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Generation of tumor-targeted human T lymphocytes from induced pluripotent stem cells for cancer therapy

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Abstract

Progress in adoptive T-cell therapy for cancer and infectious diseases^{1–2} is hampered by the lack of readily available, antigen-specific, human T lymphocytes. Pluripotent stem cells could provide an unlimited source of T lymphocytes, but the therapeutic potential of human pluripotent stem cell-derived lymphoid cells generated to date remains uncertain^{3–6}. Here we combine induced pluripotent stem cell (iPSC)⁷ and chimeric antigen receptor (CAR)⁸ technologies to generate human T cells targeted to CD19, an antigen expressed by malignant B cells, in tissue culture. These iPSC-derived, CAR-expressing T cells display a phenotype resembling that of innate $\gamma\delta$ T cells. Similar to CAR-transduced, peripheral blood $\gamma\delta$ T cells, the iPSC-derived T cells potently inhibit tumor growth in a xenograft model. This approach of generating therapeutic human T cells ‘in the dish’ may be useful for cancer immunotherapy and other medical applications.

Current approaches to adoptive T-cell therapy require the labor-intensive generation of T-cell lines from carefully selected donors or the genetic engineering of autologous T cells from each individual patient, hindering the facile and broad use of T cells with pre-determined antigen specificity. Having rapid access to unlimited antigen-specific T lymphocytes with optimized therapeutic features would greatly advance the scope and delivery of T-cell therapies. Previous studies support the feasibility of generating T lymphocytes from human

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Note: Any Supplementary Information and Source Data files are available in the [online version of the paper](#).

Contributions

M.T. designed the study, planned and performed experiments, analyzed and interpreted data and wrote the manuscript; C.C.K. performed experiments, helped in data analysis and edited the manuscript; G.C. analyzed and interpreted microarray data; V.D.F. helped with experiments and data analysis; F.P. helped with experiments and data analysis; M.G. chose the statistical methods and performed statistical analyses; M.S. designed the study, analyzed and interpreted data and wrote the manuscript.

Competing interests

The authors declare no competing financial interests.

embryonic stem cells (ESCs) and iPSCs *in vitro*, although the yield of lymphoid cells has been low and their nature only partially defined^{3,4}. More specifically, the functional characterization of T cells derived from ESCs and iPSCs is complicated by not knowing their antigen specificity and HLA restriction. For example, T cells generated *in vitro* from ESCs or iPSCs have an unpredictable T-cell receptor (TCR) repertoire because TCR gene rearrangements are random³ and the cells are positively selected by unclear mechanisms during their *in vitro* differentiation. This limitation can be circumvented by using iPSCs bearing a rearranged endogenous TCR of known antigen specificity^{5–6}. Unfortunately, this approach requires laborious cloning of antigen-specific T cells and is limited to antigens for which patient-specific T cells can be detected. Furthermore, as TCRs recognize antigens presented by specific HLA molecules, the clinical use of T cells that recognize antigen through an endogenous TCR is constrained by the need to match their specificity to the HLA of the recipient patient.

Genetic engineering of T lymphocytes to express CARs has recently emerged as a promising approach to rapidly generate tumor-targeted T cells endowed with enhanced anti-tumor properties⁸. For example, CARs redirect T-cell specificity in HLA-independent fashion, thereby eliminating the need to consider HLA restriction and overcoming some tumor escape mechanisms⁸. We previously demonstrated that human T cells expressing a CAR targeted to the CD19 antigen, which is expressed on the vast majority of leukemias and lymphomas, can eradicate B-cell malignancies in mice⁹. Importantly, second-generation CARs, combining both activation and co-stimulatory signaling domains, enhanced T-cell expansion and *in vivo* persistence^{8–10}. We and others, recently demonstrated in clinical trials that second-generation CD19 CAR-modified T cells efficiently induce complete remissions in patients with acute or chronic lymphoblastic leukemias^{11–14}.

Here we hypothesized that genetic engineering of iPSCs with second-generation CARs⁸ would be an efficient strategy to concomitantly harness the unlimited availability of iPSCs and to generate phenotypically defined, functional and expandable T cells that are genetically targeted to a tumor antigen of interest (Fig. 1a). To this end, we generated iPSC clones (T-iPSCs) by transducing peripheral blood T lymphocytes (PBL) from a healthy volunteer with two retroviral vectors each encoding two of the reprogramming factors KLF4, SOX2, OCT-4 and C-MYC (Supplementary Fig. 1a)⁷. Multiple randomly selected T-iPSC clones were analyzed, and their pluripotency (Supplementary Fig. 1b–g) and T-cell origin (Supplementary Fig. 2a, b) were confirmed. Clone T-iPSC-1.10 was stably transduced with a bicistronic lentiviral vector encoding 19–28z (1928z-T-iPSC), a second-generation CAR specific for CD19 (ref. 14), and the fluorescent marker mCherry (Supplementary Fig. 3a–c). To direct the differentiation of 1928z-T-iPSC to the T-lymphoid lineage, we first optimized a serum-and feeder-free *in vitro* differentiation protocol for the generation of hematopoietic precursors through embryoid body formation (Fig. 1b). Similar to previous reports^{3,4,15}, we found that CD34⁺ cells from day 10 embryoid bodies expressed the highest levels of key transcription factors for lymphoid differentiation (Supplementary Fig. 4a), specifically showing increased expression of Notch 1 and CD127 (IL7R α) in the CD34⁺CD43[–] subset compared to CD34[–]CD43[–] cells (Supplementary Fig. 4b). We therefore dissociated day 10 embryoid bodies and transferred the hematopoietic precursors onto Delta-like 1–expressing OP9 (OP9-DL1) feeder cells to induce T-lymphoid

differentiation in an established co-culture system in the presence of the cytokines stem cell factor (SCF), Flt3L and interleukin (IL)-7 (Fig. 1b). mCherry expression was ascertained throughout the differentiation process and we did not detect substantial silencing of mCherry expression (Fig. 1b). As early as day 25 of differentiation, we could detect CD7⁺CD3⁺TCR $\alpha\beta$ ⁺ cells (Supplementary Table 1). These cells harbored the same TCR β and γ chain rearrangements as the parental T-iPSC-1.10 line (Supplementary Fig. 2c). By day 30, CD3⁺TCR $\alpha\beta$ ⁺ cells typically accounted for ~80% of the cultures (Fig. 1c and Supplementary Table 1), and all of them expressed the CD19-specific CAR on their surface; day 30 cells are referred to hereafter as 1928z-T-iPSC-T cells (Fig. 1c). A substantial fraction expressed CD8 α ($10.4 \pm 3.5\%$) and CD56 ($20.7 \pm 9.5\%$), whereas very few cells expressed low amounts of CD4 and almost no cells expressed detectable CD8 β (Fig. 1c and Supplementary Table 1). Further surface phenotyping showed most cells to be CD5^{low} and negative for CD122 and TCR $\gamma\delta$ (Fig. 1c, Supplementary Fig. 5a, b and Supplementary Table 1).

Taking advantage of the CD19-specific CAR, we evaluated the functional response of 1928z-T-iPSC-T cells to cell-bound CD19. 1928z-T-iPSC-T cells harvested on days 30–35 of differentiation were cultured on NIH-3T3–based artificial antigen-presenting cells (AAPCs) expressing the CD19 antigen (3T3-CD19) where indicated⁹. The 1928z-T-iPSC-T cells rapidly bound to 3T3-CD19 cells, forming clusters and eliminating the 3T3-CD19 monolayer (Fig. 1d). No such adhesion was observed when 1928z-T-iPSC-T cells were placed on CD19-negative 3T3 cells (Fig. 1d). Exposure to 3T3-CD19 cells also prompted 1928z-T-iPSC-T-cell surface expression of T-cell activation markers CD25 and CD69 (Fig. 1e) and secretion of type 1 cytokines such as IL-2, tumor-necrosis factor (TNF)- α and interferon (IFN)- γ (Fig. 1f). These results show that 1928z-T-iPSC-T cells displayed canonical features of T-lymphocyte function and specificity for the CD19 antigen.

To better elucidate the phenotype of 1928z-T-iPSC-T cells, we carried out a gene expression microarray, and we compared the mRNA expression profile of days 30–35 1928z-T-iPSC-T cells to that of naive B cells, CD4 T cells, CD8 T cells, CD3⁺CD56⁺T cells and natural killer (NK) cells isolated from peripheral blood. We also compared the profile to freshly isolated or *in vitro*-activated peripheral blood $\gamma\delta$ T cells. Hierarchical clustering using the set of genes with most variable mRNA expression (s.d. > 0.75) showed that 1928z-T-iPSC-T cells were distinct from B cells and more closely related to the other T-lymphoid subsets and NK cells (Fig. 2a). Next, we directly compared 1928z-T-iPSC-T cells to particular lymphoid cell subsets by correlating mRNA expression levels of the most variable genes in our data set. This pairwise correlation analysis indicated that 1928z-T-iPSC-T cells were more similar to fresh or activated (for 7 d) $\gamma\delta$ T cells (Supplementary Fig. 6a). This correlation was further confirmed upon examining expression of key lymphoid differentiation genes. The 1928z-T-iPSC-T cells expressed genes characteristic of the T-lymphoid lineage (e.g., *GATA3*, *CD38*, *CD3e*, *LEF1*, *LCK* and *BCL11B*) at levels comparable to those of peripheral blood $\gamma\delta$ T cells; however, the 1928z-T-iPSC-T cells did not express many genes characteristic of the NK cell lineage (e.g., *CD94*, *CD16* and killer-cell immunoglobulin-like receptors) (Fig. 2b, Supplementary Fig. 5 and Supplementary Fig. 6b). Moreover, we detected pronounced expression of *FASLG*, *TYROBP*, *CCL20*, *TNFSF11* (*RANKL*), *CXCR6* and *RORC*, genes that are highly expressed in $\gamma\delta$ T cells versus $\alpha\beta$ T cells and/or NK cells¹⁶, in 1928z-T-

iPSC-T cells (Fig. 2b and Supplementary Fig. 6b). The innate immune cell property of 1928z-T-iPSC-T cells is further supported by their expression of the transcription factor PLZF and the surface marker CD161 (Fig. 2c). Interestingly, 1928z-T-iPSC-T cells also showed high cytotoxic potential as indicated by high expression of TNFSF10 (TRAIL), GNLY, GZMB, FASL, LTA and low expression of co-inhibitory or exhaustion markers PD1, CTLA-4 and LAG3 (Fig. 2b). The majority of the CD3⁺ cells had a CD45RA⁺CD62L⁻CCR7⁻ effector memory phenotype (T_{EMRA}), although a small percentage (~6%) had a more naive CD45RA⁺CD62L⁺ phenotype (Fig. 2d). We did not detect expression of CD27 or CD28 receptors on the surface of 1928z-T-iPSC-T cells (Supplementary Fig. 5c).

iPSC-derived T cells will be therapeutically relevant only if they can be expanded while retaining functional properties. We therefore set out to expand 1928z-T-iPSC-T cells using 3T3-CD19 cells and characterize the expanded T cells. Starting from 3×10^6 1928z-T-iPSC, $\sim 1\text{--}2 \times 10^5$ 1928z-T-iPSC-T cells were obtained by day 30 of differentiation. We were able to expand those 1928z-T-iPSC-T cells 10- to 50-fold (mean = 20, s.d. = 15, $n = 6$) after one stimulation and up to ~1,000-fold after three weekly stimulations (Fig. 2e). Therefore, although the differentiation efficiency at day 30 is around 0.05, we were able to increase it to 0.5–1.0 by 1 week and up to 50.0 after 3 weeks of expansion. The expanded cells maintained their effector memory phenotype and upregulated the expression of natural cytotoxicity receptors such as NKp44, NKp46 and NKG2D (Fig. 2e). Interestingly, the expanded cells upregulated expression of T-lymphoid lineage-specific genes (*ZAP70*, *GATA3*, *CD3δ*, *CD3ε*, *TRGC2*) and downregulated expression of RORC, indicative of a switch toward a type 1 (Tbet/IFN-γ expressing) phenotype (Fig. 2f, g). CD161 surface expression was also reduced after expansion (Fig. 2d, e). In aggregate these findings suggest that CAR-mediated proliferation polarized the 1928z-T-iPSC-T cells toward a type 1 response. A similar phenotype switch has been shown for RORC-expressing T17 γδ T cells¹⁷, IL-17-producing fetal innate lymphoid cells¹⁸ and murine TCR-transgenic Th17 cells¹⁹, which polarize to type 1 cells after antigen or cytokine stimulation.

We first evaluated the cytotoxic potential of expanded 1928z-T-iPSC-T cells using an *in vitro* ⁵¹Cr release assay with EL4 murine lymphoma cells expressing CD19 or ovalbumin (nonspecific negative control) as targets⁹. Expanded 1928z-T-iPSC-T cells displayed high antigen-specific cytotoxic activity, even at low effector-to-target (E/T) ratios (Fig. 3a). To investigate the anti-tumor activity of 1928z-T-iPSC-T cells *in vivo*, we established a xenogeneic tumor model. Nonobese diabetic–severe combined immunodeficient NOD-SCID IL2Rγ^c^{null} mice were inoculated with the CD19⁺ Raji human Burkitt lymphoma cell line expressing a fluorescent luciferase fusion protein. For comparison to 1928z-T-iPSC-T cells, we transduced TCR-αβ and TCR-γδ peripheral blood lymphocytes from the same donor as the T-iPSC line expressing the 1928z CD19 CAR.

These three T-cell populations showed some phenotypic similarities and some differences. When we expanded 1928z-T-iPSC-T cells for a week, they displayed a T_{EMRA} phenotype (CD45RA⁺CD27⁻CD28⁻CCR7⁻), similar to the expanded 1928z-γδ T cells. In contrast, a sizeable fraction (33%) of 1928z-αβ T cells displayed a CD45RA⁻CD27⁺CD28⁺CCR7⁺ phenotype indicative of central memory cells (Fig. 3b). CAR expression was much lower on

1928z-T-iPSC-T cells (mean fluorescence intensity (MFI) = 395) than on 1928z- $\gamma\delta$ (MFI = 1,212) or 1928z- $\alpha\beta$ cells (MFI = 2,010) (Fig. 3b), which may influence therapeutic activity²⁰.

As shown by bioluminescent imaging, infusion of 1928z-T-iPSC-T cells delayed tumor progression to an extent similar to that induced by peripheral blood 1928z- $\gamma\delta$ cells (Fig. 3c and Supplementary Fig. 7), and resulted in a significant survival advantage compared to tumor-bearing mice that were not treated with T cells (log-rank $P = 0.042$, Cox proportional hazards regression $P = 0.036$; Fig. 3d). Bioluminescence imaging further revealed that 1928z-T-iPSC-T and 1928z- $\gamma\delta$ T cells initiated tumor regression more rapidly than 1928z- $\alpha\beta$ cells (Fig. 3c). However, although initially slower at inducing tumor regression, the 1928z- $\alpha\beta$ T cells did eventually induce complete tumor regression (Fig. 3c). These findings demonstrate that CAR⁺ T-iPSC-T cells can lyse tumor cells *in vitro*, elicit strong anti-tumor responses *in vivo* and provide a survival benefit in tumor-bearing animals, to the same degree as their closest natural counterparts.

The iPSC and CAR technologies, combined as shown here, potentially provide an unlimited source of T lymphocytes targeted to a chosen antigen, independent of HLA restriction. Under the present conditions, starting from T-iPSCs encoding a rearranged endogenous $\alpha\beta$ TCR, we determined that the generated T cells have the properties of $\gamma\delta$ T cells^{17,21}, although they express their endogenous $\alpha\beta$ TCR on their surface. A similar lineage diversion has been observed in mice expressing TCR α and β transgenes^{22–24}, wherein T cells distinct from wild-type NK, NK T cells, or CD4⁺ or CD8⁺ $\alpha\beta$ T cells displayed $\gamma\delta$ T-cell features, including expression of CD8 α and low expression of CD5, CD122 and NK1.1 (ref.22). T cells differentiated *in vitro* from human CD34⁺ hematopoietic progenitors genetically engineered to express an antigen-specific TCR display an NK cell-like phenotype²⁵. Together these observations suggest a possible effect of premature expression of TCR $\alpha\beta$, which may skew development toward innate lymphoid-like lineages. Although T-iPSC-derived expanded T cells have been reported to have a CD3⁺CD7⁺CD5^{low}TCR $\alpha\beta$ ⁺CD56⁺ phenotype associated with expression of CD8 α but not CD8 β , they were not identified as $\gamma\delta$ -like T cells⁶. Interestingly, we observed expression of the pre-rearranged endogenous TCR $\alpha\beta$ on day 15 of differentiation on OP9-DL1 cells (Supplementary Table 1), earlier than in some other reports describing T-cell differentiation from human ESC- or iPSC-derived T cells^{3,4}. Importantly, we observed the same kinetics of T-cell development as in T-iPSC-1.10 in two other independent T-iPSC lines bearing different TCR rearrangements (Supplementary Fig. 2b and Supplementary Fig. 8), but not in cord blood-derived iPSC or in ESCs (data not shown). Altogether, these prior observations^{22–25} and ours suggest that early expression of a transgenic or endogenous TCR $\alpha\beta$ influences the T-cell differentiation process. In addition, some subtle features of our 1928z-T-iPSC-T cells, such as their CD8 α ⁺CD8 β [−] phenotype, expression of CD161 and low expression of CD5, are shared between adult $\gamma\delta$ T cells^{17,21} and innate-like T cells generated in fetal development^{18,26}. Together with previous reports, these observations suggest that these lymphoid cells may originate from a fetal cell-like hematopoietic stem cell intermediate committed to innate-like lymphopoiesis^{4,27} and that *in vitro* differentiation from pluripotent stem cells may be intrinsically skewed toward embryonic characteristics²⁸. Notably, the CAR, which effectively supported T-cell expansion, did not seem to influence

the acquisition of the $\gamma\delta$ phenotype, as non-CAR-transduced T-iPSC-1.10 cells also yielded TCR $\alpha\beta$ -expressing, $\gamma\delta$ -like T cells (Supplementary Fig. 9). A complete understanding of the maturation of T-iPSC-derived T lymphocytes will be needed to further optimize their development and differentiation, generate different T-cell lineages and shape their functional attributes.

Tumor specificity is one of the essential characteristics of T lymphocytes used in adoptive T-cell therapy. Using the protocol described here, any HLA-independent antigen specificity can be imparted to any iPSC through an appropriate CAR⁸, without requiring the establishment of a patient-specific T-cell clone. To our knowledge no previously published study reported that genetic modification of human pluripotent stem cells with a receptor for antigen is an effective approach to generate T cells with therapeutic potential. 1928z-T-iPSC-T cells delayed tumor progression *in vivo* to a similar extent as peripheral blood-derived 1928z- $\gamma\delta$ -T cells from the same donor. $\gamma\delta$ T cells have some advantageous properties, such as low graft-versus-host reactivity and the ability to infiltrate solid tumors^{29,30}. Their anti-tumor activity has been demonstrated in several clinical settings, mainly against hematological malignancies³⁰.

CAR-modified T-iPSC-derived T cells may be especially valuable in situations where autologous or allogeneic T cells are not available. This is, for example, the case in immune-deficient patients such as HIV-infected or highly immunosuppressed patients with malignancies; in these scenarios autologous T-cell isolation and expansion is problematic or impossible. CAR-T-iPSC-T cells may also be useful in patients from whom the isolation of autologous tumor-infiltrating T lymphocytes has failed³¹, while providing the additional benefit of targeting alternative antigens recognized by CARs⁸. Other patients who could benefit from CAR-T-iPSC-T cells include those with acute leukemia who relapse after allogeneic hematopoietic cell transplantation and for whom the use of allogeneic donor lymphocyte infusions (DLI) is problematic. The efficacy of DLI in those patients is minimal, yet fraught with the risk of graft-versus-host disease³². CAR-T-iPSC-T cells could thus represent an additional option for patients who do not respond to DLI or for whom DLI use is not indicated due to high risk for graft-versus-host disease.

Several steps can be taken to avert the risks of immunological complications in the context of an off-the-shelf allogeneic CAR-T-iPSC-T therapy. The alloreactivity of T-iPSC-derived T cells, which express an endogenous TCR (Fig. 1a), can be eliminated by either disrupting the TCR, using target site-specific nucleases after T-cell differentiation³³, or by generating T-iPSCs from virus-specific T cells, which due to their recognition of a pathogen-derived antigen, are less likely to cause graft-versus-host disease³⁴. Allojection of CAR-iPSC-T cells (which express HLA molecules) can be minimized by generating iPSCs from common HLA haplotypes (to ensure their histocompatibility with matched unrelated recipients³⁵) or by repressing HLA expression through additional genetic modification³⁶. Finally, the risk of insertional oncogenesis secondary to gene transfer can be decreased by integrating the CAR cDNA and other genes, such as suicide genes and noninvasive imaging reporters³⁷, at genomic safe harbor sites³⁸.

In summary, the combination of iPSC and CAR technologies that we describe here offers a potential new source of off-the-shelf T cells of predetermined antigen specificity. Considering the versatility of pluripotent stem cells and CAR engineering, this system may facilitate production of different T-cell subpopulations with additional genetic modifications and specificities suitable for a range of therapeutic indications.

ONLINE METHODS

Generation of 1928z-T-iPSC

Peripheral blood lymphocytes (PBL) were collected from a volunteer donor after informed consent was obtained. PBLs were activated with phytohemagglutinin (PHA, 2 µg/ml) and transduced with two tri-cistronic excisable Moloney murine leukemia virus-based (SFG) retroviral vectors, each one encoding reprogramming factors and a different fluorescent marker (f-Citrine-P2A-cMYC-E2A-SOX2 and f-vexGFP-P2A-OCT4-T2A-KLF4) (Supplementary Fig. 1a). Transduced cells were seeded on MEF feeder cells and cultured in T-cell medium (RPMI-1640 supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin and 100 ng/ml streptomycin). The medium was changed to human ESC medium (DMEM/F12 with 20% of knockout serum replacement, 1 mM l-glutamine, 1% nonessential amino acids, 10 µM 2-mercaptoethanol and 8 ng/ml basic fibroblast growth factor (bFGF)) on day 5 after transduction and was then refreshed daily. T-iPSC colonies appeared at ~22–25 days after transduction. Clone T-iPSC-1.10 was stably transduced with a lentiviral vector encoding 19–28z, a second-generation CAR, and a fluorescent marker (mCherry) linked by a 2A peptide (Supplementary Fig. 3a). The 1928z-T-iPSC line was established after sorting for high expression of the mCherry marker. All T-iPSC lines were maintained in culture on MEF feeder cells with human ESC medium and passaged every 3–4 D. T-iPSC lines were tested for mycoplasma contamination every 2 months.

Characterization and assessment of pluripotency of T-iPSCs

To determine the reprogramming vectors' copy numbers (VCN) we isolated genomic DNA from the T-iPSC lines and performed multiplex quantitative PCR (qPCR) using sets of primers and probes specific for the SFG vector and for the human albumin gene (Supplementary Table 2). To determine absolute VCN, we generated a standard curve using serial dilutions of a plasmid containing both SFG vector and albumin gene amplicons. Reactions were carried out in triplicate in an ABI 7500 detection system (Applied Biosystems).

For assessment of expression of endogenous pluripotency genes, total mRNA from T-iPSC was isolated with Trizol (Invitrogen). Reverse transcription was performed with Superscript III (Invitrogen) and qRT-PCR was performed with previously described primers³⁸ using SYBR Green. Reactions were carried out in duplicate in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Expression was calculated by relative quantification using the DDCT method with GAPDH as endogenous control.

For flow cytometric analysis T-iPSCs were stained with the following fluorophore-conjugated antibodies: SSEA-3-AlexaFluor647 (MC-631) purchased from Biolegend,

SSEA-4-AlexaFluor647 (MC813-70), Tra-1-81-AlexaFluor647 (TRA-1-81), Tra-1-60-AlexaFluor647 (TRA-1-60) and HLA-ABC-PE (Cat# 555553) purchased from BD Biosciences. All flow cytometry analysis was done on a LSRII cytometer (BD Biosciences) and analyzed using FlowJo software, Ver. 9.5.2 (TreeStar).

For teratoma formation assays, undifferentiated T-iPSCs were suspended in human ESC medium containing 10 μ M of the Rho-associated kinase (Rock) inhibitor Y-27632 (Tocris). Approximately 2×10^6 cells were injected subcutaneously into 6- to 12-week-old female NOD-SCID IL2R γ ^{null} mice obtained from the MSKCC Mouse Genetics Core facility. Five to six weeks later, teratomas were surgically dissected and fixed in 4% formaldehyde. Paraffin-embedded samples were stained with hematoxylin and eosin for histological analysis.

For karyotyping, standard G-banding analysis was done at the MSKCC molecular cytogenetics core facility. Chromosome analysis was done on a minimum of 12 4,6-diamidino-2-phenylindole (DAPI)-banded metaphases.

For the assessment of silencing of the reprogramming vectors, qRT-PCR was done using primers and probes that detect GFP-derivative (vexGFP and mCitrine) transcripts as previously described³⁸. Reactions were carried out in duplicate in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Expression was calculated by relative quantification using the DDCT method with GAPDH as endogenous control.

TCR β and γ chain rearrangement

Genomic DNA was isolated from T-iPSCs and 1928z-T-iPSC-T cells using Qiagen DNeasy Blood and Tissue kit (Qiagen). PCR was performed using multiplex primer kits (Invivoscribe Technologies, San Diego, CA) specific for a majority of clonal TCR β and γ chain rearrangements. Capillary electrophoresis and PCR product fragment analysis was performed at MSKCC Genomic's Core Facility using an ABI 3730 DNA analyzer. Data were analyzed using Peak Scanner software (ABI, Foster City, CA).

T-cell differentiation from 1928z-T-iPSCs and expansion of 1928z-T-iPSC-T cells

For the differentiation of 1928z-T-iPSCs to hematopoietic precursors, we used an optimized serum-and feeder-free in vitro differentiation protocol. Briefly, undifferentiated T-iPSC colonies were treated with dispase (Worthington) for 6 min and transferred to low-attachment plates to allow for the formation of embryoid bodies (EBs) in embryoid body differentiation medium (StemPro-34, Invitrogen, with 2 mM l-glutamine, 1% nonessential amino acids, 10 μ M 2-mercaptoethanol, 100 U/ml penicillin and 100 ng/ml streptomycin and 50 mg/ml ascorbic acid). The formation of embryoid bodies was facilitated by an overnight incubation in the presence of 30 ng/ml of hBMP-4. Embryoid bodies were then cultured with BMP-4 and hbFGF (5 ng/ml) until day 4 to allow for mesoderm induction. Next, hematopoietic specification and expansion was achieved in the presence of hVEGF (20 ng/ml) and a cocktail of hematopoietic cytokines (hSCF 100 ng/ml, hFlt3L 20 ng/ml, hIL-3 20 ng/ml and bFGF 5 ng/ml) as indicated. Day 10 embryoid bodies containing hematopoietic progenitor cells were dissociated by treatment with Accutase for 20 min and single cells were then seeded on OP9-DL1 monolayers³⁹ to allow for their T-lymphoid

differentiation in OP9 medium (α -MEM with 20% FBS, 2 mM l-glutamine, 1% nonessential amino acids, 10 mM 2-mercaptoethanol, 100 U/ml penicillin and 100 ng/ml streptomycin and 50 mg/ml ascorbic acid) supplemented with SCF 10 ng/ml, IL-7 5 ng/ml and Flt3L 10 ng/ml. For the stimulation and expansion of 1928z-T-iPSC-T cells, we used previously described CD19-expressing 3T3 cells as artificial antigen presenting cells (3T3-CD19)^{9,40}. The generated 1928z-T-iPSC-T cells were seeded on a monolayer of irradiated 3T3-CD19 in a 3:1 E/T ratio in T-cell medium with IL-7 (10 ng/ml) and IL-15 (10 ng/ml). All recombinant factors were purchased from R&D Systems (Minneapolis).

Flow cytometric analysis

The following conjugated antibodies were used for flow cytometric phenotyping and analysis: CD34-PECy.7 (8G12), CD43-FITC (1G10), CD7-V450 (MT701), CD8b-PE (2ST8.5H7), CD69-PECy.7 (FN50), CD161-FITC (DX12), CD16-PerCPCy5.5 (3G8), TCR γ d-FITC (11F2), CD122-FITC (TU27), CD94-PE (HP-3D9) purchased from BD Biosciences, CD3-PE/FITC/Pacific Blue (UCTH1), CD5-PE (5D7), CD4-PECy.7 (S3.5), CD8a-PE/FITC (3B5), CD25-APC (3G10), CD62L-PE (Dreg-56), CD27-APC (0323), CD28-PE (10F3), goat-anti-mouse- AlexaFluor647 purchased from Invitrogen, TCR α b-APC (IP26), CD56-PECy.7 (CMSSB) and CD45RA-PerCPCy5.5 (HI100) purchased from eBioscience, NKp44-PE (P44-8), NKp46-PE (9E2), NKG2DAPC (1D11), CD158a/h-PE (HP-MA4), CD158b-PE (OX27) purchased from BioLegend, PLZF-APC (20102) and CCR7-FITC (150503) purchased from R&D. All antibodies were used in a 1:20 dilution. Dead cells were excluded from analysis in all experiments by staining with DAPI. All flow cytometry analysis was done on a LSRII cytometer (BD Biosciences) and analyzed on FlowJo software, Ver. 9.5.2 (TreeStar).

Cytokine release and cytotoxicity assays

To measure cytokine production 6×10^4 1928z-T-iPSC-T cells were seeded on irradiated CD19- or 3T3-CD19 cells in a 3:1 ratio (E/T ratio) per well of a 96-well plate in T-cell medium with IL-7 (10 ng/ml) and IL-15 (10 ng/ml). Culture supernatants were collected after 24 h and the concentration of type I and/or type II cytokines was quantified with a Luminex assay kit (Invitrogen) according to manufacturer instructions.

Cytotoxic potential of 1928z-Ti-T cells was evaluated in standard ⁵¹Cr release assays. Target cells were labeled with ⁵¹Cr and co-cultured with 1928z-T-iPSC-T cells at decreasing effector/target (E/T) ratios. After 4 h of culture, supernatant was removed and radioactivity released from chromium was measured. Specific lysis was determined by subtracting background radioactivity of target cells not cultured with T cells and dividing by the radioactivity measured from target cells completely lysed by treatment with 0.2% Triton X-100. The murine lymphoma cell line EL4, engineered to express ovalbumin (EL4-OVA) or human CD19 (EL4-CD19)⁴¹, was used as target.

Microarray procedure and gene expression analysis

Whole PBLs were isolated from two healthy donors by Ficoll density centrifugation after informed consent was obtained. The following subpopulations: CD3⁺CD4⁺, CD3⁺CD8⁺, CD3⁺CD56⁺, CD3⁻CD56⁺ (NK) and CD3⁺TCR γ δ ⁺ (γ δ T cells) were purified (98%) from

PBL by cell sorting. Total mRNA was extracted from 1928z-T-iPSC-T cells at days 30–35 of differentiation and from the sorted PBL subpopulations using TRIzol™ Reagent (Invitrogen Life Technologies, Paisley, UK). Microarray analyses were performed at the MSKCC Genomics Core facility using 75 ng of total RNA as the starting material, amplified and labeled following the standard Affymetrix protocol (Affymetrix, Santa Clara, CA, USA). The labeled complementary RNA was then fragmented and hybridized to Affymetrix GeneChip arrays HG-U133 plus 2.0.

For the gene expression analysis the raw data (Affymetrix CEL files) produced using HG U133-Plus 2.0 platform were used. For comparison purposes, additional rawdata files obtained on the same platform were downloaded from the NCBI repository GEO database: five samples of normal naive B cells (GSE12195), five samples of $\alpha\beta$ CD4⁺ cells (GSE15659), one sample of resting $\alpha\beta$ CD8⁺ cells (GSE8059), one sample of resting NK cells (GSE8059), 12 samples of TCRV γ 9 $\gamma\delta$ T cells (GSE27291), before activation and after activation with BrHPP/IL-2 (bromohydrin pyrophosphate and IL-2) for 6 h or 7 d. Robust Multi-array Average (RMA) procedure was applied to all CEL files and comparisons of different samples were performed upon z-scores normalization. Gene-centric expression values were obtained using a CDF file based on remapping of probes to the human genome. Gene expression levels were compared both between single samples and by grouping samples of the same type in an unbiased way. Similarity between samples was evaluated by Pearson's correlation coefficient computed between a selected list of probes: 1,163 probes were selected based on their variability across samples (s.d. > 0.75) and consistency among 1928z-T-iPSC-T cells (s.d. < 1). Correlations between groups were computed after averaging probe expression levels of single samples of the same type. Using the computed set of correlations, we performed hierarchical clustering of the single samples. The clustering was performed using the R package hclust with the default settings (Euclidean distance). Second, a comparison between the analyzed samples on a selected panel of genes was performed.

Quantitative real-time PCR

Total mRNA was extracted using TRIzol™ Reagent (Invitrogen Life Technologies, Paisley, UK). Reverse transcription was done using the Superscript III First-Strand Synthesis supermix for qRT-PCR (Invitrogen). Quantitative-PCR for specific genes were done using the respective probe-based TaqMan Gene Expression assays (Applied Biosystems). Reactions were carried out in duplicate in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Expression was calculated by relative quantification using the DDCT method with GAPDH as endogenous control.

Isolation and retroviral transduction of $\gamma\delta$ and $\alpha\beta$ -T cells

PBL were isolated from the same donor as the T-iPSC. TCR $\gamma\delta$ T cells were isolated with magnetic cell sorting (negative selection) using the TCR $\gamma\delta$ + T-cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Next, TCR $\gamma\delta$ T cells were stimulated with 5 mM zoledronic acid (Zometa, Novartis) and 1,000 IU/ml IL-2 for 48 h. The TCR $\alpha\beta$ fraction of PBLs (obtained as the positive fraction after negative selection of TCR $\gamma\delta$ T cells) was activated with PHA 2 mg/ml for 48 h. Synthesis of the 1928zCAR-encoding 1928z-

IRES-LNGFR vector has been described⁴¹. Retroviral producers were prepared from plasmid-transfected H29 cell supernatants as previously described⁴¹. Activated $\gamma\delta$ and $\alpha\beta$ T cells were transduced with retroviral supernatants on two consecutive days by spin-infection in retronectin (Takara)-coated oncoretroviral vector-bound plates. Cells were fed every 3 d with T-cell medium supplemented with 1,000 IU/ml or 20 IU/ml of IL-2 for $\gamma\delta$ and $\alpha\beta$ T cells, respectively.

In vivo tumor model

6-to 12-week-old male NOD-SCID IL2R γ null mice, obtained from the MSKCC Mouse Genetics Core facility, were inoculated i.p. with 10⁵ Raji human CD19⁺ Burkitt lymphoma cells expressing a green fluorescent protein-firefly luciferase fusion protein (GFP/Luc) as previously described^{9,40}. Four days later 4×10^5 expanded (1-week stimulation on irradiated 3T3-CD19) 1928z-T-iPSC-T cells or CAR-transduced syngeneic $\alpha\beta$ or $\gamma\delta$ T cells were injected i.p. along with IL-2 (50,000 U/mouse) and IL-15 (0.25 mg/mouse). Only mice that had equal tumor burden ($2 \times 10^6 \pm 0.5 \times 10^6$ photons/sec) before T-cell injection were used. Mice with lesser or greater tumor burden were excluded from the study. Tumor-bearing mice retained in the study were randomized to the different treatment groups (at least four mice per group). No blinding method was used. T-cell dose was based on the percentage of CAR⁺ cells as measured by pre-injection flow cytometric analysis. IL-2 administration was continued daily and IL-15 every 2 d for 2 weeks. Tumor burden was monitored twice per week by in vivo bioluminescence imaging (IVIS 100 Imaging System). Living Image software Version 4.3.1 was used to acquire and quantify the bioluminescence imaging data sets. All animal experiments were conducted in accordance with protocols approved by MSKCC Institutional Animal Care and Use Committee (IACUC) and following National Institutes of Health guidelines for animal welfare.

Statistical methods

No pre-specified effect size was used to determine sample sizes. The use of statistical tests was chosen according to the nature of the data. We used the Wilcoxon rank-sum test (Mann-Whitney U test) to compare the tumor burden across multiple groups. This test was chosen because of its robustness to the underlying distribution of the observations. Comparison of survival curves was done using the log-rank test. Partial likelihood ratio test from a Cox regression model was also used to compare the survival between 1928z-T-iPSC-T and no treatment groups after ensuring that the data were consistent with the proportional hazards assumption ($P = 0.15$ using the weighted-residuals test⁴²). As we were unable to fit a Cox model for the remaining treatment groups due to the paucity of events, the reported P-values are those provided by the log-rank test. Statistical significance was defined as $P < 0.05$. Statistical analyses were done on Prism software (GraphPad) (tumor burden comparison and log-rank) or R (microarray analysis and Cox proportional hazards regression).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Differentiation of 1928z CAR-engineered T-iPSCs into CD19-specific functional T lymphocytes. **(a)** The study concept. Peripheral blood lymphocytes are reprogrammed to pluripotency by transduction with retroviruses encoding c-MYC, SOX2, KLF4 and OCT-4 (ref. 7). The resulting T-iPSCs are genetically engineered to express a CAR and are then differentiated into T cells that express both the CAR and an endogenous TCR. **(b)** In vitro T-lymphoid differentiation protocol. T-iPSCs were stably transduced with a bicistronic lentiviral vector encoding the 19–28z CAR and the fluorescent marker mCherry. mCherry⁺ CAR⁺ T-iPSCs are differentiated in three steps: (i) mesoderm formation (days 1–4), (ii) hematopoietic specification and expansion (days 5–10) and (iii) T-lymphoid commitment (days 10–30). Fluorescence microscopy images (below) show mCherry expression was maintained throughout the differentiation process. Scale bars, 100 μ M. **(c)** Flow cytometric

analysis of 1928z-T-iPSC-derived cells at day 30 of differentiation. Representative plots are of at least five independent differentiations. **(d)** 1928z-T-iPSC-T cells were seeded into cultures of 3T3 cells or 3T3 cells expressing CD19 (3T3-CD19). Co-cultures shown 24 h after T-cell seeding; formation of T-cell clusters and elimination of the 3T3-CD19 monolayer are visible. Scale bars, 100 μ m. **(e)** Flow cytometric analysis of CD25 and CD69 expression on the surface of 1928z-T-iPSC-T cells 48 h after exposure to 3T3 or 3T3-CD19 cells. **(f)** Luminex multiplex cytokine analysis of culture supernatant 24 h after seeding of 1928z-T-iPSC-T cells on 3T3 or 3T3-CD19 cells. Data are presented as mean of two independent experiments $\pm$ s.d.

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Figure 2.

Phenotypic profiling of 1928z-T-iPSC-T cells before and after CD19-induced expansion. **(a)** Unsupervised hierarchical clustering of 35 total transcriptomes, generated by an mRNA gene expression microarray, from 1928z-T-iPSC-T cells at days 30–35 of differentiation (1928z-T-iPSC-T) and other human lymphoid cell subsets isolated for this study [CD3⁺TCR $\gamma\delta$ ⁺ cells ($\gamma\delta$ -T), CD3⁺CD56⁺ cells, CD8⁺ cells and CD4⁺ cells] or downloaded from the NCBI repository GEO database (naive B cells, TCRV γ 9 $\gamma\delta$ T-cells before activation ($\gamma\delta$ -T GEO) and after activation with BrHPP/IL-2 (bromohydrin pyrophosphate/interleukin-2) for 6 h ($\gamma\delta$ -T 6h activ) or 7 days ($\gamma\delta$ -T 7d activ) and resting NK cells). **(b)** Heatmap comparing the expression of indicated mRNA transcripts expressed in lymphoid and/or NK cells. Transcripts are classified according to known function and expression patterns. **(c)** Intracellular expression of the transcription factor PLZF (red histogram), compared to isotype control (black histogram), and surface expression of CD161 and CD3 in 1928z-T-iPSC-T cells, as assessed by flow cytometry. **(d)** Expansion of 1928z-T-iPSC-T

cells after weekly stimulations with 3T3-CD19 cells in the presence of IL-7 (10 ng/ml) and IL-15 (10 ng/ml) for 4 weeks. Absolute cell numbers are shown. Arrows indicate restimulations with freshly irradiated 3T3-CD19 AAPCs. **(e)** Flow cytometric analysis of cell surface molecules and cytotoxic receptors in gated CD3⁺ 1928z-T-iPSC-T cells before and 7 d after expansion on 3T3-CD19 AAPCs. **(f, g)** qRT-PCR analysis of the expression of the indicated mRNA transcripts in 1928z-T-iPSC-T cells before and 7 d after expansion on 3T3-CD19 AAPCs. Data were normalized to the values of endogenous GAPDH and pre-expansion expression levels were used as reference. Graphs represent average of intra-assay technical triplicates. Error bars, mean $\pm$ s.d.

Figure 3.

1928z-T-iPSC-T cells lyse CD19-positive tumor cells in vitro and in vivo. **(a)** In vitro ^{51}Cr release assay of 7 d-expanded 1928z-T-iPSC-T cells (effectors) and the murine lymphoma cell line EL-4 expressing ovalbumin (EL4-OVA) or human CD19 (EL4-CD19) (targets). E/T, effector/target ratio. Representative of two independent experiments. **(b)** Flow cytometric analysis of 1928z-T-iPSC-T cells and syngeneic 1928z-transduced $\gamma\delta$ (1928z- $\gamma\delta$) and $\alpha\beta$ (1928z- $\alpha\beta$) T cells before their injection into tumor-bearing mice. Bottom: black histogram, untransduced cells; red histogram, transduced cells. Representative plots of two independent experiments. **(c)** NOD-SCID IL2R γ null mice were inoculated intraperitoneally with CD19+ Raji human Burkitt lymphoma cell line expressing a green

fluorescent protein–firefly luciferase fusion protein (GFP/Luc). Four days later, T cells (4×10^5) described in b were injected intraperitoneally. No treatment indicates mice that were injected with tumor cells but not T cells. Tumor burden was measured biweekly by bioluminescent imaging. Images of representative time points are shown. Images of three mice from each group were intentionally selected to show mice relapsing after treatment. Disappearance of a mouse from the sequence of images indicates death of that mouse. **(d)** Kaplan-Meier curve representing the percent survival of the experimental groups described in b (1928z-T-iPSC-T: $n = 4$, 1928z- $\gamma\delta$: $n = 5$, 1928z- $\alpha\beta$: $n = 7$, no treatment: $n = 6$). Color-coded arrows depict death events not related to tumor growth in the corresponding groups. Statistical analysis between the treated experimental and the untreated control group, depicted here, was done using the log-rank test and $P < 0.05$ was considered significant.