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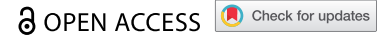


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ORIGINAL RESEARCH



Comparing CAR and TCR engineered T cell performance as a function of tumor cell exposure

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ABSTRACT

Chimeric antigen receptor (CAR) T cell therapies have resulted in profound clinical responses in the treatment of CD19-positive hematological malignancies, but a significant proportion of patients do not respond or relapse eventually. As an alternative to CAR T cells, T cells can be engineered to express a tumor-targeting T cell receptor (TCR). Due to HLA restriction of TCRs, CARs have emerged as a preferred treatment moiety when targeting surface antigens, despite the fact that functional differences between engineered TCR (eTCR) T and CAR T cells remain ill-defined. Here, we compared the activity of CAR T cells versus engineered TCR T cells in targeting the B cell malignancy-associated antigen CD20 as a function of antigen exposure. We found CAR T cells to be more potent effector cells, producing higher levels of cytokines and killing more efficiently than eTCR T cells in a short time frame. However, we revealed that the increase of antigen exposure significantly impaired CAR T cell expansion, a phenotype defined by high expression of coinhibitory molecules and effector differentiation. In contrast, eTCR T cells expanded better than CAR T cells under high antigenic pressure, with lower expression of coinhibitory molecules and maintenance of an early differentiation phenotype, and comparable clearance of tumor cells.

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T cell receptor; chimeric antigen receptor; CAR; TCR; comparison; solid tumors; antigen exposure; exhaustion; activation-induced cell death; tumor load

Introduction

Adoptive transfer of genetically engineered T cells has curative potential for the treatment of cancer.¹ Prominently, CD19-targeting chimeric antigen receptor (CAR) T cell therapies have demonstrated impressive clinical results in the treatment of B cell malignancies. While initial studies reported complete response rates of up to 90% in pediatric ALL,² reported initial response rates with CD19-targeting CARs are lower in other B-cell malignancies such as in CLL³ or large B cell lymphoma.⁴ Furthermore, a substantial fraction of patients eventually suffer from relapse.^{5,6} These relapses can include not only antigen-negative relapses but also antigen-positive relapses, indicating low CAR T cell persistence or incomplete clearance of tumor cells due to CAR T cell dysfunction. Moreover, CAR T cell therapy is associated with a high degree of toxicity, as exemplified in the frequent occurrence of cytokine release syndrome or neurotoxicity.⁷ These observations demonstrate that not only further improvements of CAR T cell therapies are needed, but also there is room for alternative or complementary approaches.

T cells can also be engineered to express a transgenic T cell receptor (TCR),^{8,9} termed engineered TCR (eTCR) T cells. Clinically, eTCR T cells have been studied less extensively than CAR T cells. Most clinical trials with eTCR T cells that have been performed used NY-ESO-1-specific T cells and produced responses in the treatment of solid tumors such as melanoma, synovial sarcoma,¹⁰ and multiple myeloma.¹¹ More

recently, E7-specific TCR-engineered T cells yielded encouraging results in the treatment of metastatic human papilloma virus-associated cancers.¹² However, clinical efficacy of eTCR T cells in treating B cell malignancies is yet to be tested.

While CAR T cells are redirected to surface antigens via an antibody-based targeting moiety, eTCR T cells express a heterodimeric receptor that recognizes antigen-derived peptides presented in the context of HLA. On the one hand, this necessity of antigen presentation provides the opportunity to target antigens that are derived from intracellular proteins. This enlarges the pool of antigens theoretically targetable by different TCRs, while CARs are typically restricted to surface antigens. On the other hand, HLA restriction of TCRs limits the patient pool that is treatable with one individual TCR. Because of this HLA restriction, CARs currently pose a preferred choice when targeting surface antigens. However, it is unclear how eTCR T and CAR T cells perform in a side-by-side comparison when targeting tumor cells that can be targeted by both treatment modalities.

Within this context, we compared the efficacy of CAR- and TCR-transduced T cells in targeting tumor cells that express the B cell malignancy-associated antigen CD20. Using a panel of long-term expanded ALL cells that naturally differ in their CD20 expression level, we show that CAR T cells had stronger initial effector functions than eTCR T cells. We also show that a sustained strong CAR T cell expansion was limited to settings of weak to moderate antigen exposure. In contrast and despite moderate immediate effector functions, eTCR T cells

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outperformed CAR T cells under high antigenic pressure. Our results indicate the delicate balance needed for optimal T cell activation and have implications for the conceptual advancement of both CAR and TCR therapeutics.

Materials and Methods

Cell culture

Peripheral blood was obtained from healthy donors after informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll. CD8 + T cells were isolated from frozen PBMCs using magnetic-activated cell sorting (MACS) according to the manufacturer's instructions (aCD8 microbeads, Miltenyi Biotec, Bergisch Gladbach). CD8 + T cells were activated with 0.8 $\mu\text{g}/\text{ml}$ PHA and autologous feeder cells at an E:T ratio of 1:3. T cells were cultured in IMDM supplemented with 5% FCS (Gibco, Life technologies, Carlsbad, CA), 5% human serum, 100 IU/ml IL-2, 1.5% 200 mM L-glutamine (Lonza, Switzerland), and 1% 10,000 U/ml penicillin/streptomycin (Lonza, Switzerland). Acute lymphoblastic leukemia cells were expanded and long-term cultured from primary bulk leukemic cells in serum-free medium as described earlier.¹³ For some experiments, ALL CM cells were used which had been previously lentivirally transduced with pCDH-EF1-Luc2-P2A-tdTomato (ALL CM tdTom) to allow for staining free FACS gating.

Generation of viral supernatants and retroviral transduction

The 1E9 TCR, described in Ref.¹⁴, was cysteine modified, constant domain murinized, codon optimized and cloned into the pMP71 flex vector. 1E9 TCR is a HLA-A*02:01-restricted, high-avidity TCR isolated from an individual that was negative for HLA-A*02:01. CAR constructs were cloned into pLZRS-P2A-dNGFR vectors. The FMC63-28z CAR sequence was taken from Ref.¹⁵, accession number HM852952. For ofatumumab- and rituximab-based CARs, amino acid sequences for heavy and light chain antigen binding domains were extracted from patents no. US 7,850,962 B2 and no. US 5,843,439, respectively. scFvs were generated in a V1-Vh configuration using a 4GS linker, and a CD8a leader sequence was cloned upstream. To generate 28z CARs, CD28 hinge, transmembrane, and signaling domains together with the CD3z domain were fused to Ofa or RTX scFvs using overlapping primer PCR analogous to the FMC63-28z construct. For BBz CARs, sequences encoding for Ofa or RTX scFvs were fused to CD8a hinge and transmembrane domains, 4-1BB costimulatory, and CD3z signaling domain, following the design of clinically used FMC63-BBz CAR.¹⁶

For generation of pMP71 viral supernatants, Phoenix-A cells were transfected with respective pMP71-TCR constructs together with pCL-amp help vector using Fugene transfection agent (Promega, Madison, WI). Medium of transfected

Phoenix cells was changed after 24 h, and viral supernatants were harvested and frozen at -80°C 48 h after initial transfection. For generation of pLZRS supernatants, Phoenix A cells were transfected with respective pLZRS constructs and retroviral supernatant was harvested following selection of transfected Phoenix-A cells with puromycin. For retroviral transduction, 24-well flat-bottom suspension culture plates (Greiner Bio-One) were coated with 30 $\mu\text{g}/\text{ml}$ Retronectin (Takara, Japan) and blocked with 2% human serum albumin (Sanquin, Amsterdam, The Netherlands). Virus supernatant was thawed and spun on Retronectin-coated wells at 3000 g for 20 minutes at 4°C . Viral supernatant was then removed, and 0.3×10^6 T cells were added to each well. After 24 h, transduced T cells were transferred to tissue culture-treated culture flasks and expanded.

Generation of CAR and eTCR T cells

Activated CD8 T cells were transduced 48 h after activation. 4 days after transduction, transduction efficiency was measured by FACS by staining for dNGFR or mTCR expression, respectively. On day 5 after transduction, T cells were enriched for transgene expression by MACS enrichment using anti-APC microbeads (Miltenyi Biotec, Germany) following staining of T cells with APC-conjugated anti-dNGFR or anti-mTCR antibodies, respectively. Purity after MACS was verified using FACS analysis. Cells were used for downstream analysis if purity exceeded $>90\%$. Enriched T cells were used for assays between days 11 and 15 after activation or as indicated.

Flow cytometry

Unless noted differently, 50,000 cells were washed and stained in 96-well u-bottom plates. Sytox Blue (Thermo Fisher, US) was used as viability dye in a 1:1000 dilution. For experiments involving absolute quantification of events, 10 μl (10,000) of Flow-Count Fluorespheres (Beckman Coulter, US) were added to the acquisition tubes. Data showing cell counts were normalized to the amount of acquired beads. Data were acquired on LSRII or Fortessa X-20 flow cytometers (BD Biosciences, USA) and analyzed using FlowJo V10 software.

T cell reactivity assays

For IFN γ secretion, 5,000 T cells were incubated with target cells in varying E:T ratios in 384-well plates (Greiner Bio-One, Austria) overnight in 60 μl of TCM. 12.5 μl of supernatants were harvested and serially diluted at 1:5 and 1:25. IFN γ was measured using IFN γ ELISA (Sanquin, The Netherlands). Values of IFN γ concentrations were back calculated accounting for respective dilution factors. If values were outside of the linear range of the standard in the 1:5 dilution, values generated with the 1:25 dilution were used for figures.

To assess cytotoxicity as measured by chromium release, target cells were labeled with 100 μCi $\text{Na}_2^{51}\text{CrO}_4$ for 1 h at 37°C and washed 3 times. Labeled target cells were then incubated in triplicate with T cells in varying E:T ratios in TCM for 6 h at 37°C. 25 μl of respective supernatants were then harvested, transferred to 96-well LumaPlates (PerkinElmer, US), and allowed to dry overnight. Chromium release was measured using a 2450 Microbeta² plate counter (PerkinElmer, US). Toxicity was calculated using the following formula

$$\%(\text{killing}) = \frac{\text{test release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} * 100.$$

Spontaneous release depicts release of target cells without effector cells, and maximum release was determined after incubation of target cells with 1% Triton-X.

CRISPR/Cas9-mediated knockout of TRAC/BC genes

CRISPR/Cas9-mediated knockout of endogenous TCR genes TRAC/BC was performed as described previously [17; 18]. In brief, RNPs targeting TRAC or TRBC were generated by hybridizing *Streptococcus pyogenes* Cas9 protein (IDT Technologies) with tracrRNA and respective TRAC/BC-targeting sgRNAs. For endogenous TCR knockout, T cells were electroporated with pooled TRAC/BC RNPs using the NEON transfection system two days after activation. Mock electroporated T cells were electroporated without the addition of RNPs. Retroviral transduction followed 24 h after electroporation.

pRT-PCR

RNA isolation and production of cDNA generation were performed as described previously.¹⁴ Gene expression using quantitative RT-PCR was measured on a Lightcycler 480 (Roche) using Fast Start TaqDNA Polymerase (Roche) and EvaGreen (Biotum). Gene expression of *CD19* and *MS4A1* (*CD20*) is depicted as relative gene expression to the average expression of housekeeping genes *GUSB*, *PSMB4*, and *VPS29*.

CFSE dilution

To assess antigen specific proliferation, T cells were labeled with 1 μM CFSE and rested for four hours at 37°C. 20,000 CFSE-labeled T cells were then incubated with 40,000 irradiated target cells in duplicates and cultured in 96-well plates over the course of 4 days in the presence of 100 IU/ml IL-2 and assessed via flow cytometry.

Activation-induced cell death

Activation-induced cell death (AICD) was determined using a CellEvent Caspase-3/7 Green Flow Cytometry Assay Kit (ThermoFisher, US). 10,000 T cells were incubated in different E:T ratios with ALL CM tdTom in 96-well round bottom plates. After 16 h, Caspase-3/7 detection reagent was added (1:100) followed by an incubation period of 25 minutes at 37°C. After that, Sytox ADDvanced was added (1:100) followed by

another incubation period of 5 minutes at 37°C. Cells were then transferred to FACS tubes and assessed by flow cytometry. The gating strategy is given in supplemental Fig. S4A. To calculate AICD, the following formula was used:

$$\text{AICD} = \frac{\%(\text{Casp37pos}(E : T)) - \%(\text{Casp37pos}(\text{TCM}))}{100\% - \%(\text{Casp37pos}(\text{TCM}))}$$

Proliferation stress test

3,000 T cells were incubated in 96-well round bottom plates with varying E:T ratios up to 1:27 (81,000 target cells) over the course of 7 days in the presence of 100 IU IL-2/ml. Half of the total medium volume (100 μl) was refreshed on days 3 and 6. On day 7, cells were spun down and resuspended in SytoxBlue live/dead marker (1:1000) and 10,000 Flow-Count Fluorospheres (Beckman Coulter, US) were added. In the experiment using ALL RL, ALL BV, and ALL CM as target cells, a staining step using APC-conjugated anti-CD19 antibody was included. In the experiments using only ALL CM tdTom as target cells, no counterstain was added.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (Version 8.4.2). Respective statistical tests used are indicated in figure legends. Samples were paired when appropriate. Indicated significance levels used are $p < .05$ *, $p < .01$ **, and $p < .001$ ***.

Study approval

Healthy donor and patient material from the Leiden University Medical Center Biobank for Hematological Diseases were used in this study. The study was approved by the Institutional Review Board of the Leiden University Medical Center (approval number B16.039). Materials were obtained after written informed consent in accordance with the Declaration of Helsinki.

Results

CAR T cells have stronger short-term effector functions than eTCR T cells

To generate CAR constructs that target CD20, we cloned single-chain fragments (scFvs) derived from clinically used anti-CD20 antibodies rituximab (RTX) or ofatumumab (Ofa) and built them into second-generation CARs incorporating a CD28 costimulatory domain (Figure 1a). The CD20-directed TCR, termed 1E9 TCR, is a high-avidity TCR recognizing the CD20-derived peptide SLFLGILSV presented in HLA-A*02:01 and was described earlier.¹⁴ The 1E9 TCR was cysteine modified and constant domain murinized to enhance preferential pairing and surface expression. All constructs were cloned into retroviral vectors. CAR constructs were also equipped with truncated NGFR (dNGFR) as a marker gene.¹⁹ Activated primary human CD8 T cells were retrovirally

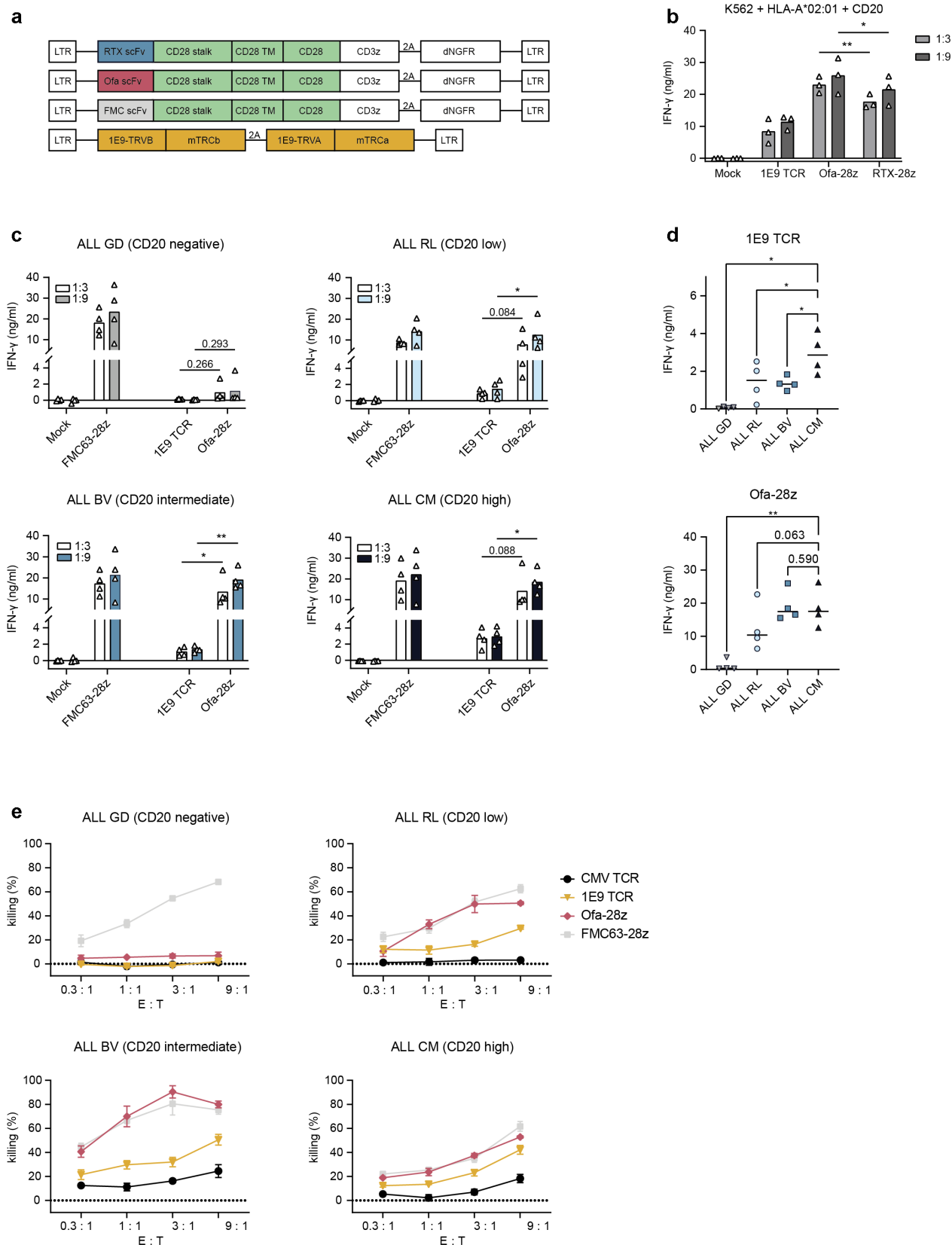


Figure 1. CAR T cells have stronger short-term effector functions than eTCR T cells. Retrovirally transduced and MACS purified CD8⁺ CAR T and eTCR T cells were assessed for effector functions in targeting CD20-expressing malignancies. (a) Overview of used constructs. CAR constructs and the 1E9 TCR construct were cloned into retroviral expression systems. CD20-targeting CARs were designed on either ofatumumab (Ofa)- or rituximab (RTX)-derived scFvs combined with CD28 stalk, transmembrane, and signaling domains. FMC63-28z CAR was used as a CD19-targeting control. 1E9 TCR is constant domain murinized and cysteine modified. (b) Specificity of CD20-targeting constructs was assessed by IFN γ ELISA after overnight coculture. 5000 T cells were incubated for 16 h with target cells at indicated E:T ratios. Data points show averaged duplicate values from three different experiments using individual donors. (c) 5000 T cells were cocultured overnight with long-term expanded ALL cells that cells that naturally differ in their CD20 expression. E:T ratios are as indicated. Data depict averaged duplicate values from four experiments using T cells derived from independent donors. (d) Comparison of cytokine secretion as a function of target cell exposure at an E:T ratio of 1:9 as depicted in (c). (e) Representative killing efficacy of CAR- and TCR-transduced T cells in 6 h ⁵¹Cr release assay. 2500 target cells were labeled for 1 h with Na₂⁵¹CrO₄ and incubated with T cells at indicated E:T ratios. Statistics in (b), (c), and (d) show Fisher's least significant difference test with comparisons as indicated. Comparisons were paired for donors.

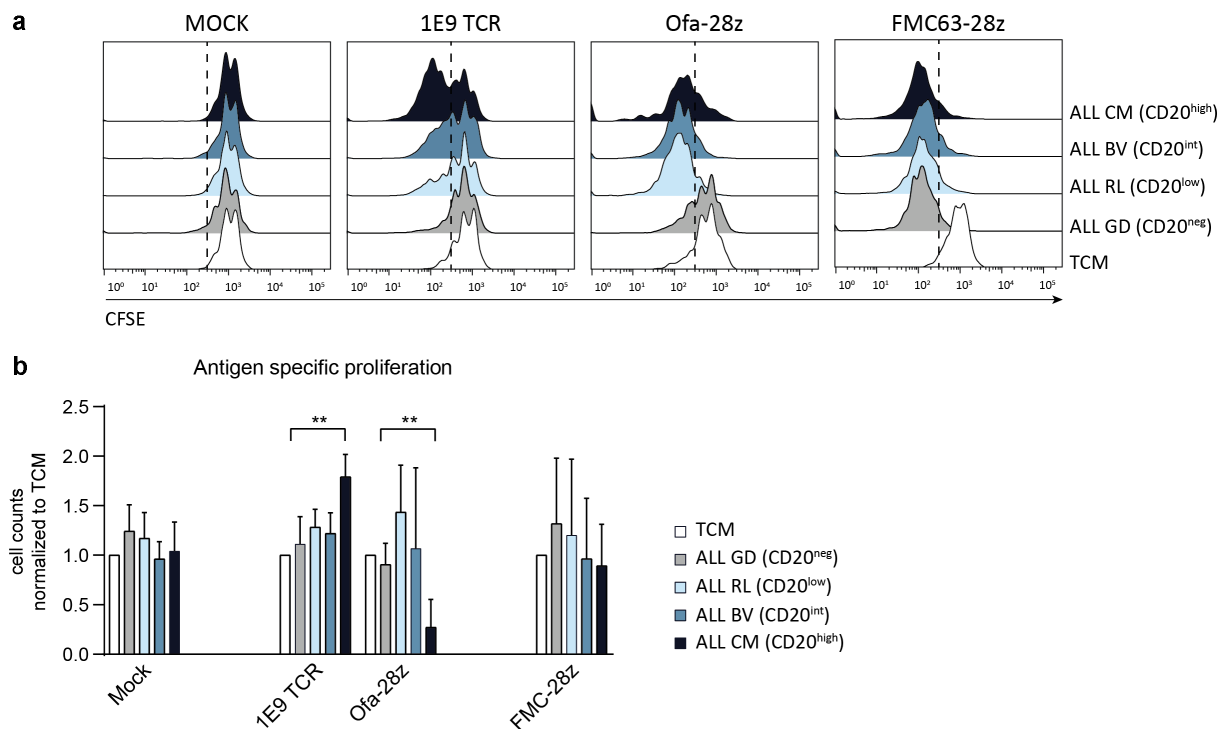


Figure 2. Both CAR T cells and TCR T cells proliferate when encountering target cells, but CD20 CAR T cell expansion correlates negatively with target antigen expression. To assess antigen specific proliferation of T cells as a function of target antigen expression, T cells were labeled with CFSE and incubated with irradiated target cells for 4 days at an E:T ratio of 1:2 in the presence of IL-2. CFSE dilution was assessed by flow cytometry. (a) Representative histograms of CFSE dilution after 4 days of coculture with indicated target cells. (b) Antigen-specific expansion as T cell counts after 4 days, normalized to T cells counts without target cells. Data pooled from different experiments using 3 individual donors, performed in duplicates. Error bars show SD. Statistics depict Fisher's LSD test, comparing 1E9 eTCR T cell or Ofa-28z CAR T cell counts when encountering CD20-negative ALL GD and CD20-high ALL CM target cells.

transduced and purified for transgene expression using MACS enrichment for dNGFR or murine TCR (mTCR), respectively (suppl. Figure S1A). T cells transduced with the CD19-targeting FMC63-28z CAR and Mock (dNGFR only)-transduced T cells were included as controls. First, we assessed the specificity of CD20-directed constructs against the myeloid cell line K562 retrovirally transduced to express HLA-A*02:01 and CD20 (Figure 1b). We found antigen-specific cytokine secretion of 1E9 TCR T cells and Ofa-28z and RTX-28z CAR T cells. For subsequent experiments, we used Ofa-28z CAR T cells as they appeared superior to RTX-28z CAR T cells in terms of antigen-specific cytokine secretion (Figure 1b). Next, we compared short-term effector functions of 1E9 TCR T cells to Ofa-28z CAR T cells when targeting a panel of long-term expanded primary ALL cells¹³ that naturally differ in their CD20 expression (ALL GD CD20-negative, ALL RL CD20-low, ALL BV CD20-intermediate, and ALL CM CD20-high)¹⁴ but are positive for CD19 as evidenced by flow cytometry and gene expression data (suppl. Fig. S1B and S1C). All ALL cells stained positive for HLA-ABC and HLA-A*02 (suppl. Fig. S1B). We observed that Ofa-28z CAR T cells secrete significantly more IFN γ than 1E9 TCR T cells when encountering any of the CD20-

positive ALL cells at an E:T ratio of 1:9 (Figure 1c). All ALL cells were recognized by CD19-targeting FMC63-28z CAR T cells. IFN γ secretion by 1E9 TCR T cells was highest when encountering CD20-high ALL-CM, while for Ofa-28z CAR T cells, cytokine secretion was highest and comparable for both ALL BV and ALL CM (Figure 1d). In terms of cytotoxicity, we also observed that Ofa-28z CAR T cells very efficiently killed both high and low CD20-expressing ALL cells and that this was much more efficient than 1E9 TCR T cells in a 6 h ⁵¹Cr release assay (Figure 1e), indicating that for cytotoxicity of Ofa-28z CAR T cells, low levels of CD20 are sufficient. In contrast to CARs, introduced TCRs have to compete with the endogenous TCR for binding of surface molecules, particularly CD3. Furthermore, some TCRs are suboptimally expressed depending on their variable chain.²⁰ We therefore wondered whether knockout of endogenous *TRAC/BC* genes would result in enhanced 1E9 functionality, as this was demonstrated to be the case for some TCRs.¹⁷ *TRAC/BC* KO 1E9 TCR T cells indeed showed higher expression of mTCR and improved tetramer binding (suppl. Fig S2A and S2B), but functionality was not improved (suppl. Fig. S2C), indicating sufficient expression of the 1E9 TCR. Taken together, these

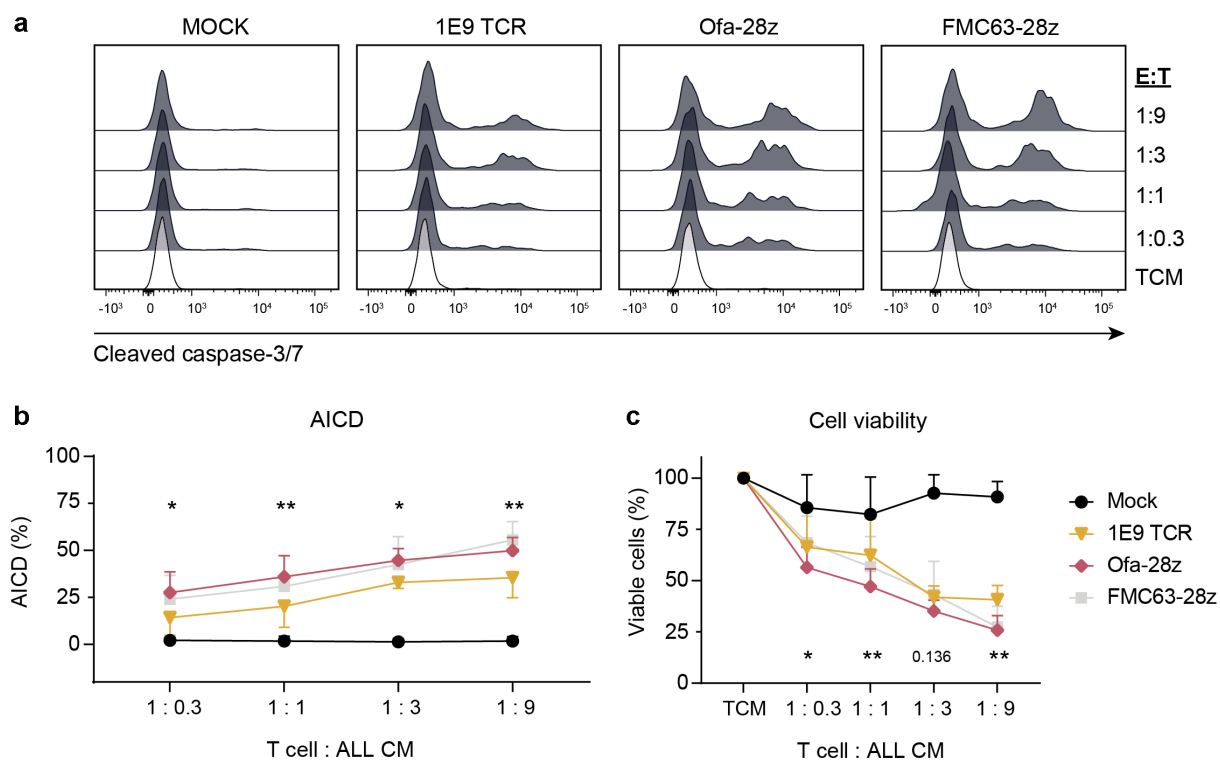


Figure 3. CAR T cells are more susceptible to activation-induced cell death than eTCR T cells. CAR T and TCR T cells were incubated with varying ratios of CD20-high ALL CM to assess activation-induced cell death (AICD). (a) Exemplary FACS plot after staining for the presence of cleaved caspase-3/7. (b) AICD levels after overnight culture as normalized frequencies of T cells staining positive for activated Caspase-3/7 and (c) number of viable T cells normalized to nonstimulating conditions after overnight coculture. Pooled data from four experiments using different donors, performed in duplicates. Statistics depict pairwise comparisons of 1E9 TCR and Ofa-28z T cells per E:T ratio using repeated measure ANOVA (matched for donors) and Fisher's LSD test. Error bars depict SD.

results show that CD20-targeting CAR T cells secreted more cytokines after overnight coculture and killed target cells more efficiently within a short time frame than 1E9 TCR T cells.

Both CAR T cells and eTCR T cells proliferate when encountering target cells, but Ofa-28z CAR T cell expansion correlates negatively with target antigen expression

Next, we explored how eTCR and CAR T cells proliferate upon antigen encounter. We assessed dye dilution of CFSE-labeled T cells after coculture for 4 days with irradiated target cells in the presence of IL-2 (Figure 2a). For 1E9 eTCR T cells, CFSE dilution correlated positively with CD20 surface expression of target cells, resulting in more dividing eTCR T cells when encountering target cells with higher expression of CD20 (ALL CM>BV>RL>GD = TCM). FMC63-28z CAR T cells proliferated strongly when encountering any of the CD19-positive target cells. Ofa-28z CAR T cells also proliferated strongly when encountering CD20-low and CD20-high positive target cells. Interestingly, however, when quantifying viable T cell counts at the end of the experiment, we found an inverse correlation of Ofa-28z CAR T cell counts with CD20 expression on target cells (Figure 2b;

representative experiment shown in suppl. Figure S3). Exposure to CD20-high ALL CM reproducibly resulted in significantly reduced numbers of viable Ofa-28z CAR T cells, a finding in discrepancy with strong proliferation as shown in Figure 2a. In contrast – and despite lower proliferation based on CFSE dilution – 1E9 TCR T cells expanded when encountering CD20-high ALL CM, resulting in significantly increased T cell counts as compared to without antigenic stimulation.

CAR T cells are more susceptible to activation-induced cell death than eTCR T cells

We found CD20 CAR T cell counts to be decreased when encountering target cells that have a high CD20 expression, despite strong proliferation based on CFSE dilution. We hypothesized that high antigenic exposure triggers activation-induced cell death (AICD) of CAR T cells. We therefore analyzed AICD in relation to various levels of antigen exposure. We cocultured eTCR and CAR T cells overnight with CD20-high ALL CM in E:T ratios ranging from 1:0.3 to 1:9. AICD was measured by staining for cleaved Caspase-3/7 as a marker of apoptotic cells (Figure 3a; gating strategy in supplemental Fig. S4A). Without antigenic stimulation, the frequency of apoptotic T cells was comparable in all T cell populations

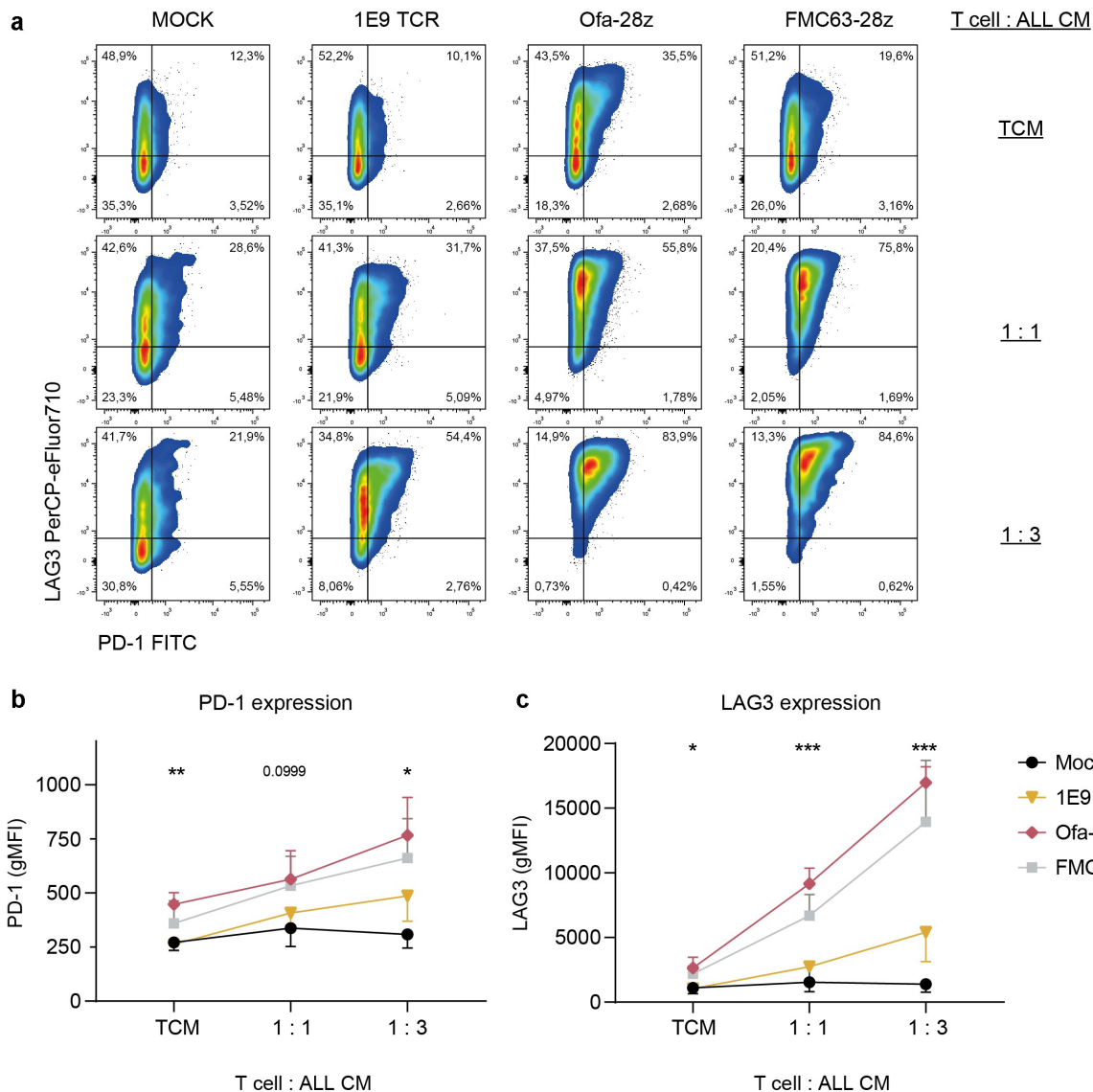


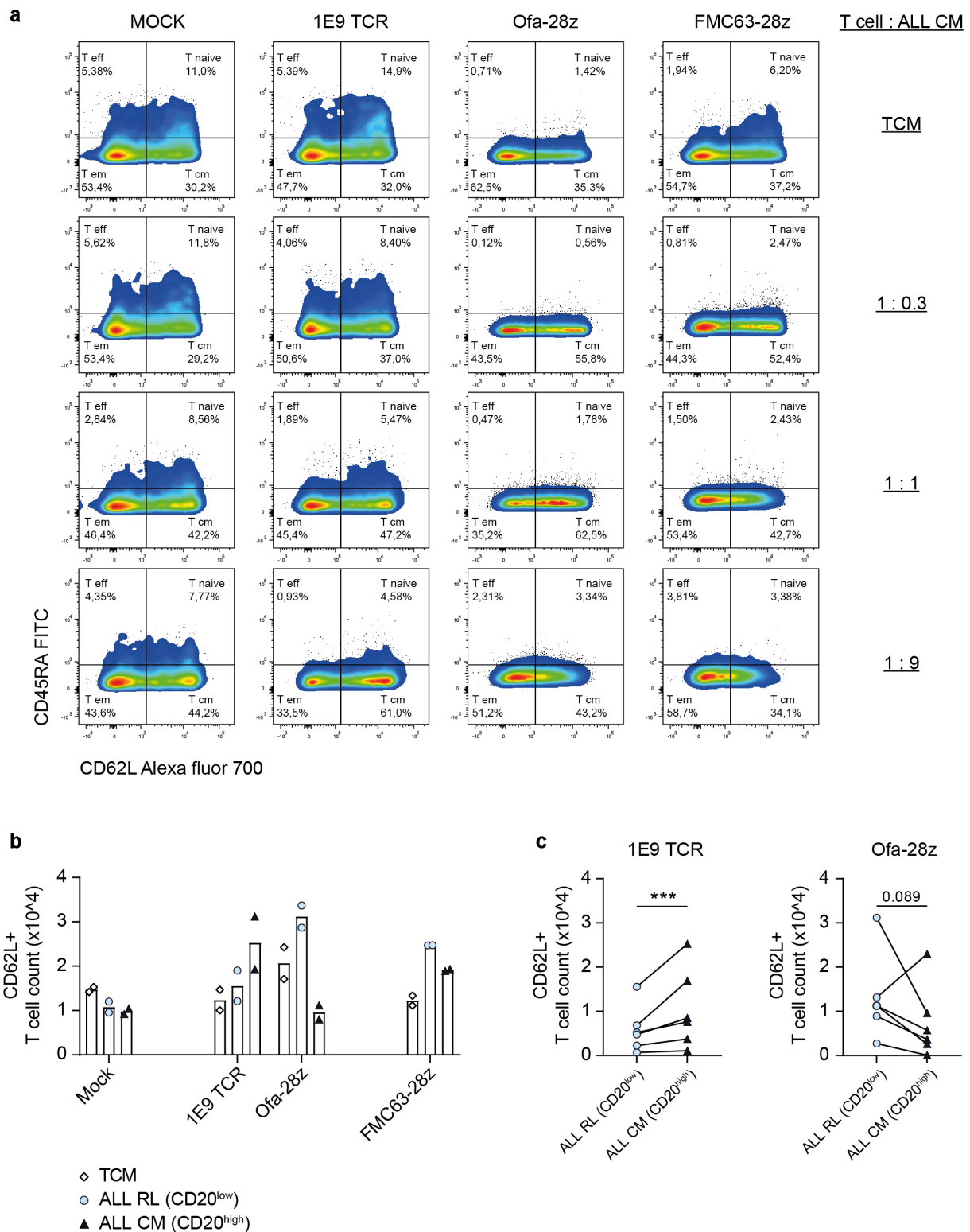
Figure 4. eTCR T cells express lower levels of coinhibitory molecules after activation than CAR T cells. T cells were incubated with ALL CM (CD20 high) at indicated E:T ratios for 72 h hours and assessed for expression of coinhibitory molecules PD-1 and LAG3. (a) Representative FACS plots of PD-1 and LAG3 expression 72 h after coculture. Gates were set based on FMO controls. (b/c) gMFI of PD1 (b) and LAG3 (c) on T cells 72 h after coculture. Pooled data from four experiments using different donors; pairwise comparisons were performed using repeated measures ANOVA (paired for donors) and Fisher's LSD test.

(supplemental Fig. S4B). After antigenic stimulation, we saw an increase of AICD in 1E9 TCR T cells, Ofa-28z CAR T cells, and FMC63-28z CAR T cells that correlated positively with target cell exposure, while AICD levels of Mock transduced T cells remained negligible (Figure 3a). Over all E:T ratios, AICD was significantly higher in Ofa-28z CAR T cells as compared to 1E9 TCR T cells (Figure 3b), resulting in fewer viable Ofa-28z CAR T cells than 1E9 TCR T cells after overnight coculture (Figure 3c). AICD of FMC63-28z CAR T cells followed a pattern that was comparable to Ofa-28z CAR T cells. In conclusion, Ofa-28z CAR T cells were more susceptible to AICD than 1E9 TCR T cells. Nevertheless, given the fact that AICD levels were still considerably high in eTCR

T cells, differences in AICD alone are unlikely to fully explain the differences observed in total T cell expansion after exposure to ALL CM as described in Figure 2.

CAR T cells express higher levels of coinhibitory molecules PD-1 and LAG3 after activation compared to eTCR T cells

It is conceivable that CAR T cells at first proliferate strongly, but that high exposure to antigen induces phenotypical changes that eventually compromise their potential to sustain their proliferative capacity and/or survival. Next to AICD, these phenotypical changes could include the upregulation of coinhibitory molecules such as PD1 or LAG3²¹ or changes in T cell differentiation subsets.^{22,23}



To assess the expression of coinhibitory molecules on eTCR and CAR T cells after activation, we cocultured TCR and CAR T cells with ALL CM at E:T ratios of 1:1

or 1:3 for 72 h and assessed the expression of PD-1 and LAG3 by flow cytometry (Figure 4a). Interestingly, CAR T cells already displayed elevated levels of PD-1 and LAG3

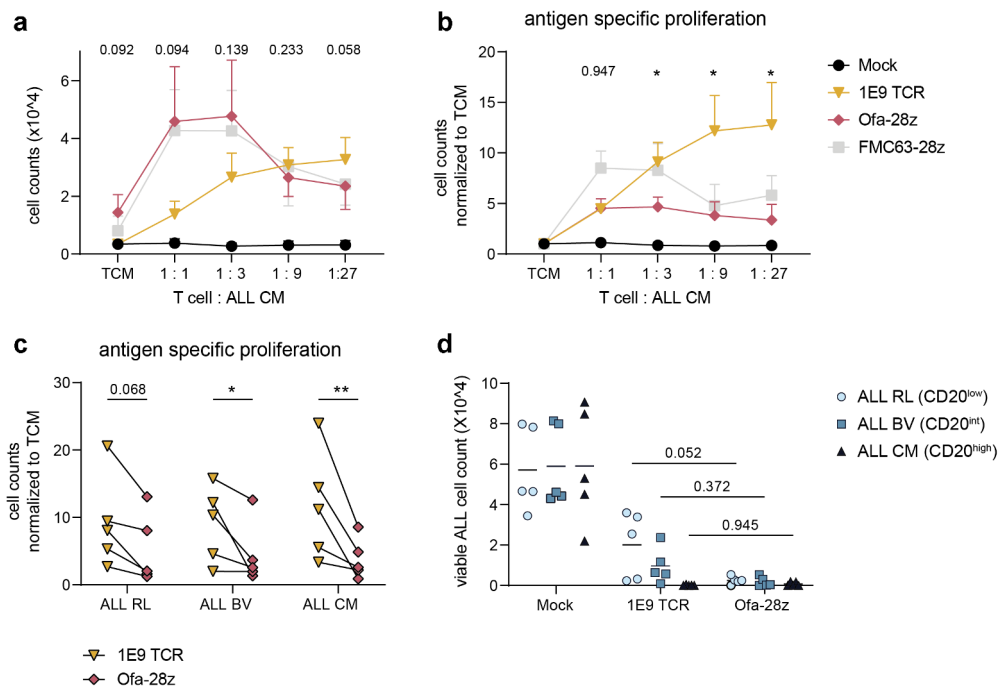


Figure 6. eTCR T cells outperform CAR T cells under high antigenic pressure. T cells were incubated with nonirradiated target cells at different E:T ratios for 7 days in the presence of 100IU/ml IL-2. (a) T cell counts after 7 days of coculture. Summary data of 5 different experiments using different donors. (b) Antigen-specific proliferation (normalized cell count to TCM) derived from (a). (c) Antigen-specific proliferation of 1E9 TCR T cells and Ofa-28z CAR T cells after encounter of nonirradiated ALL RL, ALL BV, or ALL CM. (d) Remaining viable ALL cells after 7 days of coculture for the same experiments shown in (c). Error bars in (a) and (b) depict SEM. Statistics in (a)-(d) compare 1E9 TCR and Ofa-28z using repeated measures ANOVA matched for donors and Fisher's LSD test.

expression in the absence of antigenic stimulation (Figures 4b and 4c). After antigen exposure, both 1E9 TCR T cells and Ofa-28z CAR T cells upregulate PD-1 and LAG3 (Figure 4a). Notably, we observed differences in the extent of upregulation. Regarding PD-1, Ofa-28z CAR T cells express significantly higher levels of PD-1 at an E:T ratio of 1:3 as compared to 1E9 TCR T cells (Figure 4b). This is even more prominent concerning the expression of LAG3: while most (>90%) eTCR T cells as well as CAR T cells stained positive for LAG3 after exposure to an E:T ratio of 1:3 (Figure 3a), the expression level of LAG3 as indicated by gMFI was around threefold higher on Ofa-28z CAR T cells as compared to 1E9 TCR T cells (Figure 4c). FMC63-28z CAR T cells followed a pattern comparable to that of Ofa-28z CAR T cells. Taken together, the expression of PD-1 and LAG3 increases proportionally with antigen exposure on both eTCR T cells and CAR T cells, but to a greater extent on CAR T cells as compared to eTCR T cells.

eTCR T cells, but not CAR T cells, maintain an early differentiation phenotype in settings of increased target cell exposure

Next, we assessed whether antigen exposure changes the differentiation phenotype of CAR and eTCR T cells. We measured expression of CD62L and CD45RA on CAR T and eTCR T cells 72 h after exposure to antigen by flow cytometry (Figure 5a).

Without any stimulation, eTCR T cells showed a differentiation pattern that is comparable to Mock T cells. In contrast, CAR T cells were further differentiated with substantially reduced frequencies of CD45RA+CD62L+ naïve-like T cells. This was most prominent in Ofa-28z CAR T cells with less than 2% CD45RA+CD62L+ T cells compared to >10% in eTCR T and Mock T cells and >6% in FMC63-28z CAR T cells. Upon antigen exposure, eTCR T cells preferentially differentiated from naïve-like T cells toward CD45RA-CD62L+ central memory-like T cells, with the highest relative frequency of central memory-like T cells present in the condition of highest antigen exposure (E:T = 1:3). For CAR T cells, we also saw a preferential relative increase of a CD45RA-CD62L+ central memory-like population in the setting of low antigen exposure (E:T = 1:0.3 and 1:1). However, at an E:T ratio of 1:3, we observed a relative decrease of the frequency of central memory-like T cells in favor of a CD45RA-CD62L- effector memory-like T cell phenotype of Ofa-28z CAR T cells. These results indicate that low antigen exposure supports a central memory phenotype of CAR T cells, while higher antigenic exposure drives effector differentiation. This is contrary to eTCR T cells, where an increase of target cell exposure resulted in preferential differentiation toward a central memory phenotype.

To obtain a better understanding of the expansion of central memory T cells and to compensate for differences in the differentiation phenotype observed without antigenic stimulation, we proceeded to sort eTCR and CAR T cells on expression of CD62L. CD62L+ T cells were then incubated with target

cells ALL RL (CD20 low) and ALL CM (CD20 high) at an E:T ratio of 1:3 for 72 h and subsequently quantified using flow cytometry. For Mock T cells, counts of CD62L⁺ T cells remained largely unchanged, irrespective of target cell exposure. For 1E9 TCR T cells, the number of CD62L⁺ cells increased, correlating with the antigen positivity of target cells (Figure 5b). Ofa-28z CAR T cells were also able to increase the number CD62L⁺ cells when encountering CD20 low ALL RL. Upon encounter of CD20 high ALL CM, however, the count of CD62L⁺ T cells actually decreased as compared to without antigenic stimulation. Exposure to ALL CM resulted in fewer CD62L⁺ Ofa-28z CAR T cells as compared to ALL RL in 5 out of 6 donors tested, while for 1E9 TCR T cells, counts of CD62L⁺ T cells were consistently higher after exposure to ALL CM compared to ALL RL (Figure 5c). FMC63-28z CAR T cells, on the other hand, were able to increase the number of CD62L⁺ cells after exposure not only to ALL RL but also to ALL CM (Figure 5b and data not shown). These observations, based on relative as well as absolute frequencies of CD62L⁺ T cells, suggest that Ofa-28z CAR T cells failed to maintain a central memory pool upon exposure to high amounts of antigen, while 1E9 TCR T cells actively expanded central memory-like T cells.

Incorporation of a 4-1BB costimulatory domain does not rescue the sensitivity of CD20-targeting CAR T cells to high antigenic stimuli

In our previous experiments, we made use of CAR T cells incorporating a CD28 costimulatory domain. Several reports have suggested that CARs incorporating a 4-1BB costimulatory domain instead can be circumstantially superior to CD28-based CAR T cells due to lower expression of coinhibitory molecules, retention of an early differentiation phenotype, and resistance to apoptosis and that this is partially attributable to lower CAR tonic signaling.^{24–27} CAR tonic signaling is defined as constitutive activation of CAR T cells in the absence of stimulatory antigen and is increasingly perceived as one of the major complicators of effective CAR design.²⁸ Therefore, we also designed two additional CD20 targeting CAR constructs incorporating a 4-1BB costimulatory domain (supplemental Fig. S5A) and assessed whether these CD20-targeting BBz CAR T cells would perform differently under varying degrees of antigen exposure. First, we assessed the tonic signaling phenotype by staining for activation markers in the absence of antigenic stimulation. All CAR T cell populations showed elevated expression of activation markers CD25 and CD54, as well as of coinhibitory molecules PD-1 and LAG3 one week after transduction as compared to Mock T cells, with CD20-targeting CAR T cells showing a trend of higher expression of activation or coinhibitory markers as compared to CD19-targeting FMC63-28z CAR T cells (supp. Fig. S5B). After antigen exposure, BBz-based CD20-targeting CAR T cells yielded lower T cell counts after exposure to CD20-high ALL CM (supp. fig. S5C), upregulated coinhibitory molecules strongly (supp. Fig. S5D), and differentiated toward an effector memory phenotype (supplemental Fig. S5E) after exposure to increasing amounts of stimulator cells. Taken together, CD20-targeting

BBz-based CAR T cells did not show an ameliorated tonic signaling phenotype and responded comparably to increased antigen exposure as did Ofa-28z CAR T cells.

eTCR T cells outperform CAR T cells in proliferation stress tests

Our previous results showed that eTCR T cells and CAR T cells responded differently to different levels of antigen exposure. We therefore wanted to challenge eTCR T cell and CAR T cell performance in terms of proliferation and tumor population control after exposure to a wider array of antigen levels over a longer period of time. We performed an antigen stress test where we cocultured transduced T cells for a culture period of seven days with nonirradiated target cells using a E:T ratio range between 1:1 and 1:27. CAR T cells expanded strongly in the setting of low antigen exposure in E:T ratios of 1:1 and 1:3 over the course of seven days, yielding higher numbers of viable CAR T cells compared to 1E9 eTCR T cells (Figure 6a). However, increasing target cell exposure had an advert effect, resulting in substantially reduced CAR T cell counts at E:T ratios of 1:9 and 1:27. In contrast, eTCR T cells responded better to higher antigen loads, expanding stronger with increased target cell exposure and reproducibly yielding the highest number of viable T cells at an E:T ratio of 1:27 (Figure 6a). We also observed CAR T cell expansion in the absence of antigenic stimulation (TCM condition), a hallmark of CAR tonic signaling. Antigen-specific proliferation was significantly higher in 1E9 TCR T cells than in Ofa-28z at E:T ratios of 1:3, 1:9, and 1:27 (Figure 6b). Finally, we compared eTCR T and CAR T cell performance when targeting ALL RL (CD20 low), ALL BV (CD20 intermediate), or ALL CM (CD20 high) as target cells at an E:T ratio of 1:27. Antigen-specific proliferation was higher for 1E9 TCR T cells compared to Ofa-28z CAR T cells when encountering ALL CM as well as ALL BV, and a similar trend could be observed when targeting ALL RL (Figure 6c). With respect to tumor clearance, Ofa-28z CAR T cells seemed to clear ALL RL and ALL BV more effectively than 1E9 TCR T cells, although residual viable tumor cells were still detected. Clearance of ALL CM was near-complete and comparable between eTCR and CAR T cells. Taken together, CAR T cells appeared to outperform eTCR T cells in settings of low antigen exposure, whereas eTCR T cells performed better than CAR T cells at high antigenic pressure.

Discussion

In the present study, we provide a functional comparison of CAR and eTCR T cells in targeting CD20-expressing malignancies. We found that the efficacy of eTCR T cells and CAR T cells is a function of antigen exposure. Over the course of several days, CAR T cells appeared to outperform eTCR T cells in settings of low antigen exposure, while under high antigenic pressure, eTCR T cells performed better than CAR T cells. Our data suggest that CAR T cells receive a strong signal even by low amounts of target antigen, resulting in rapid tumor cell clearance and subsequently vigorous proliferation. However,

continuous presence or initial abundance of antigen, as is the case when encountering an excess of target cells, forces CAR T cell differentiation coinciding with a progressive loss of central memory like T cells. Together with elevated expression of PD1 and LAG3, CAR T cells are then unable to counterbalance higher levels of AICD, lastly resulting in reduced numbers of CAR T cells. eTCR T cells, on the other hand, retain their ability to maintain a central memory pool and only limitedly upregulate coinhibitory molecules, thereby retaining their potential to proliferate productively and to counterbalance AICD, even under high antigenic pressure.

While a number of studies have aimed to compare TCR and CAR T cells, the present study is the first to perform a functional comparison at different levels of antigen exposure from a translational perspective, i.e. assessing functionality versus tumor cells whose targetability is defined by the expression of an antigen that is targetable by both treatment modalities. Previous studies have outlined differences using different model systems and assessed functionality mostly at the lower signaling threshold or within a short time frame. Harris et al. used CAR constructs recognizing HLA peptides in a similar affinity range than a corresponding peptide MHC-specific TCR.²⁹ They found that, in murine T cells, eTCR T cells were much more sensitive than CARs, recognizing 10–100 fold less antigen than CAR T cells. This was also confirmed by another recent study that compared human CAR T cells and virus-specific T cells on a supported lipid bilayer system.³⁰ Using a ROR1-specific CAR and polyclonal CMV-specific T cells, the authors also found that CMV-specific T cells were more sensitive than CAR T cells for their cognate antigen. In the present study, we did not specifically examine the lower signaling threshold. We found relatively low reactivity of eTCR T cells against CD20 low ALL RL, while CAR effector functions were high despite low expression of CD20. It is likely that – in terms of absolute numbers – the amount of stimulatory antigen is magnitudes higher for full length surface CD20 as compared to CD20-derived peptide-HLA, therefore permitting more effective CAR T cell activation than TCR T cell activation. Since we did not quantify absolute numbers of stimulatory antigens, we cannot draw conclusions from our data on CAR T cell or eTCR T cell sensitivity on a molecular level.

Next to that, a study by Davenport et al. suggested that CAR T cells are effective serial killers, killing more target cells in a shorter time frame than eTCR T cells.³¹ However, they only assessed a short time window of less than 1 h. This is congruent with our finding of more effective killing in a 6 h ⁵¹Cr release assay of CAR T cells. On the other hand, in our long-term coculture assays using nonirradiated target cells, eTCR T cell performance in terms of target cell killing was actually comparable to that of CAR T cells, arguing that the total number of target cells being killed might actually be higher per eTCR T cell than per CAR T cell, despite slower kinetics.

Previous reports have suggested that CARs incorporating a 4–1BB costimulatory domain have an ameliorated tonic signaling phenotype, express lower levels of coinhibitory molecules, and are more likely to maintain an early differentiation

phenotype as compared to CD28-based CARs.^{24–27} We therefore considered the possibility that the observed overactivation-sensitive CAR T cell phenotype was attributable to the use of a CD28 costimulatory domain. However, CD20 directed CARs incorporating 4–1BB still showed a pronounced tonic signaling phenotype. Furthermore, Ofa-BBz and RTX-BBz CAR T cells did perform comparably to Ofa-28z CAR T cells under increased antigenic pressure in terms of sustained proliferation, upregulation of coinhibitory molecules, and differentiation. Worthy of note is the fact that we used a retroviral expression system: a previous report has demonstrated that the expression of BBz-based CARs under control of an LTR promoter can result a distinct tonic signaling phenotype, resulting in CAR T cell dysfunction.³² Hence, it might be worth exploring the performance of RTX-BBz and Ofa-BBz CARs under high antigenic pressure using a different, e.g., lentiviral expression system.

While earlier research has tried to optimize CAR T cell function by including more costimulatory or cytokine signaling domains, a number of recent preclinical studies have suggested that “normalizing” rather than augmenting second-generation CAR T cell signaling can actually potentiate CAR T cell function *in vivo*.^{33–36} This is in line with our findings that second-generation CAR T cells are susceptible to overactivation and that further improvements of CAR designs are needed. eTCR T cells, on the other hand, make use of the endogenous physiological signaling machinery, thereby potentially self-limiting input signal and conclusively coping better with strong antigenic stimulation.

To view our data from a translational point of view, it is interesting to outline aspects of the clinical experience with CAR T cell therapies. One long-term study suggested that, in ALL, a durable complete remission after CD19-targeted CAR T cells correlates with a favorable ‘*in vivo* E:T ratio’ of CAR T cells and tumor cells, i.e. the ratio of peak CAR T cell expansion to tumor burden.⁶ While eTCR T cells are yet to be tested clinically in the treatment of most hematological malignancies, our data suggest that eTCR T cells might remain functional even in unfavorable E:T ratios. Furthermore, eTCR T cells secreted lower amounts of cytokines as compared to CAR T cells. Although it is unclear how this would translate to clinical efficacy, a lower or delayed cytokine secretion might translate to milder treatment-related toxicity of eTCR T cells as compared to CAR T cells. This consideration is at least partially supported by the findings of a recent study that tested the performance of a CD19-directed CAR T cell product incorporating a different, fully human scFv in treating B cell lymphoma.³⁷ The authors reported that, while their product secreted lower levels of cytokines such as IFN γ *in vitro*, observed toxicities were significantly less frequent and response rates were comparable to those achieved with FMC63-28z CAR T cells.

Taken together, this report outlines that the effectivity of CAR and eTCR T cells may be highly contextual. Our data suggest that eTCR T cells, while secreting lower levels of cytokines and initially killing less efficiently than compared to CAR T cells, cope better with high antigen loads. Given these

functional differences, TCR-engineered T cells targeting surface molecules may add to the portfolio of cellular therapy of B cell malignancies, encouraging further advancement in vitro and in vivo and ultimately clinical testing of TCR-transduced T cells.

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Author contributions

T LAW designed, performed, analyzed, and interpreted experiments and wrote the manuscript. AKW, DFGR, and RSH provided technical assistance and performed in vitro experiments. MHM provided assistance for data visualization and critically revised the manuscript. EvD, JL, and JK generated original CD20-targeting CAR constructs and performed preliminary experiments. JHFF critically revised the manuscript and supervised the study. MHMH conceptualized and supervised the study, critically revised the manuscript, and provided funding.







Disclosure statement

The authors report no conflict of interest.

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