV, spleen focus forming virus promoter. **c**. Down-regulation of cell surface CD3 expression measured by flow cytometry in Jurkat cells exposed to increasing concentrations of *TRBC*-ZFN IDLVs (measured by HIV Gag p24 equivalents). The percentage of $CD3^{neg}$ cells is indicated. UT, Untransduced cells. **d**. Level of targeted gene disruption measured by a mismatch-selective Cel1 endonuclease assay on DNA amplified from the cells shown in c, before (bulk) and after sorting for presence (+) or absence (−) of CD3 on the cell surface. The schematic on the right shows the expected size of the intact amplicon (upper wild type bands, denoted w/t, in the gel) and the Cel1 cleavage products of heteroduplexes carrying mismatches around the ZFN target site introduced by non-homologous end joining (NHEJ) (lower bands in the gel; note that the two cleavage products co-migrate at ~200 bp for *TRBC2*). Other bands are non-specific amplification products. The calculated level of targeted gene disruption in *TRBC1* and *TRBC2* is shown on bottom. **e.** Down-regulation of cell surface CD3 expression and level of targeted gene disruption (**f**) in primary human lymphocytes exposed to increasing concentrations of *TRBC*-ZFN IDLVs stimulated with baCD3/CD28, and cultured with 5ng ml^{-1} IL-7 and IL-15. Legend as in c, d. Other bands in the *TRBC*2 gel derive from a single nucleotide polymorphism in that locus. **g**. The *TRBC1* and *TRBC2* DNA in ZFN-treated lymphocytes was amplified, cloned and sequenced to confirm ZFN cleavage. Sequence alignment revealed several nucleotide insertions and/or deletions (indels) encompassing the ZFN target region. Left column indicates the number of clones retrieved while the right column indicates the number of nucleotides deleted or inserted.

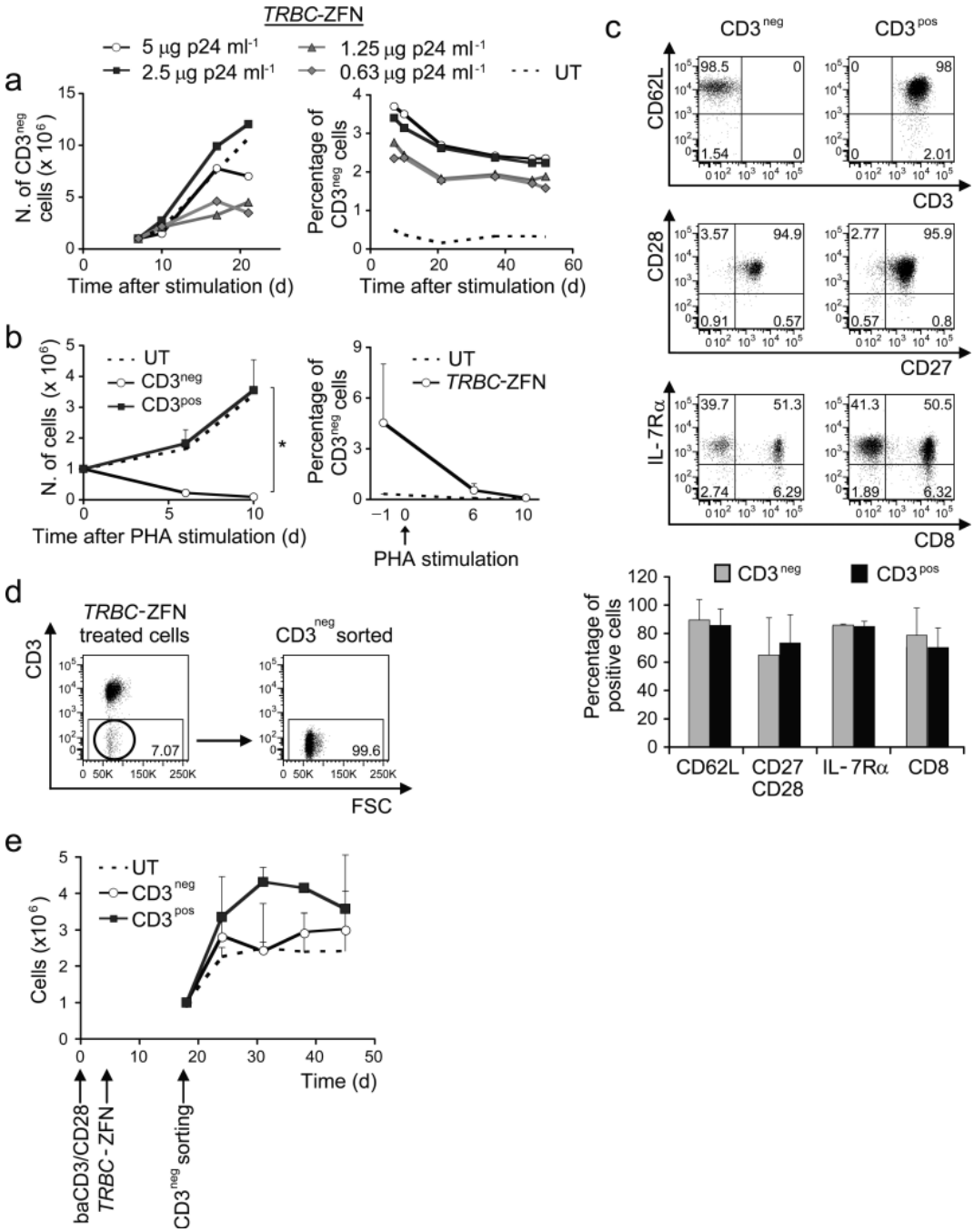

Figure 2. Expansion and purification of CD3^{neg} primary T lymphocytes

a. Left: Expansion of CD3^{neg} cells generated by exposure to increasing concentrations of *TRBC*-ZFN IDLVs in the presence of 5ng ml^{-1} IL-7 and IL-15. CD3^{pos}, untransduced (UT) lymphocytes from the same donors are shown as controls. Right: Stability of CD3^{neg} phenotype in culture. **b.** Left: Resistance of CD3^{neg} cells to polyclonal TCR-mediated stimulation. Lymphocytes were activated with PHA 2 µg ml^{-1} 20 days after exposure to *TRBC*-ZFN and cultured with 5ng ml^{-1} IL-7, IL-15 and 300IU ml^{-1} IL-2. CD3^{pos} and CD3^{neg} cells were quantified by cytometry every 4-6 days and average + SD are shown

(*n=2, *, p<0.05*). Right: average + SD of percentage of $CD3^{neg}$ lymphocytes before and after PHA stimulation. **c.** Representative plots (upper panel) and average histograms (lower panels) showing the expression of CD62L, CD28, CD27 and IL-7Rα in $CD3^{neg}$ and $CD3^{pos}$ gated cells. To measure IL-7Rα expression, cells were washed and cultured in the absence of cytokines for 18 hours before staining with fluorochrome-conjugated antibodies. **d.** Sorting of $CD3^{neg}$ cells to near homogeneity and expansion of sorted $CD3^{neg}$ cells (**e**) compared to that of $CD3^{pos}$ cells and untransduced (UT) lymphocytes in the presence of IL-7 and IL-15.

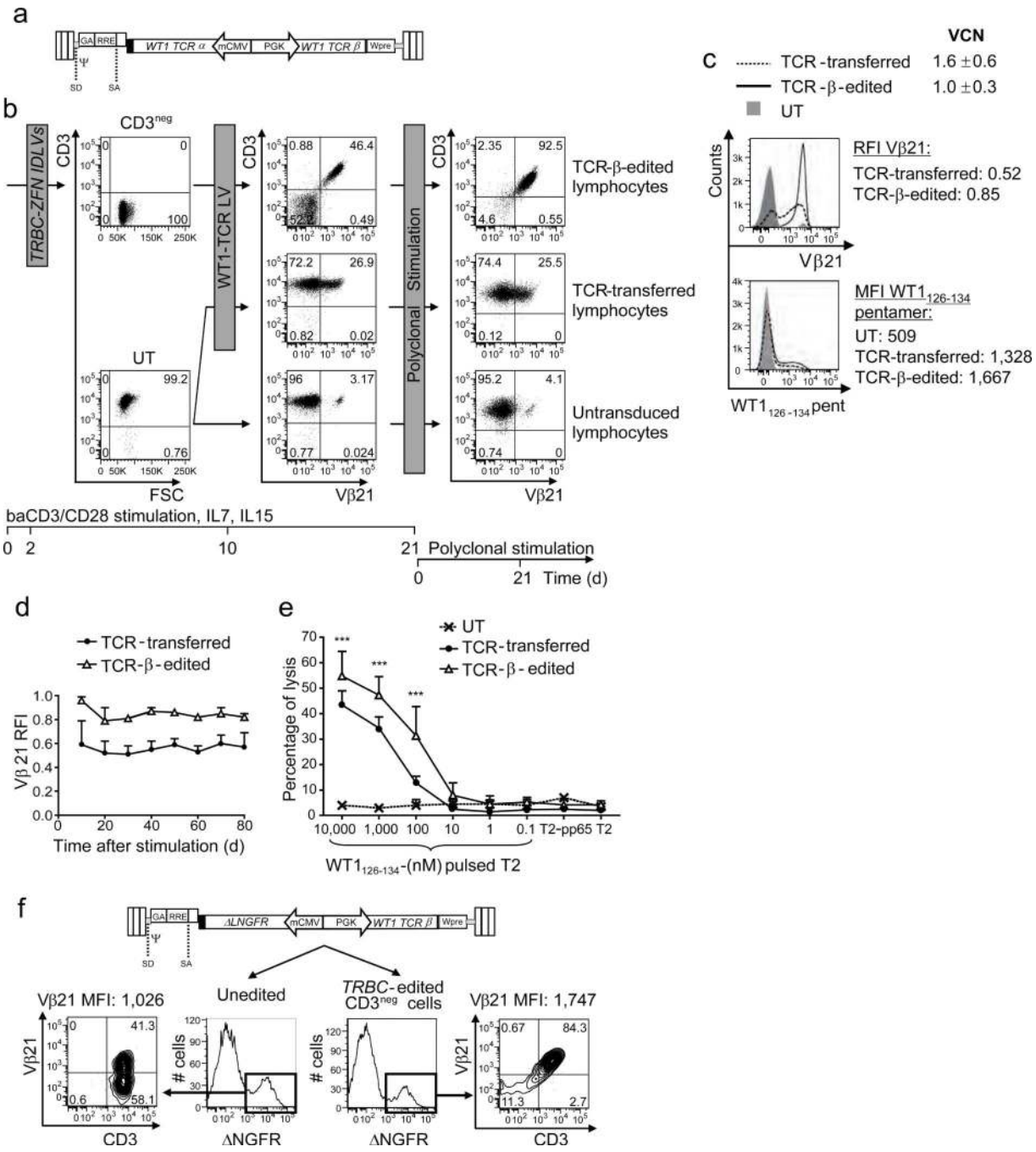

Figure 3. TCR β-gene editing results in high avidity tumor-specific T lymphocytes

$CD3^{neg}$ lymphocytes, obtained by treatment with *TRBC*-ZFN-IDLVs followed by cell sorting, and untreated lymphocytes (UT) were transduced with WT1-TCR LV (MOI 65), expanded and assayed for WT1-TCR expression and activity. **a.** Schematics of bidirectional LV coordinately expressing the two WT1-specific TCR chains. A PGK promoter, flanked upstream and in opposite orientation by a minimal core promoter (derived from the human cytomegalovirus, minCMV), drive transcription of both codon-optimized cysteine-modified TCR chains. Other legends as in 1b. **b**. Representative dot plots showing surface expression

of WT1-specific TCR β chain (Vβ21) and CD3 at each step of the experiment. Percentage of events measured in each quadrant are shown. The timeline is shown on the bottom. **c.** Vβ21 expression (upper histograms), and $WT1_{126\text{-}134}$ pentamer binding (lower histograms) are shown in representative TCR-β-edited and unedited $CD8^{pos}$ T cells after WT1-TCR LV gene transfer, and in control untransduced lymphocytes treated with the same culture conditions. Vector copy number (VCN) measured by quantitative PCR in TCR-transferred and TCR-β-edited cells is shown. **d.** Stability of surface expression of Vβ21 TCR chain. Vβ21 relative fluorescence intensity (RFI) is calculated as the ratio of the mean fluorescence intensity (MFI) of Vβ21 measured in genetically modified lymphocytes to the MFI of Vβ21 in naturally expressing T cells. **e.** Cytotoxic assay with TCR-β-edited and TCR-transferred cells. Functional activity is measured by a ^{51}Chromium release assay for lysis of labeled T2 cells pulsed with increasing concentrations of the $WT1_{126\text{-}134}$ HLA-A2 restricted peptide, or with the irrelevant CMV-derived $pp65_{495\text{-}503}$ HLA-A2 restricted peptide (10 μM) as negative control, at an Effector/Target (E/T) ratio of 12. Results are represented as average + SD of percentage of lysis (****, $p<0.001$, $n=6$*). **f.** Mispairing of cysteine-modified TCR β chain and endogenous α chain. $CD3^{neg}$ cells sorted from *TRBC*-disrupted lymphocytes (right panels) and unedited cells (left panels) were transduced by a bidirectional LV encoding the β chain of the WT1-specific TCR and the truncated low affinity NGF receptor (ΔLNGFR) cDNA under the control of the bidirectional PGK promoter. Transduction efficiency was assessed as percentage of $\Delta LNGFR^{pos}$ lymphocytes (histograms). Vβ21 expression was measured on $\Delta LNGFR^{pos}$ cells (plots). The MFI of Vβ21 is shown. Results of 1 representative of six experiments are shown.

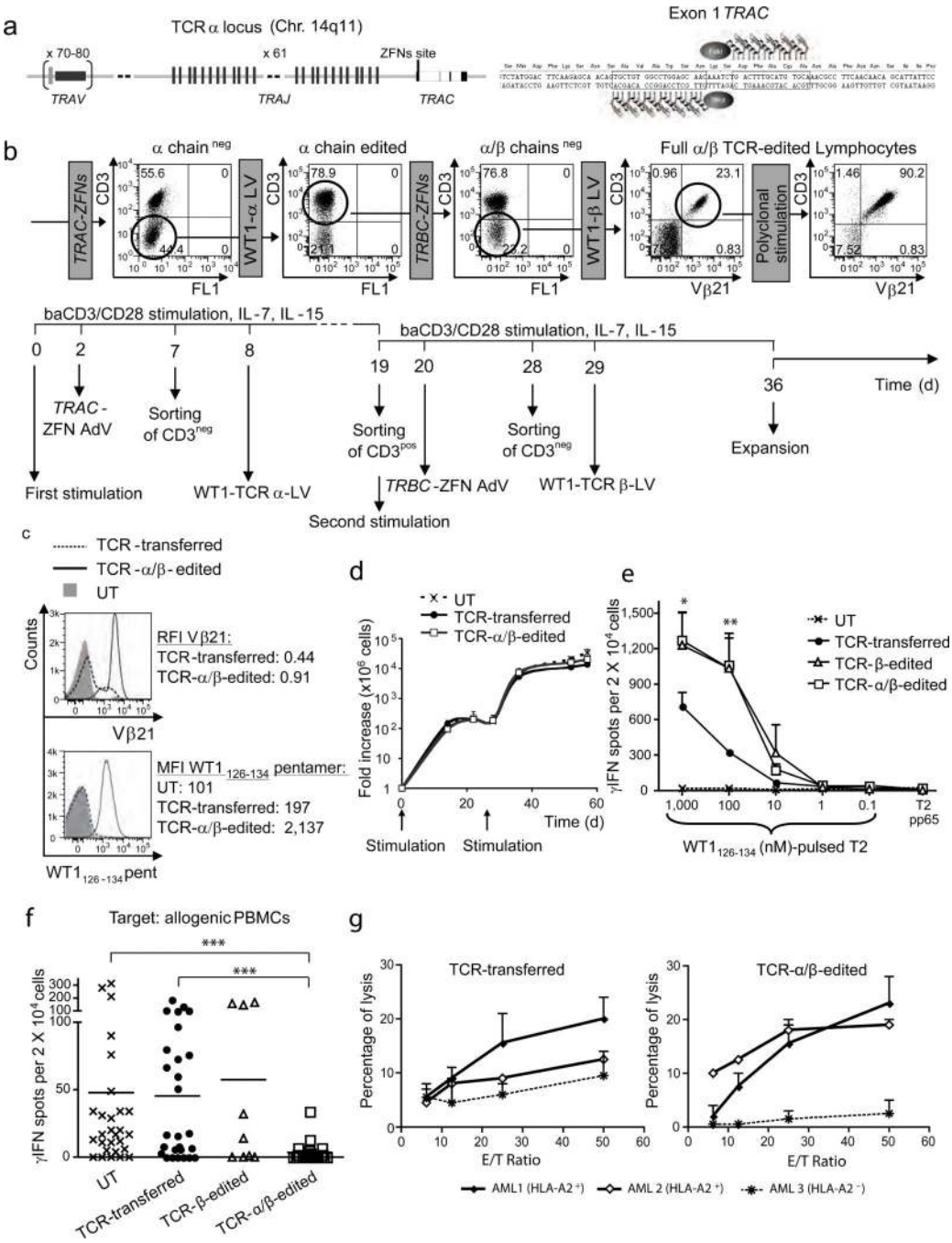

Figure 4. Full TCR a/β-gene editing results in high avidity tumor-specific lymphocytes with sharply reduced alloreactivity

a. Left: diagram of the human locus encoding for the TCR α chain; *TRAV*, variable region genes; *TRAJ*, junction region genes; *TRAC*, constant region gene. Right: the genomic DNA sequences in *TRAC* bound by each *TRAC*-ZFN. **b.** Schematic, timeline and representative flow cytometry analysis at each step of a protocol optimized for full TCR gene editing. Activated T lymphocytes were treated with *TRAC*-ZFN-AdV (MOI 10^3), and $CD3^{neg}$ lymphocytes sorted and transduced with WT1-TCR-α LV (MOI 65). After one cycle of polyclonal stimulation, α-edited lymphocytes were treated with *TRBC*-ZFN-AdV (MOI

10^4) and $CD3^{neg}$ cells sorted and transduced with WT1-TCR-β LV (MOI 100). The full TCR-edited lymphocytes were then expanded for functional analysis. Surface expression of CD3 and Vβ21 is shown, with the percentage of events measured in each quadrant. **c.** Vβ21 TCR expression (upper histograms) and $WT1_{126\text{-}134}$ pentamer binding (lower histograms) are shown in $CD8^{pos}$ T cells with TCR α/β chains disrupted and transduced with the WT1-TCR chains (TCR-α/β-edited), unedited WT1-TCR LV transduced cells (TCR-transferred), and untransduced lymphocytes treated with the same culture conditions. Vβ21 RFI and $WT1_{126\text{-}134}$ pentamer MFI are indicated on the right for cells gated on Vβ21 and CD8. **d.** TCR-transferred and TCR-α/β-edited T lymphocytes were plated at 0.2×10^5 ml^{-1} and activated with anti-CD3 antibody (30 ng ml^{-1}), irradiated allogeneic PBMCs (1×10^6 cells ml^{-1}) and irradiated allogeneic EBV cell lines (0.2×10^6 ml^{-1}), and IL-2 (300 IU ml^{-1}). IL-2 was replaced every 3-4 days, and cells were counted by trypan blue exclusion. Every step of activation is indicated by an arrow. **e-g**. Functional activity of genetically modified lymphocytes. Three weeks after polyclonal stimulation TCR-α/β-edited, TCR-β-edited, TCR-transferred (sorted for high expression of Vβ21) and untransduced lymphocytes were tested in γIFN ELISpot against: (**e**) T2 cells pulsed with increasing concentrations of the $WT1_{126\text{-}134}$ HLA-A2 restricted peptide, or with the irrelevant CMV-derived $pp65_{495\text{-}503}$ HLA-A2 restricted peptide (10 μM) as negative control; (**f**) irradiated allogeneic PBMC. All assays were performed at a responder/stimulator ratio of 1. Specific spots are shown on the y axis as spots produced in presence of stimulators minus spots produced by effectors alone. (**g**) TCR-α/β-edited and Vβ21-sorted TCR-transferred cells were challenged with $HLA\text{-}A2^{pos}$ and $HLA\text{-}A2^{neg}$ AML primary blasts, harvested from leukemic patients, in a cytotoxic assay. **, p<0.05, **,p<0.01, ***, p<0.001.*

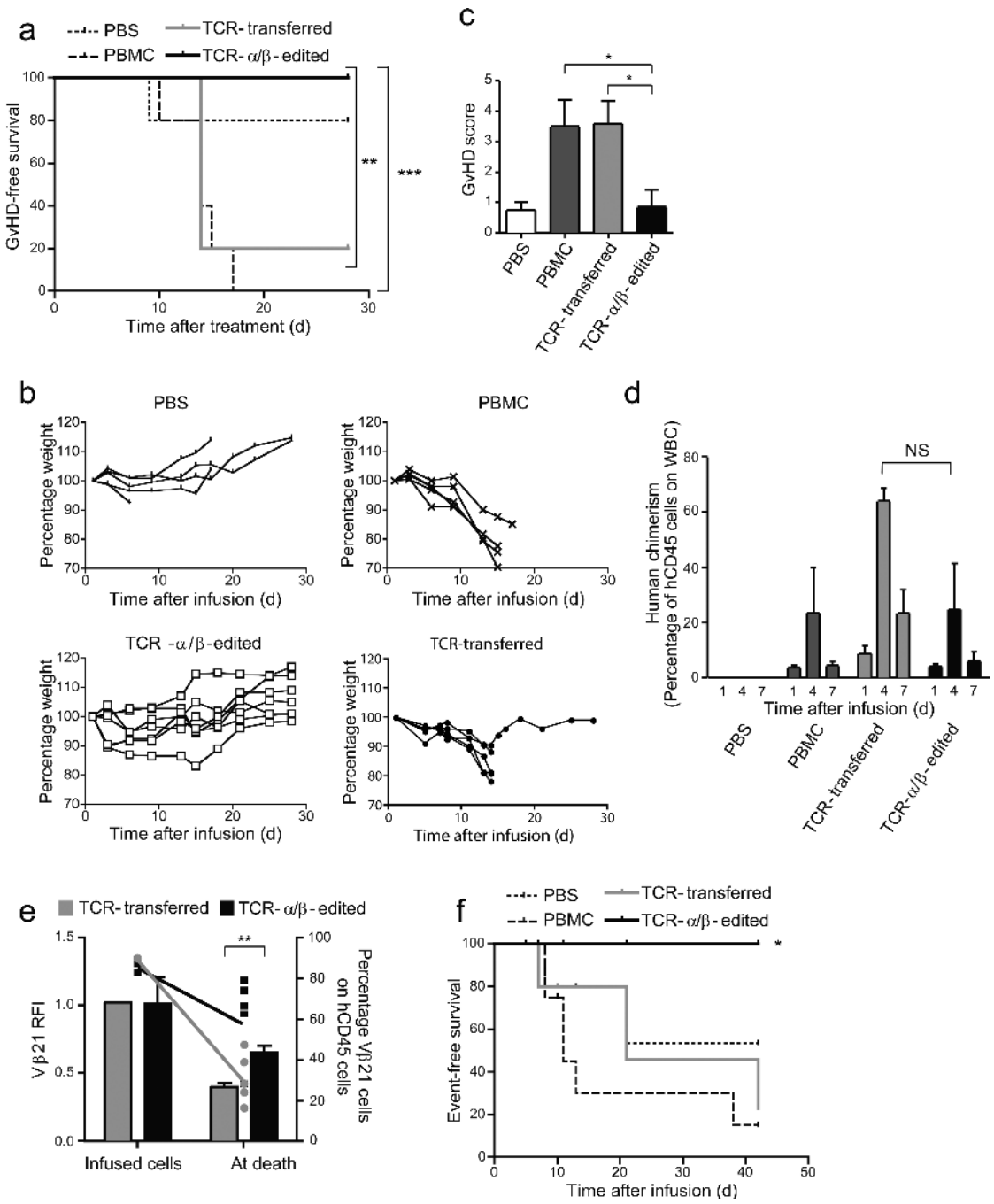

Figure 5. TCR a/β-gene edited lymphocytes inhibit leukemia development without inducing GvHD in immunodeficient mice

NSG mice were sub-lethally irradiated (175 cGy) prior to the i.v. transfer of PBS (*n=5*), 10×10^6 PBMC (*n=6*), 10×10^6 TCR-transferred cells sorted for Vβ21 expression (*n=5*), and 10×10^6 matched TCR-a/β-edited lymphocytes (*n=7*). After infusion, mice were followed for survival without severe GvHD (**a**) and weight loss (**b**). Mice were monitored and scored for clinical signs of GvHD (**c**) according to the following criteria: weight loss (0-2), hunching (0-2), activity (0-2), fur texture (0-2), and skin integrity (0-2). Human chimerism was

assessed in peripheral blood and calculated as [huCD45^{+}/(huCD45^{+} + mCD45^{+})] × 100 at days 1, 4 and 7 after infusion (**d**). Expression of WT1-specific TCR was quantified before infusion and at sacrifice on gated human cells (hCD45^{+}) and expressed as percentage of Vβ21^{pos} cells (lines, **e**) and Vβ21 RFI (columns, **e**), calculated as the ratio of the Vβ21 MFI measured on genetically modified lymphocytes to the MFI of Vβ21 in naturally expressing T cells. TCR-transferred cells, gray. TCR-α/β-edited cells, black. **f.** NSG mice were sub-lethally irradiated (175 cGy) prior to the i.v. transfer of 5-10 ×10^{6} AML blasts from HLA-A2^{pos} patients, and PBS (*n=11*), 10×10^{6} untransduced lymphocytes (UT, *n=8*), 10×10^{6} TCR-transferred cells sorted for Vβ21 expression (*n=10*), and 10×10^{6} TCR-α/β-edited lymphocytes from the same donors (*n=8*). After infusion, mice were followed for event free survival (survival in the absence of GvHD and/or leukemia). **, p<0.05, **, p<0.01, ***, p<0.001.*