PLOS ONE

RESEARCH ARTICLE

Promoter choice: Who should drive the CAR in T cells?

**Ali Hosseini Rad S. M.*, Aarati Poudel☯, Grace Min Yi Tan☯, Alexander D. McLellan*

Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand

☯ These authors contributed equally to this work.
* a.hosseini.rad@postgrad.otago.ac.nz (RSMAH); alex.mclellan@otago.ac.nz (ADM)

OPEN ACCESS

Citation: Rad S. M. AH, Poudel A, Tan GMY, McLellan AD (2020) Promoter choice: Who should drive the CAR in T cells? PLoS ONE 15(7): e0232915. https://doi.org/10.1371/journal.pone.0232915

Editor: Joe W. Ramos, University of Hawai'i at Manoa, UNITED STATES

Received: April 22, 2020

Accepted: July 7, 2020

Published: July 24, 2020

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was funded by The Cancer Society New Zealand (19.11) and The University of Otago Research Grant Committee (2019). ADM and AR received salary support from the Royal Society Marsden fund 18-UOO-188. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Chimeric antigen receptor (CAR) T cell therapy is an effective treatment for B cell malignancies, with emerging potential for the treatment of other hematologic cancers and solid tumors. The strength of the promoter within the CAR cassette will alter CAR-polypeptide levels on the cell surface of the T cell–impacting on the kinetics of activation, survival and memory cell formation in T cells. In addition to the CAR, promoters can be used to drive other genes of interest to enhance CAR T cell function. Expressing multiple genes from a single RNA transcript can be effectively achieved by linking the genes via a ribosomal skip site. However, promoters may differ in their ability to transcribe longer RNAs, or could interfere with lentiviral production, or transduction frequencies. In this study we compared the ability of the strong well-characterized promoters CMV, EF-1, hPGK and RPBSA to drive functional expression of a single RNA encoding three products: GFP, CAR, plus an additional cell-survival gene, Mcl-1. Although the four promoters produced similarly high lentiviral titres, EF-1 gave the best transduction efficacy of primary T cells. Major differences were found in the ability of the promoters to drive expression of long RNA encoding GFP, CAR and Mcl-1, highlighting promoter choice as an important consideration for gene therapy applications requiring the expression of long and complex mRNA.

Introduction

Promoters are of critical importance for expressing optimal levels of the transgene in CAR T cells for the production of functional proteins or non-coding RNA [1–5]. It is also clear that high expression of the CAR can result in antigen-independent CAR signaling, resulting in T cell exhaustion and sub-optimal anti-tumor responses, or lead to the inappropriate recognition of tumor antigen on self-tissue [1, 2]. In addition, controlling CAR T cell signaling is critical for proper memory cell formation [6]. Because surface expression of the CAR may be limited by mRNA levels, the choice of promoter is critical [1, 2].

There have been limited studies that directly compare the efficiency of different promoters for driving long mRNA comprising multiple genes within CAR T cells [1, 2, 7]. Recent studies investigating promoter performance in mouse or human T cells were usually limited to either the CAR, a single gene of interest alone, or single fluorescent reporter genes of limited size [1,

Competing interests: Authors have no competing interests

2, 7–9]. For the generation of lentiviral particles for transduction, using multiple internal promoters or internal ribosome entry sites (IRES) for multiple genes may interfere with transcription or reverse transcription of viral genomic RNA (vgRNA), impacting upon lentiviral particle titre, and/or on the efficiency of integration into the target cell [8, 10]. Therefore, strategies that employ single promoters to drive multiple genes may be preferred for CAR T cell engineering [9].

Although all current, clinically-approved second and third generation CAR T cells rely on the expression of a single gene encoding a single polypeptide, it may be advantageous to express longer RNA containing the CAR, together with one or more genes of interest. For example, endogenous growth factors or membrane bound or secreted cytokines could improve T cell expansion and survival [6, 11]. Alternatively, markers of transduction efficiency or death switches could be incorporated into the CAR element [4, 12–14]. Promoter choice for such applications is crucial to obtain optimised gene expression of multiple, linked genes.

Because requirements for driving short versus long RNA might be distinct in CAR T cell genetic elements, we investigated the ability of several promoters to drive an extended downstream genetic sequence comprised of GFP, anti-Her2-CAR and an additional cell survival gene Myeloid leukemia cell differentiation protein (Mcl-1), an anti-apoptotic Bcl2 family member. Mcl-1 aids in T cell development, mitochondrial function and lifespan and appears to a suitable candidate for enhancing CAR T cell performance [15, 16]. Mcl-1 inhibits the action of pro-apoptotic BIM / BAK / BAX at the mitochondrial membrane and is expressed throughout T cell differentiation and is essential for memory T cell formation [16–20].

The individual elements were tested at protein level and for functional activity. The results demonstrated clear differences in the ability of these internal promoters to drive expression of multiple CAR-cassette associated transgenes.

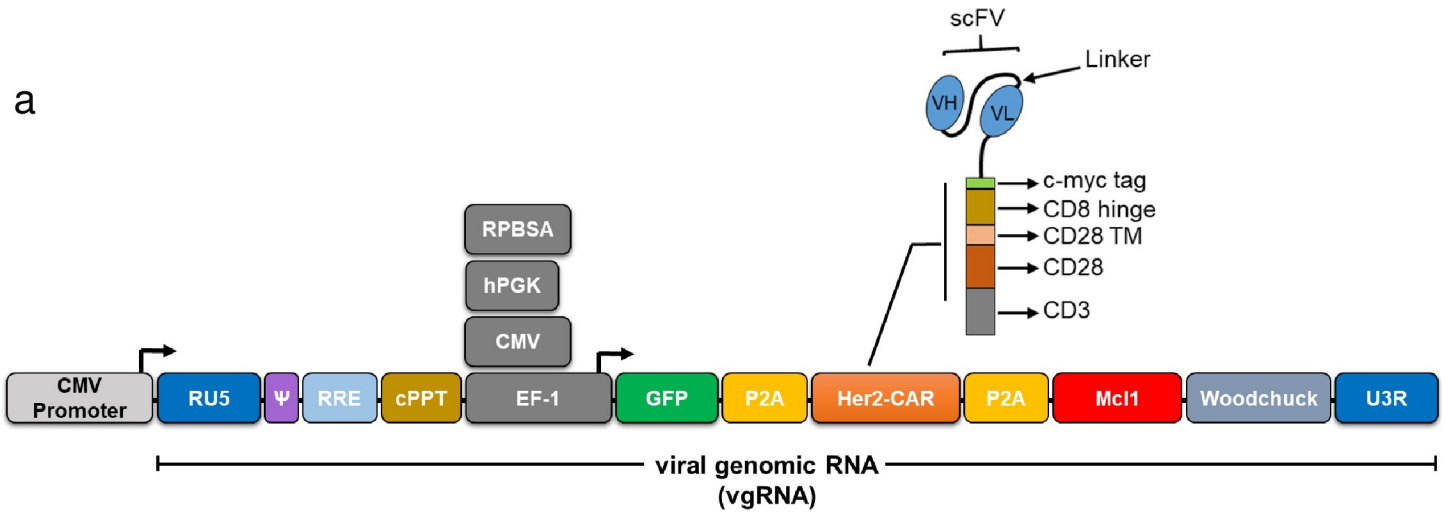
Material and methods

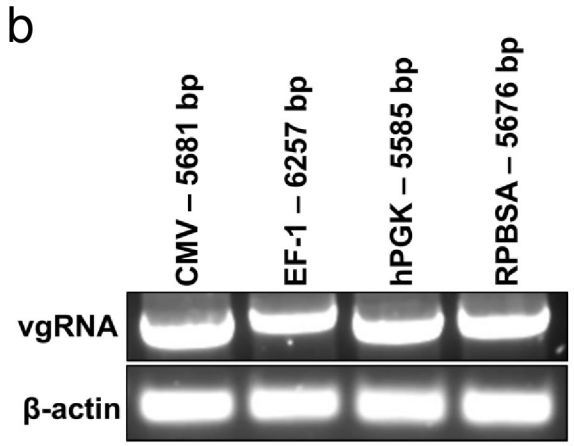
Plasmid construction

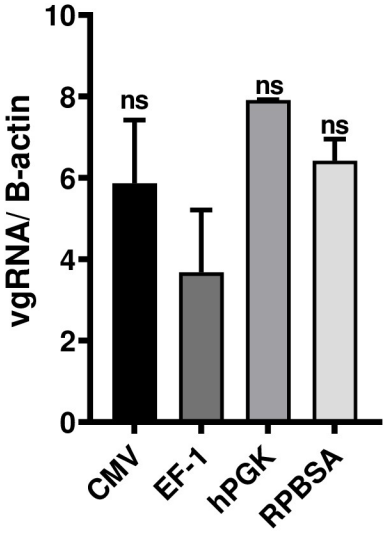
The third-generation lentiviral vector pCCLsin.cPPT.hPGK.GFP.WPRE (pCCLsin) and VSV-G-based packaging plasmids were a kind gift from Prof. Dr. Naldini and have been described elsewhere [21]. The anti-Her-2 CAR FRP5, anti-CD19 CAR FMC63 (with–EQKLISEEDL–c-myc tag between scFv and CD8 hinge) and codon-optimized human Mcl-1 (cop-Mcl-1) were synthesized as gene blocks (IDT Technologies). Both CAR constructs are second generation CAR with CD28 costimulatory domains (Fig 1A). Sap I Type IIs restriction enzyme cloning was utilized for scarless assembly of the eGFP-P2A-CAR-P2A-Mcl-1. This cassette was then cloned into the BamHI and SalI sites of the pCCLsin (Fig 1A). Promoters were amplified with 5' EcoRV and 3' BamHI sites from respective plasmids: CMV from pcDNA3.1(-), EF-1 from Sleeping Beauty (pSBbiRP) and RPBSA from Sleeping Beauty (pSBtet-GP) and ligated upstream of the GFP-CAR-mcl1 cassette. Codon optimized Leucine Zipper CD95 (LZ-CD95L) gene was synthesized by IDT with EcoRI and BamHI sites and cloned into pcDNA3.1(-) (Addgene #104349).

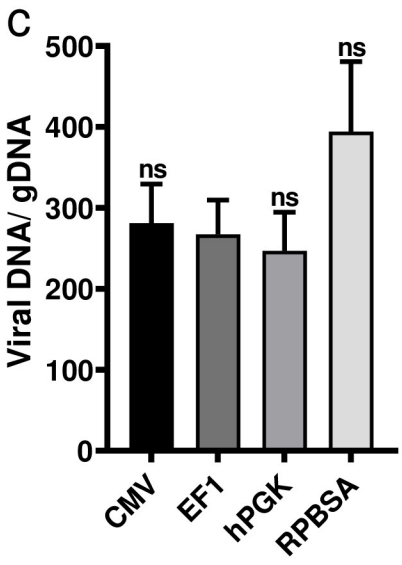
Cell culture

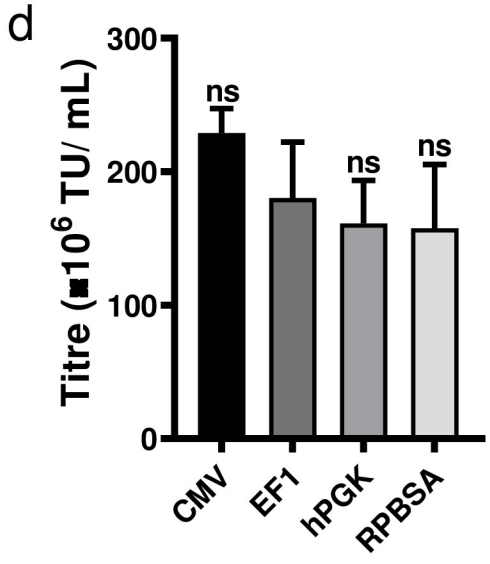
Cell lines were cultured in a humidified atmosphere at 37˚C, 5% CO_2 (or with 8% CO_2 for LV-Max and Expi293F). Human embryonic kidney 293T (ATCC CRL-1573) and MCF-7 (ATCC HTB-22) cell lines were cultured in high glucose Dulbecco's Modified Essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Pan-Biotech GmbH), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Gibco). MCF-7 and HEK293T cells were transfected using Lipofectamine 3000 according to manufacturer's protocol. Human


a
scFV
Linker
VH
VL
c-myc tag
CD8 hinge
CD28 TM
CD28
CD3
RPBSA
hPGK
CMV
CMV Promoter
RU5
Ψ
RRE
cPPT
EF-1
GFP
P2A
Her2-CAR
P2A
Mcl1
Woodchuck
U3R
viral genomic RNA
(vgRNA)


b
CMV – 5681 bp
EF-1 – 6257 bp
hPGK – 5585 bp
RPBSA – 5676 bp
vgRNA
β-actin


vgRNA/ B-actin
10
8
6
4
2
0
ns
ns
ns
CMV
EF-1
hPGK
RPBSA


c
Viral DNA/ gDNA
500
400
300
200
100
0
ns
ns
ns
CMV
EF1
hPGK
RPBSA


d
Titre (■10^6 TU/ mL)
300
200
100
0
ns
ns
ns
CMV
EF1
hPGK
RPBSA

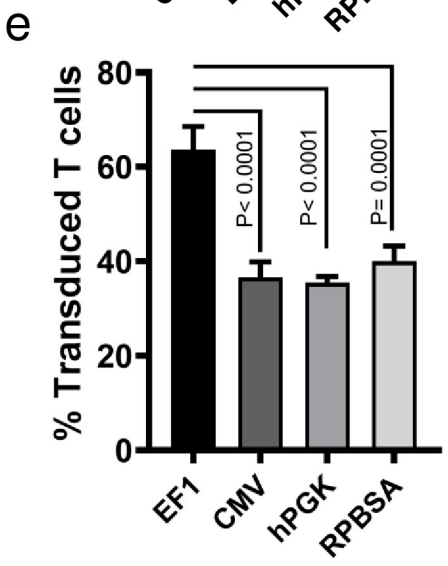

e
% Transduced T cells
80
60
40
20
0
P< 0.0001
P< 0.0001
P= 0.0001
EF1
CMV
hPGK
RPBSA

Fig 1. The effect of internal promoters in producing functional lentiviral particles. (a) Schematic illustration of the pCCLsin backbone bearing four different internal promoters (CMV, EF-1, hPGK and RPBSA) for driving a long RNA consist of GFP-P2A-Her2CAR-P2A-Mcl-1, (b) HEK293T cells were transfected with lentiviral constructs containing different promoters along with packaging plasmids. At 24 h post transfection, total RNA was extracted and 1 μg of RNA was converted to cDNA. PCR was carried out using specific primers binding to PPT and woodchuck region. Agarose gel electrophoresis displays the PCR product band of each construct. Lower band displays the PCR product of β-actin serving as a loading control. The ratio between viral genomic RNA (vgRNA) to β-actin was quantified and presented in the bar graph (right) using Image Studio Lite. There was no statistically significant difference between promoters ($P>0.05$). (c) Shows the ratio between integrated viral cassettes to gDNA 48 h post-transduction. Genomic DNA was extracted from cell lysates and qPCR was performed using Gag for integrated lentivirus and β2 microglobulin and β-actin as a housekeeping genes for host gDNA quantification. There was no statistically significant difference between promoters ($P>0.05$). (d) Comparison of the viral particle titration of four different constructs through analysis of the percent GFP expression in HEK293T cells using flow cytometry. Bar graph values represent the titre unit/mL (TU/mL) from three independent repeats. There was no statistically significant difference between promoters ($P>0.05$). (e) Transduction efficiency of primary T cells for the four lentivectors. CD3 / CD28 stimulated human primary T cells were transduced at MOI 40 and cells were analyzed for GFP expression at 72 h post-transduction by flow cytometry. A representative experiment, and all GFP MFI values relating to graph, are presented in Fig 3A. Dead cells were excluded with Zombie NIR viability dye gating at analysis. Bar graph values represent the mean values ± SD from three independent repeats. scFv; single-chain variable fragment, VH; variable heavy chain, VL; variable light chain, TM; transmembrane domain.

https://doi.org/10.1371/journal.pone.0232915.g001

peripheral blood mononuclear cells (PBMC) were isolated from healthy donors. The University of Otago Human Ethics Committee (Health; Ethics Approval# H18/089) approved this study and written consent was obtained from blood donors. Frozen PBMCs were thawed and then rested overnight in T cell expansion media (Thermofisher #A1048501) supplemented with 50 U/mL of hIL-2 (Peprotech, #200–02), L-glutamine and 10 U/mL penicillin and streptomycin (Gibco), prior to CD4 and CD8 T cells isolation using EasySep Human T cell isolation kit (STEMCELL Technology, #17951). Isolated T cells were activated with Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher, #111.32D).

Lentiviral production, titration and T cell transduction

Lentiviral production and titration were carried out using LV-Max Viral production system (ThermoFisher #A35684) according to manufacturer's protocol. HEK293T cells were transduced at MOI 2:1 with 8 μg/mL of polybrene (Sigma-Aldrich). One day before T cell transduction, plates were coated with 40 μg/mL retronectin (TAKARA, #T100A/B) overnight at 4˚C, blocked with 2% FBS/PBS for 15 min, before adding LV at 40:1 MOI to the plate; followed by centrifugation at 800 ×g for 2.5 h at room temperature. After 48 h of activation with a 1:1 ratio of CD3/CD28 Dynabeads, T cells were added to virus-coated wells and spinoculation carried out at 500 ×g for 5 min. The next day, T cells were debeaded and cultured in media plus 50 U/mL of hIL-2. Media was changed with fresh medium supplemented with 50 U/mL hIL-2 every three days.

RNA extraction, long cDNA synthesis and RT-PCR

Total cellular RNA (containing viral genomic RNA) was extracted 48 h after transfection using NucleoSpin RNA Plus kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. Then RNA was reverse transcribed using PrimeScript™ RT Reagent Kit (Takara Bio, USA) according to manufacturer's protocol RT-PCR was performed using internal primers PPT-Fwd: `GGGTACAGTGCAGGGGAAAG` and Woodchuck-Rev: `AAGCAGCGTATCCACATAGCG` for comparison with β-actin Fwd: `CTTCCTTCCTGGGCATG` and β-actin-Rev: `GTCTTTGCGGA TGTCCAC`.

Quantification of gDNA/ integrated viral DNA ratio

At 48 h post transduction, integrated lentiviral DNA was quantified by extracting genomic DNA using Qiamp DNA Mini kit (Qiagen, Germany) and the ratio of viral genome: human gDNA were estimated using qPCR via Luna Universal qPCR Master Mix (New England Biolabs) using designed primers Gag-Fwd: `GGA GCT AGA ACG ATT CGC AGT TA`, Gag-Rev:

GGT TGT AGC TGT CCC AGT ATT TG TC, PBS-Fwd: TCT CGA CGC AGG ACT CG; PBS-Rev: TAC TGA CGC TCT CGC ACC, and β-actin forward and reverse primers described above. All reactions were run in triplicate and were presented as mean ± SD.

Western blot

Cell lysates were prepared using RIPA lysis buffer and blotting carried out using mouse monoclonal anti-EGFP antibody (Abcam, #ab184601), rabbit anti-human Mcl-1 (Abcam, #ab28147), biotin anti-c-myc (Biolegend #908805). Mouse monoclonal β-actin primary antibody (Sigma-Aldrich #A2228) was used as loading control. goat anti-mouse IgG DyLight 680 (Thermo Fisher #A3274), goat anti-rabbit IgG 800, streptavidin-800 in 1:10000 dilution as secondary antibody (#A32730 and # A32735). The membrane was scanned using an Odyssey Fc imaging system (Licor, Germany) and analyzed using Image Studio Lite software.

Mitochondrial membrane potential assay (TMRE)

Transduced T cells were incubated overnight with 1 μg/mL LZ-CD95L, then 4 μM TMRE (Invitrogen) was added at 37˚C for 30 min. DAPI (50 ng /mL) was added immediately prior to flow cytometric analysis and GFP positive cells electronically gated for quantification of TMRE and DAPI signals using the YG586/16 and BV421 channels.

Cytotoxicity and cytokine release assay

Luciferase-based cytotoxicity assay was carried out for Her2 and CD19 CAR T cells as previously described [22] at a 10:1 ratio of effector to target cells of using Firefly Luc One-Step Glow assay (Thermo Fisher #16197). For analysis of cytokine release, CAR T cells were added to target cells in a 2:1 ratio. IL-2 and IFN-γ concentration secreted in cell supernatant were measured using sandwich ELISA according to manufacturer's protocol (BD Biosciences, USA). Plates were read on a Varioskan Lux multimode microplate reader (Thermo Fisher, USA).

Flow cytometry

CAR T cells were stained with biotin anti-c-myc antibody (Biolegend #908805) detected with Streptavidin-Brilliant Violet 421 (Biolegend #405225). Antigen stimulated CAR T cells were stained for CD69 expression using APC-conjugated anti-human CD69 antibody (Biolegend #310910). Flow cytometric data was acquired using a BD LSRFortessa with BD FACSDiva software. Data was analysed with FlowJo v10.6.2 software. Cells were subject to FSc and SSc doublet discrimination and dead cells were excluded from analysis using Zombie NIR viability dye (Biolegend #423106).

Statistical analysis

All experiments were carried out at least three times, presented as mean ± standard deviation (SD) and analyzed by one-way ANOVA test with Bonferroni post-test correction. The P values of ≤ 0.05 were considered statistically significant. (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$)

Results

Compatibility of the promoter systems with a third-generation lentiviral system

The four promoters were chosen based on their widespread use in the literature and documented ability to drive high level expression of transgenes in either lentiviral vectors, or in

Sleeping Beauty transposon vectors [1, 8, 9, 21, 23]. Each of the four promoters were cloned upstream of the series of P2A-linked genes comprised of GFP, the FRP5 anti-Her2 CAR followed by human Mcl-1 (Fig 1A), a Bcl2 family member–the latter gene as a strategy to protect CAR T cells against activation-induced cell death (AICD). A first consideration for the choice of internal promoter driving transgenes within lentiviral systems is the effect on viral titration and transduction efficiency. Generally, there is a difference in the degree of transcriptional interference between the internal promoters and the promoter driving expression of genomic RNA, resulting in a lower number of full-length viral genomic RNAs (vgRNA) particularly when the CMV or EF-1 promoter is being used [10, 24]. In order to test the promoter interference, HEK293 cells were transfected with four constructs along with helper plasmids and the levels of vgRNA for four promoters were measured ($P>0.05$, Fig 1B). Similar levels of full-length transcripts were obtained using all constructs, as assessed by RT-PCR carried out with primers binding to cPPT and woodchuck regions (Fig 1B). Next, the effect of internal promoter interference with provirus production was estimated. QPCR was performed on gDNA extracted from HEK293 cells transduced with all constructs. The ratio between integrated cassette to gDNA did not show significant differences among constructs ($P>0.05$, Fig 1C), suggesting that the selected promoters do not adversely affect reverse transcription or integration steps.

Next, we determined if the choice of internal promoter affects titre and transduction of primary T cells. As shown in Fig 1D, constructs containing any of the four promoters were able to produce similar viral titres, as determined by transduction of the GFP marker into HEK293T cells. To determine if the sequences of internal promoters altered primary T cells transduction, we transduced primary T cells obtained from different donors and analyzed for GFP expression by flow cytometry three days later. EF-1 gave superior transduction efficacy compared to the other three promoters ($P\leq 0.0001$, Fig 1E).

Promoter comparison for long and complex gene expression

To determine if the promoters differed in their ability to transcribe individual gene products within a long gene, the expression of individual genes were assessed in HEK293T and primary T cells. From the data obtained with HEK293T, CMV and EF-1 were superior to hPGK and RPBSA in producing all three products (Fig 2). We next examined the strength of the four promoters in primary T cells by analyzing GFP and CAR expression. Live primary T cells were gated for GFP, for determining the intensity of CAR and GFP expression. As shown in Fig 3, EF-1 gave stronger expression of GFP and Her2 CAR compared to the other promoters. CMV was weaker in primary T cells, as compared to its activity in HEK293T cells. This could be due to the differences in the transcriptome of both cell types and / or the different techniques that have been used to measure the protein level.

Functional effect of CAR T cells in tumour and T cell engagement

We next examined the function of the CAR T cells transduced with each of the promoter constructs, measuring cytokine release (IL-2 and IFN-γ), cytotoxicity and activation following incubation of CAR T cells with the $Her2^{+}$ MCF-7 breast cancer cell line. Although the expression of CD69 as an activation marker was similarly expressed among the CAR T cells with different promoters (Fig 4A), EF-1 and CMV CAR T cells showed optimal cytokine release after engaging MCF-7 cells (Fig 4B & 4C). CAR T cells transduced with hPGK were less active and those with the RPBSA construct failed to release detectable IL-2 and IFN-γ. Cytotoxicity assay with the four constructs showed similar results with strong killing with CAR T cells expressing under the EF-1 promoter at 24 h time points (Fig 4D).

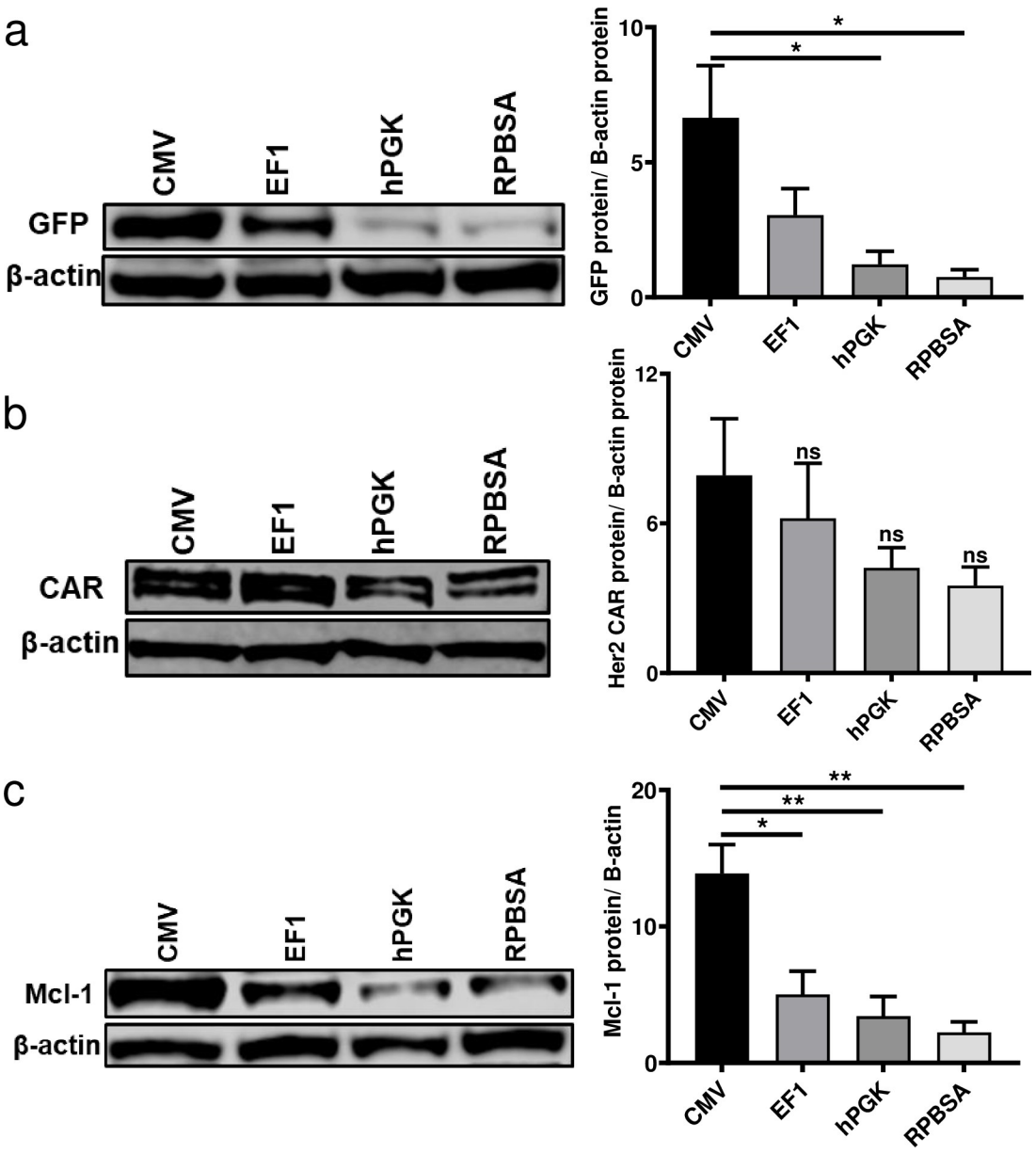


Fig 2. Protein expression from four different constitutive promoters driving long mRNA. Transfected HEK293T cells were lysed with RIPA buffer and processed for immunoblotting using antibodies to detect (a) GFP (b) c-Myc tag for Her2 CAR and (c) Mcl-1 expression with β-actin used as a loading control for the Western blots. All representative blots above are repeated three times and quantified and presented in the bar graph (right) using Image Studio Lite. Bar graph values represent the mean values ± SD from three independent repeats.

https://doi.org/10.1371/journal.pone.0232915.g002

To functionally test the relationship between the expression level of the most distal gene Mcl-1, and resistance to AICD, CAR T cells carrying four different promoters were challenged with 1 μg/mL LZ-CD95L and mitochondrial depolarisation monitored by TMRE staining and flow cytometry. In the absence of CD95L-triggering, there was little difference in cell viability or CAR T cell yields using the four different promoters (Fig 5A and data not shown). Again, EF-1 provided the most potent protection against CD95L-induced cell death (Fig 5). Note, the protection against AICD observed here could reflect a contribution of both Mcl-1, as well as

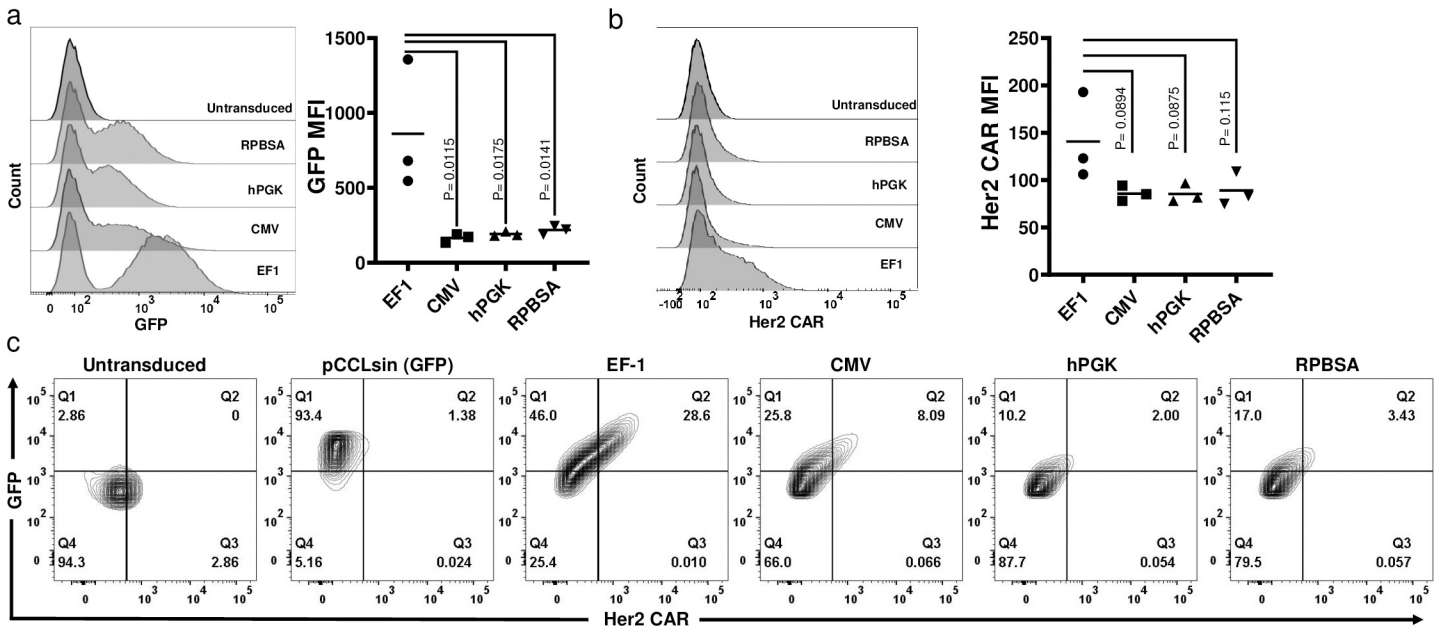


Fig 3. GFP and Her2 CAR expression of the four constructs in primary human T cells. Flow cytometry carried out to measure the expression of (a) GFP and (b) Her2 CAR (c-myc tag). Dead cells were excluded by Zombie NIR viability dye at analysis. GFP positive cells were gated and MFI assessment of CAR and GFP is shown for three individual donors are shown in graphs. (c) Live T cells positive for GFP (Y-axis) and / or Her2 CAR (anti-c-myc; X-axis).

https://doi.org/10.1371/journal.pone.0232915.g003

the pro-survival effect of the CD28 domain in the CAR. For example, CD28 has been shown to enhance the T cell survival by upregulating Bcl2-xL [25]. However, in this setting (without CAR triggering), the presence of the CD28 domain-CAR makes only a minor contribution to the observed protection against CD95L-induced cell death, as compared to the major anti-apoptotic action of Mcl-1 (manuscript in preparation).

Promoter comparison for driving short transcripts

We compared the ability of the four promoters in transcribing GFP linked to an FMC63 CD19 CAR, the most studied CAR construct and the first CAR T cell design approved by the FDA. The FMC63 CAR transcript is 1.2 kb shorter than the GFP-Her2CAR-Mcl1. Viral titres and transduction efficacies were similar among all promoters driving the shorter FMC63 CAR mRNA (Fig 6A & 6B). Protein expression of the shorter GFP-CAR constructs was enhanced in HEK293T transduced with EF-1 and CMV constructs (Fig 6C). In primary T cells, EF-1 gave the highest expression for GFP and CD19 CAR, while CMV gave a more heterogenous expression, but this was not statistically significantly lower than EF-1 (Fig 6D & 6E).

Although CD69 expression on antigen stimulated CAR T cells was similar for all promoter constructs (Fig 7A), EF-1 constructs drove higher levels of CAR triggering in terms of cytokine release and cytotoxicity. RPBSA was more effective in driving short transcripts, as compared to performance observed earlier for long and complex RNA (Fig 7B–7D), further emphasizing that promoter activity is dependent on the nature of the downstream transcript.

Core promoter elements, CpG island and TF binding sites are varying between promoters

Although all four selected promoters are assumed to be constitutive and active in most cell types, bioinformatic analysis showed that the four promoters vary in terms of core promoter

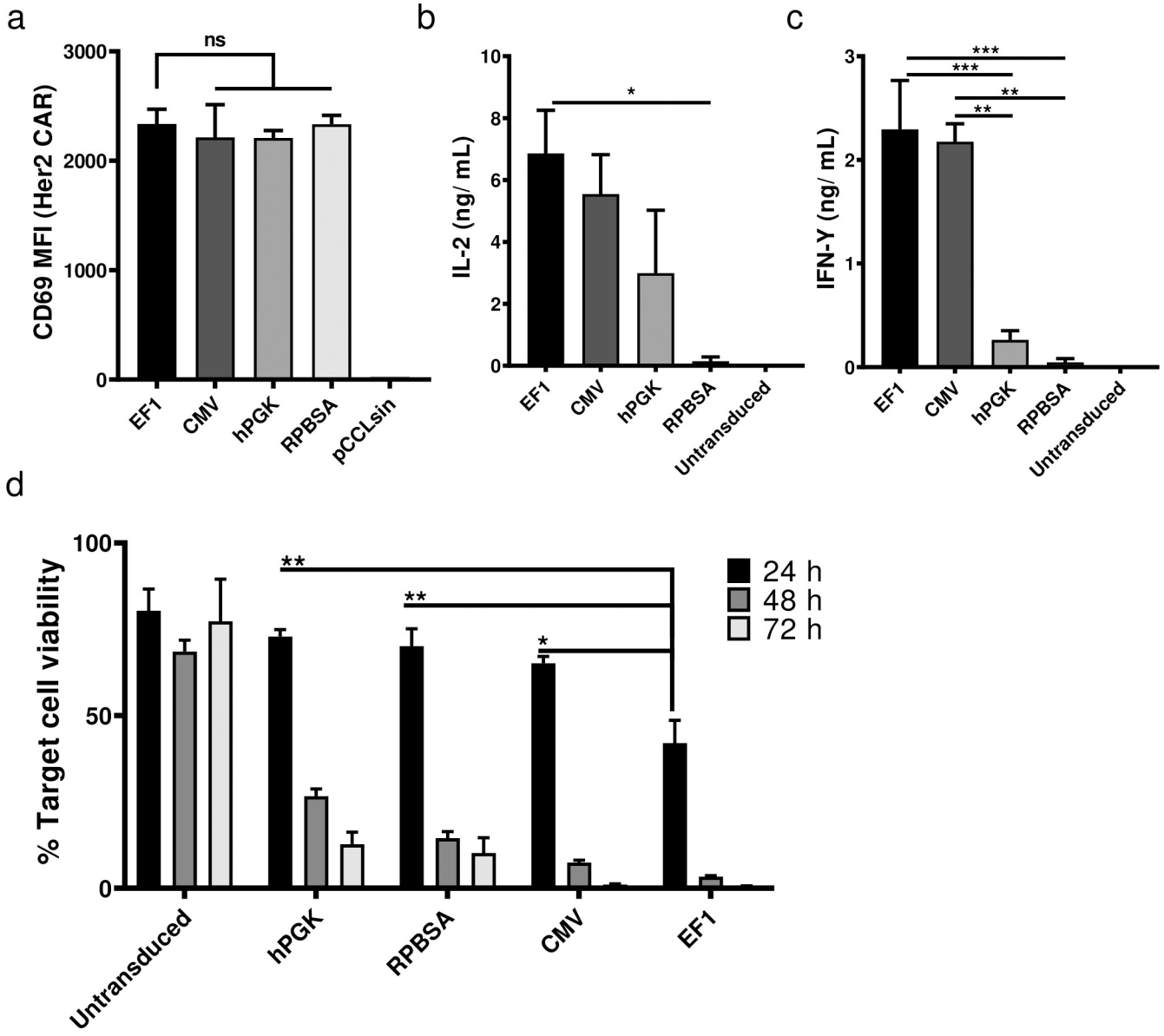


Fig 4. Comparison of anti-tumor activity using different promoters in CAR T cells. (a) Flow cytometric analysis of Her2 CAR T cells 18 h after co-culture with Her2^{+} / MCF-7 cells. Data shows the MFI of CD69 expression from three different donors. Bar graphs show the secretion of (b) IL-2 and (c) IFN-γ by different CAR T cells measured by ELISA. CAR T cells were incubated with Her-2^{+} MCF-7 cell line for 24 h before supernatants were collected. Cytokines were measured in ng/mL. (d) Luciferase based cytotoxicity assay assessed 24, 48 and after 72 h after incubation of CAR T cells with MCF-7 cells stably expressing the firefly luciferase gene. The graph shows the percent of cell viability, calculated by dividing the luciferase of the sample well over the luciferase reading of untreated MCF-7.

https://doi.org/10.1371/journal.pone.0232915.g004

elements and potential TF binding sites. While there is no universal core promoter elements for RNA polymerase II, the TATA box, initiator (Inr) element, TFIIB recognition element (BRE), downstream core promoter element (DPE) and motif ten element (MTE) are well-established core promoter elements (Fig 8A). Overall, EF-1 had more core promoter elements, such as GC box, DPE and MTE (Fig 8B, Table 1). Except for hPGK, all promoters contain a TATA box.

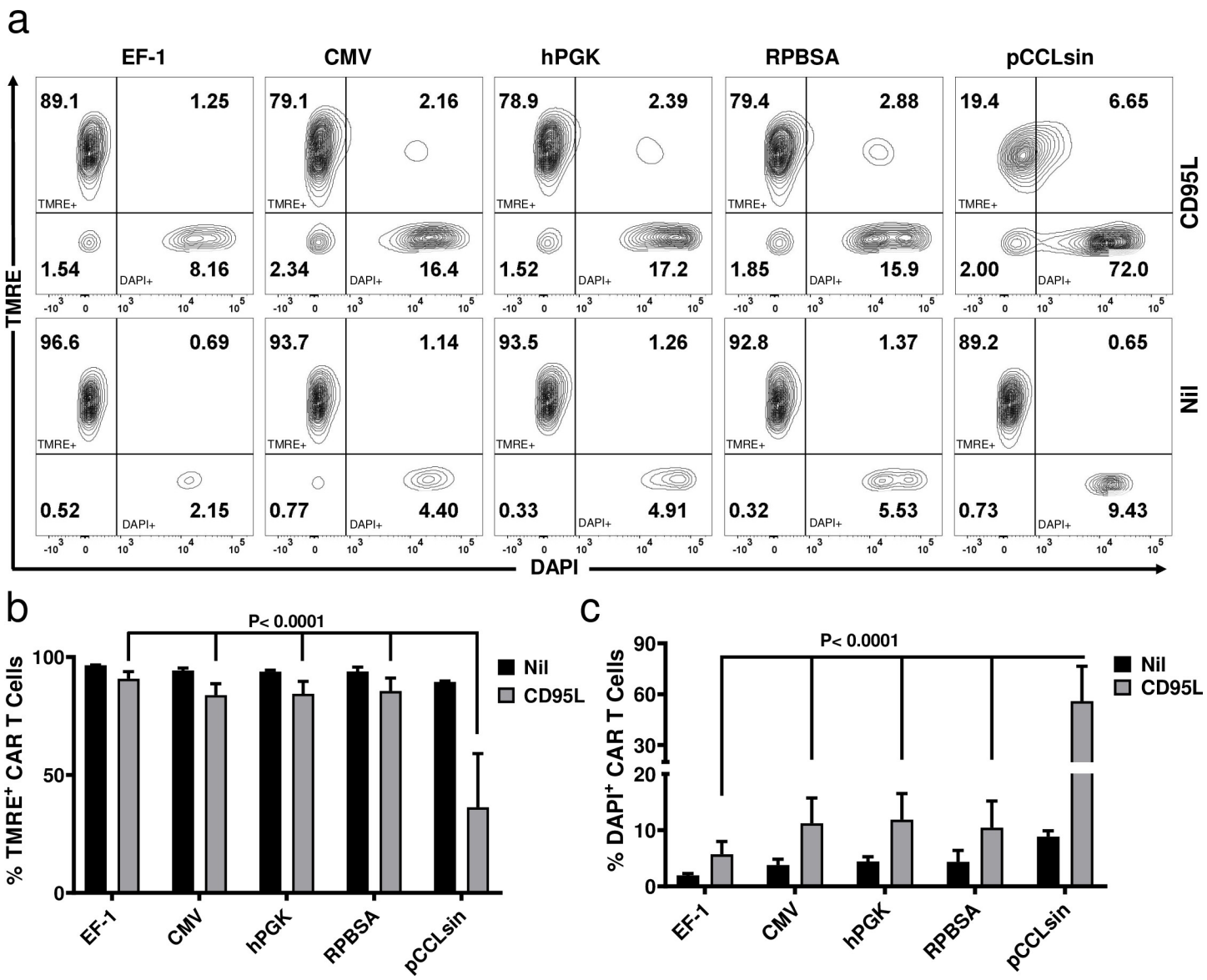


Fig 5. TMRE assay for monitoring mitochondrial membrane potential. (a) CAR T cells expressing Mcl-1 as an anti-apoptotic gene as the most distal gene in the cassette were challenged with 1 μg/mL (top) or 0 μg/mL (below) LZ-CD95L to mimic AICD. $TMRE^{+}$ events represent intact cells with healthy mitochondria, while $TMRE^{-}$ are cells with depolarised mitochondria. pCCLsin (lentivector expressing only GFP) was used as control. Graphs represent the percent of (b) TMRE and c) DAPI positive CAR T cells.

https://doi.org/10.1371/journal.pone.0232915.g005

Another feature of eukaryotic promoters is the presence of CpG islands. CpG islands could result in hypermethylation and gene silencing. However, promoters with CpG islands containing multiple Sp1 binding sites exhibit a hypomethylated state and are typically stronger promoters [26]. We therefore searched for CpG islands within our promoters using two different programs (Table 2). Except for CMV, all promoters were expected to have at least one CpG island. When we searched the Sp1 binding sites within the CpG islands, EF-1 and hPGK showed the highest number of Sp1 binding sites in their CpG islands (Table 3). EMBOSS Cpgplot program predicted two CpG island for EF-1 with 37 Sp1 binding sites. Fig 8C represents the total number of TFBS within the four promoters. Of these identified TFBS, sixteen TFs were selected based on their function and expression in T cells [27–29] and the relative

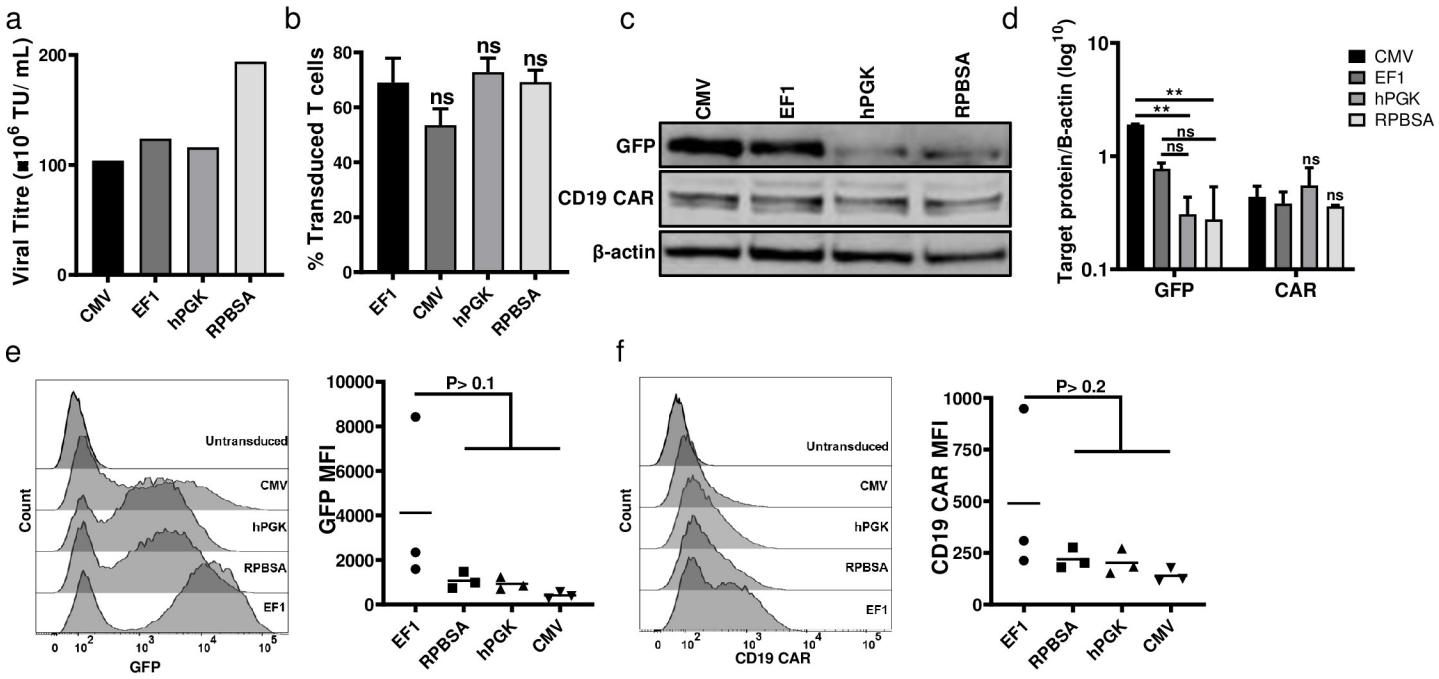


Fig 6. Comparison of four constructs for transcribing short RNA. The eGFP gene linked to FMC63 CD19 CAR was cloned under the control of the four promoters. (a) Titration and (b) transduction efficacy among four constructs (c) Western blot analysis for GFP and CD19 CAR level of HEK293T cells transduced at MOI 2:1 plus 1 μg/mL polybrene (d) Quantification representation of western blot using Image Studio Lite. Bar graph values represent the mean values ± SD from three different repeats. (e) GFP and f) CD19 CAR expression in CAR T cells by flow cytometry.

https://doi.org/10.1371/journal.pone.0232915.g006

enrichment of their corresponding TFBS in each promoter plotted (Fig 8D) [27–29]. Fig 8D highlights promoters that demonstrate a specific enrichment of binding sites for T cell-associated TF, relative to the other promoters. Essentially, the graph illustrates the number of TFBS present in each promoter, expressed as percentage of those present in all promoters. EF-1 possessed binding sites for all these TFs (Fig 8D). CMV is the next promoter enriched for T cell-specific TFs, excluding GATA3, LEF-1, STAT5 and IF-2 (Fig 8D). It should be noted that EF-1 is almost twice the length of other promoters (>600 bp), and this length allows a greater possible enrichment of TFBS and core promoter elements.

Who should drive the CAR?

In order to have a broader view in comparing the strength of each promoter, scores from 0–10 were assigned to all functional assays carried out in primary T cells (Fig 9). Scores were calculated using the following formula:

$$\text{Score} = \frac{\text{Mean of each value}}{\text{Mean of maximum value}} \times 10$$

Based on data from Fig 9A, the promoter strengths for short transcript were in the following order: EF-1 > RPBSA > hPGK > CMV. For long transcripts carrying another accessory gene (Mcl-1) in addition to GFP-CAR, the promoter strengths were as follow: EF-1 > CMV > hPGK > RPBSA (Fig 9B). Taken together, EF-1 displayed the best function in driving both short and long RNA transcripts. However, if the insert size between two LTR increases beyond 10 kb, other promoters could be considered to mitigate drops in the viral titre and transduction efficiency [30]. In our study, the largest insert utilised was 6.8 kb.

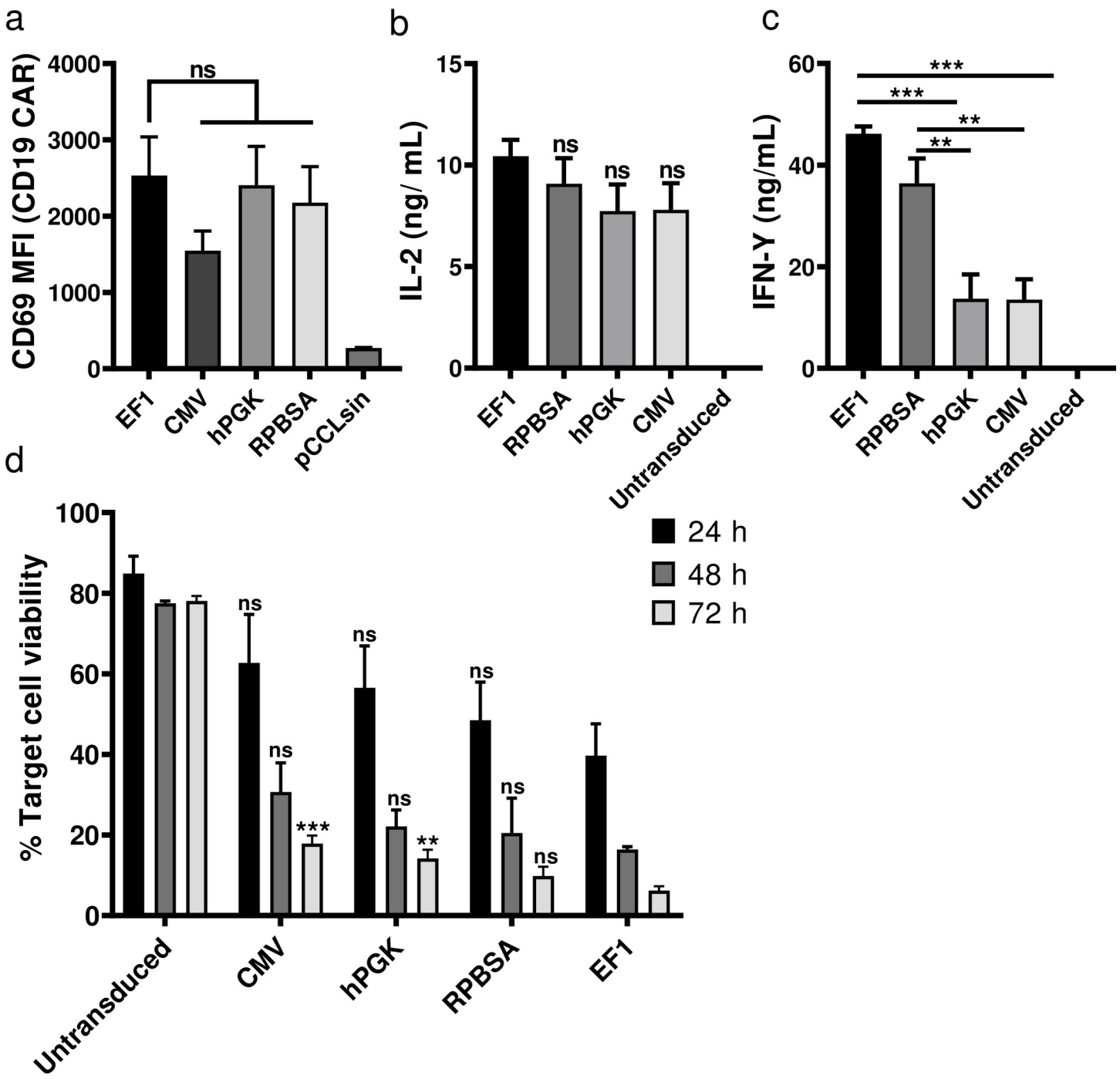


Fig 7. Functional analysis of CAR T cells expressing short RNA. (a) CD69 activation assay was carried out 18 hours after incubation of the four types of promoter-driven CD19 CAR T cells with $CD19^+$ HEK293T cells. (b, c) Cytokine release assay for secretion of (b) IL-2 and (c) IFN-γ. CAR T cells were co-cultured with $CD19^+$ HEK239T and supernatant were collected after 24 h. (d) Luciferase based cytotoxicity assay assessed 24, 48 and 72 h after incubation of CD19 CAR T cells with $CD19^+$ HEK293 cells stably expressing the firefly luciferase gene. The graph shows the percent of cell viability, calculated by dividing the luciferase of test wells divided by the luciferase signal of untreated HEK293T.

https://doi.org/10.1371/journal.pone.0232915.g007

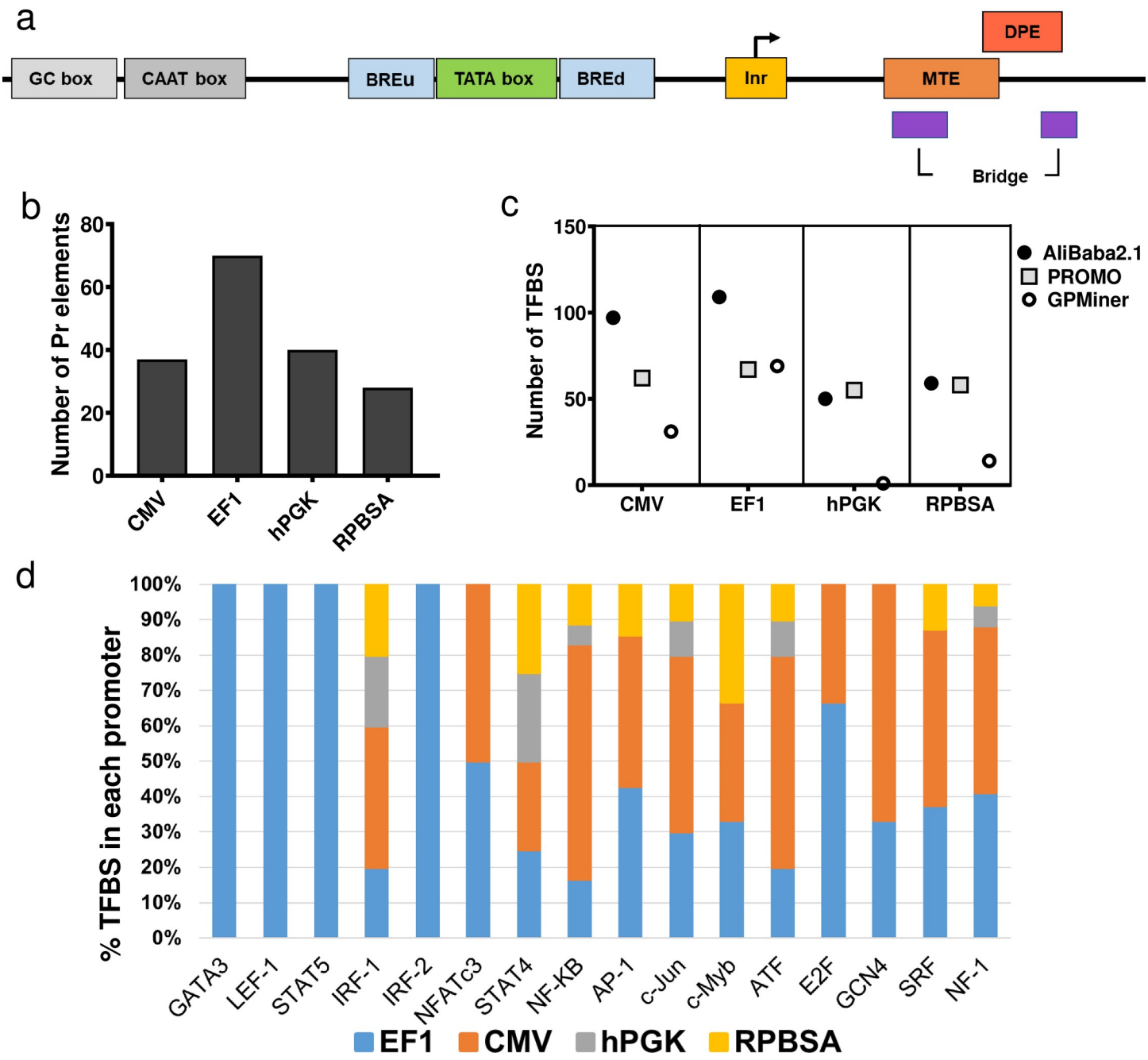


Fig 8. Structure and bioinformatic analysis of the four different promoters. (a) Structure of a typical eukaryotic core promoter and the position of core elements within a promoter were investigated in the four different promoters (b) Total number of core promoter elements predicted by YAPP, GPMiner and ElemeNT algorithms (details provided in Table 1) (c) The number of TF binding sites in promoters sequenced analyzed by AliBaba2.1, PROMO and GPMiner programs d) Enrichment of sixteen TFs highly-expressed in T cells in the four promoters. The data shows the percentage of total number predicted binding sites for the four promoters.

https://doi.org/10.1371/journal.pone.0232915.g008

Discussion

In this study, we compared four promoters for optimal expression of long RNA encoding multiple gene products in CAR T cells. Our results suggest that promoter requirements are stringent for driving long RNA, and that EF-1 is the best choice for driving short or long RNA in CAR T cells, similar to an early study [31]. In contrast to the poor results obtained here for

Table 1. The number of core promoter elements and TF binding sites predicted for four promoters with YAPP, GPMiner and ElemeNT algorithms.

Promoter	Size (bp)	Core prompter elements							
		GC box	CAAT box	BRE	TATA box	Inr	MTE	DPE	Bridge
CMV	617	3	4	-	2	10	-	6	12
EF-1	1192	11	-	2	2	7	2	24	22
hPGK	516	8	2	3	-	3	2	12	10
RPBSA	612	3	1	1	2	3	-	10	8

https://doi.org/10.1371/journal.pone.0232915.t001

hPGK and RPBSA in driving long and complex RNA, these same promoters demonstrated little difference to the so-called strong promoters CMV and EF-1 in lentiviral based systems driving shorter RNA sequences, such as CAR and fluorescent reporter genes (see Fig 6E)–consistent with other studies [1, 8, 9, 21, 23].

To determine the functional role of additional accessory genes expressed in long constructs, we utilised Mcl-1, a bcl2 family member with an essential role in T cell development, mitochondrial function and lifespan. To our knowledge, this is the first study to demonstrate that Mcl-1 is a suitable candidate for enhancing CAR T cell performance [15, 16]. Expression of mcl1 in a position distal to the CAR allowed protection from CD95-induced cell death. Interestingly, although protection was noted with all promoters, EF-1 driven-cassettes consistently gave the best protection. The fact that protection was observed with Mcl-1 driven by the weaker promoters RPBSA and hPGK contrasts with the stringent requirement for a strong promoter to drive CAR expression for optimal cytotoxicity and cytokine release.

Our analysis of promoter motifs demonstrates clear differences in transcription factor binding sites and core promoter elements between the strong (EF-1 and CMV) and weaker (hPGK and RPBSA) promoters. Although not all the predicted core promoter elements might be functional in primary T cells, the high number of the core elements can correlate with the strength of the promoter [26]. In addition EF-1 and CMV predominantly enriched for TFs specific or highly expressed in T cells [27–29, 32, 33] such as GATA3, NFATc3, NF-kB, AP1 and c-Jun, The number of transcription factor and core promoter element sites predicted within the promoters may provide some explanation for the ability of the CMV and EF-1 promoters to direct long mRNA expression (Fig 1, S1 Data). However, it should be noted that EF-1 is almost twice the length of the other promoters, therefore has the potential to house more TFBS and core promoter elements.

The activity of promoters with predicted 'ubiquitous' expression, such as the four studied here, will still depend greatly on the lineage of the host cell [34]. However, EF-1 promoter was found to be active and resistant to silencing in cells where other viral promoters may become silenced [35]. Therefore, future work will be required to determine if the superior performance

Table 2. Bioinformatic tools used for studying promoter structure and TF binding sites.

Program	Promoter element	CpG island	TF binding sites
YAPP	✓	-	-
GPMiner	✓	✓	✓
ElemeNT	✓	-	-
AliBaba2.1	-	-	✓
PROMO	-	-	✓
EMBOSS Cpgplot	-	✓	-
CpGFinder	-	✓	-

https://doi.org/10.1371/journal.pone.0232915.t002

Table 3. The number of CpG islands and Sp1 binding sites within selected promoters.

Promoter	Number of CpG islands	Position	Number of Sp1 binding sites
EF-1	1	604–868	17
CMV	0	-	-
hPGK	1	54–392	16
RPBSA	1	194–405	8

https://doi.org/10.1371/journal.pone.0232915.t003

of EF-1 and CMV in expressing long RNA sequences can be extrapolated to other cell primary cell types.

In our study, the lower expression of CAR within a long mRNA transcript driven by the RPBSA and hPGK translated into lower lytic function for a Her2-expressing tumor cell line. Given the profound effects that CAR density has on T cell activation, our results will be useful for developing strategies to titrate CAR expression at the T cell. Promoter choice would be expected to be a critical consideration for controlling the levels of surface expressed CAR, which in turn would dictate the level of T cell activation, lytic function, as well as undesirable tonic (antigen-independent) signaling [2, 36–39]. Optimal CAR expression will be critical for minimizing tonic signaling, while optimizing signal transduction during antigen-specific signaling. In addition, lowering the level of CAR expression could contribute desirable avidity effects to T cell recognition of antigen, thereby minimizing CAR T cell activation by tumor-associated antigen on self-tissue [14]. Interestingly, despite CMV inducing a noticeably higher level expression of GFP, CAR and Mcl-1 in HEK293T cells, as compared to EF-1, functional analysis showed superior activation of primary human CAR T cells driven by EF-1 in terms of cytokine release and cytotoxicity against MCF-7. EF-1 is enriched in binding sites of TFs expressed in T cells (Fig 8C), suggesting a mechanism for the increased EF-1 activity in T cells, as compared to HEK293T cells. In addition functional experiments demonstrated that EF-1

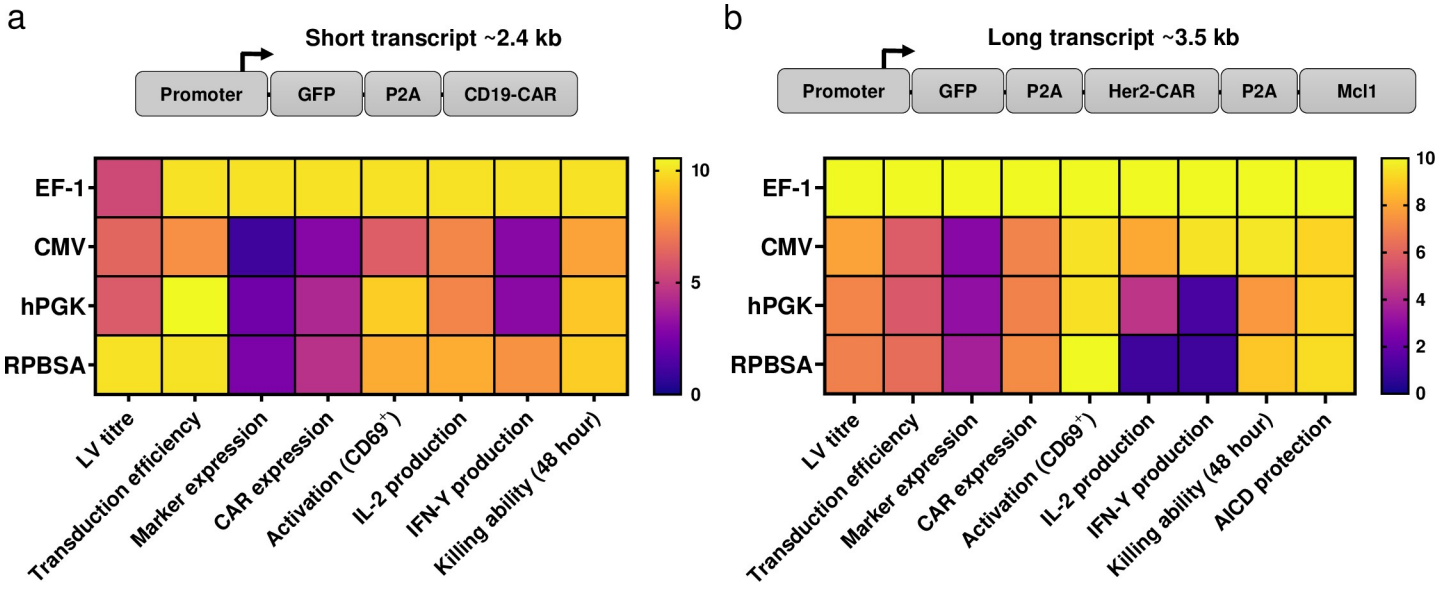


Fig 9. Heat map charts representing the strength of each promoter in functional assays for a) short transcripts and b) long transcripts. Each promoter was assigned a score from 0–10 based on the data obtained from primary T cell experiments, and values were calculated by dividing mean of each data set by mean of the maximum value obtained in the experiment and multiplied by ten.

https://doi.org/10.1371/journal.pone.0232915.g009

driven expression of Mcl-1 provided the best protection of CAR T cells to AICD induced by CD95L.

A further consideration for promoter choice is possible silencing *in vivo*. In particular, CMV can be silenced after a period of weeks post-transduction [34, 40]. However, the effects of promoter silencing might be overshadowed by the long term CAR T cell downregulation that occurs in a methylation-independent fashion following CAR triggering both *in vitro* and *in vivo* [14, 41, 42]. In conclusion, the study of long mRNA production will improve our ability to express multiple genes in CAR T cells to improve cell survival and persistence of infused CAR T cells.

Supporting information

S1 Raw images.
(PDF)

S1 Data.
(DOCX)

Acknowledgments

We thank Dr. Sarah Saunderson for critical review of the manuscript.

Author Contributions

Conceptualization: Ali Hosseini Rad S. M., Alexander D. McLellan.

Formal analysis: Ali Hosseini Rad S. M., Aarati Poudel, Grace Min Yi Tan, Alexander D. McLellan.

Funding acquisition: Alexander D. McLellan.

Investigation: Ali Hosseini Rad S. M., Aarati Poudel, Grace Min Yi Tan, Alexander D. McLellan.

Methodology: Ali Hosseini Rad S. M., Aarati Poudel, Grace Min Yi Tan, Alexander D. McLellan.

Project administration: Ali Hosseini Rad S. M.

Resources: Ali Hosseini Rad S. M., Alexander D. McLellan.

Supervision: Ali Hosseini Rad S. M., Alexander D. McLellan.

Writing – original draft: Ali Hosseini Rad S. M., Aarati Poudel, Grace Min Yi Tan, Alexander D. McLellan.

Writing – review & editing: Ali Hosseini Rad S. M., Alexander D. McLellan.

References

1. Milone MC, Fish JD, Carpenito C, Carroll RG, Binder GK, Teachey D, et al. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. Molecular therapy: the journal of the American Society of Gene Therapy. 2009; 17 (8):1453–64.
2. Frigault MJ, Lee J, Basil MC, Carpenito C, Motohashi S, Scholler J, et al. Identification of chimeric antigen receptors that mediate constitutive or inducible proliferation of T cells. Cancer immunology research. 2015; 3(4):356–67. https://doi.org/10.1158/2326-6066.CIR-14-0186 PMID: 25600436

3. Drent E, Poels R, Mulders MJ, van de Donk N, Themeli M, Lokhorst HM, et al. Feasibility of controlling CD38-CAR T cell activity with a Tet-on inducible CAR design. PloS one. 2018; 13(5):e0197349. https://doi.org/10.1371/journal.pone.0197349 PMID: 29847570
4. Stoiber S, Cadilha BL, Benmebarek MR, Lesch S, Endres S, Kobold S. Limitations in the Design of Chimeric Antigen Receptors for Cancer Therapy. Cells. 2019; 8(5).
5. Naldini L, Trono D, Verma IM. Lentiviral vectors, two decades later. Science (New York, NY). 2016; 353 (6304):1101–2.
6. McLellan AD, Ali Hosseini Rad SM. Chimeric antigen receptor T cell persistence and memory cell formation. Immunology and cell biology. 2019.
7. Gilham DE, Lie ALM, Taylor N, Hawkins RE. Cytokine stimulation and the choice of promoter are critical factors for the efficient transduction of mouse T cells with HIV-1 vectors. The journal of gene medicine. 2010; 12(2):129–36. https://doi.org/10.1002/jgm.1421 PMID: 20033928
8. Jones S, Peng PD, Yang S, Hsu C, Cohen CJ, Zhao Y, et al. Lentiviral vector design for optimal T cell receptor gene expression in the transduction of peripheral blood lymphocytes and tumor-infiltrating lymphocytes. Human gene therapy. 2009; 20(6):630–40. https://doi.org/10.1089/hum.2008.048 PMID: 19265475
9. Amendola M, Venneri MA, Biffi A, Vigna E, Naldini L. Coordinate dual-gene transgenesis by lentiviral vectors carrying synthetic bidirectional promoters. Nature biotechnology. 2005; 23(1):108–16. https://doi.org/10.1038/nbt1049 PMID: 15619618
10. Curtin JA, Dane AP, Swanson A, Alexander IE, Ginn SL. Bidirectional promoter interference between two widely used internal heterologous promoters in a late-generation lentiviral construct. Gene therapy. 2008; 15(5):384–90. https://doi.org/10.1038/sj.gt.3303105 PMID: 18283290
11. Wang D, Shao Y, Zhang X, Lu G, Liu B. IL-23 and PSMA-targeted duo-CAR T cells in Prostate Cancer Eradication in a preclinical model. Journal of translational medicine. 2020; 18(1):23. https://doi.org/10.1186/s12967-019-02206-w PMID: 31937346
12. Hoyos V, Savoldo B, Quintarelli C, Mahendravada A, Zhang M, Vera J, et al. Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety. Leukemia. 2010; 24(6):1160–70. https://doi.org/10.1038/leu.2010.75 PMID: 20428207
13. Philip B, Kokalaki E, Mekkaoui L, Thomas S, Straathof K, Flutter B, et al. A highly compact epitope-based marker/suicide gene for easier and safer T-cell therapy. Blood. 2014; 124(8):1277–87. https://doi.org/10.1182/blood-2014-01-545020 PMID: 24970931
14. Eyquem J, Mansilla-Soto J, Giavridis T, van der Stegen SJ, Hamieh M, Cunanan KM, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature. 2017; 543(7643):113–7. https://doi.org/10.1038/nature21405 PMID: 28225754
15. Andersen JL, Kornbluth S. Mcl-1 rescues a glitch in the matrix. Nat Cell Biol. 2012; 14(6):563–5. https://doi.org/10.1038/ncb2511 PMID: 22643875
16. Morciano G, Pedriali G, Sbano L, Iannitti T, Giorgi C, Pinton P. Intersection of mitochondrial fission and fusion machinery with apoptotic pathways: Role of Mcl-1. Biol Cell. 2016; 108(10):279–93. https://doi.org/10.1111/boc.201600019 PMID: 27234233
17. Kim EH, Neldner B, Gui J, Craig RW, Suresh M. Mcl-1 regulates effector and memory CD8 T-cell differentiation during acute viral infection. Virology. 2016; 490:75–82. https://doi.org/10.1016/j.virol.2016.01.008 PMID: 26855329
18. Dzhagalov I, Dunkle A, He YW. The anti-apoptotic Bcl-2 family member Mcl-1 promotes T lymphocyte survival at multiple stages. Journal of immunology (Baltimore, Md: 1950). 2008; 181(1):521–8.
19. Dunkle A, Dzhagalov I, He YW. Cytokine-dependent and cytokine-independent roles for Mcl-1: genetic evidence for multiple mechanisms by which Mcl-1 promotes survival in primary T lymphocytes. Cell death & disease. 2011; 2:e214.
20. Stewart DP, Koss B, Bathina M, Perciavalle RM, Bisanz K, Opferman JT. Ubiquitin-independent degradation of antiapoptotic MCL-1. Mol Cell Biol. 2010; 30(12):3099–110. https://doi.org/10.1128/MCB.01266-09 PMID: 20385764
21. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, et al. A third-generation lentivirus vector with a conditional packaging system. Journal of virology. 1998; 72(11):8463–71. PMID: 9765382
22. Fu X, Tao L, Rivera A, Williamson S, Song X-T, Ahmed N, et al. A simple and sensitive method for measuring tumor-specific T cell cytotoxicity. PloS one. 2010; 5(7).
23. Kowarz E, Loscher D, Marschalek R. Optimized Sleeping Beauty transposons rapidly generate stable transgenic cell lines. Biotechnol J. 2015; 10(4):647–53. https://doi.org/10.1002/biot.201400821 PMID: 25650551

24. Yee J-K, Moores JC, Jolly DJ, Wolff JA, Respess JG, Friedmann T. Gene expression from transcriptionally disabled retroviral vectors. Proceedings of the National Academy of Sciences. 1987; 84(15):5197–201.

25. Watts TH. Staying alive: T cell costimulation, CD28, and Bcl-xL. The Journal of Immunology. 2010; 185 (7):3785–7. https://doi.org/10.4049/jimmunol.1090085 PMID: 20858889

26. Butler JE, Kadonaga JT. The RNA polymerase II core promoter: a key component in the regulation of gene expression. Genes & development. 2002; 16(20):2583–92.

27. Naito T, Tanaka H, Naoe Y, Taniuchi I. Transcriptional control of T-cell development. Int Immunol. 2011; 23(11):661–8. https://doi.org/10.1093/intimm/dxr078 PMID: 21948191

28. Hosokawa H, Rothenberg EV. Cytokines, Transcription Factors, and the Initiation of T-Cell Development. Cold Spring Harb Perspect Biol. 2018; 10(5). https://doi.org/10.1101/cshperspect.a028621 PMID: 28716889

29. Rothenberg EV. The chromatin landscape and transcription factors in T cell programming. Trends Immunol. 2014; 35(5):195–204. https://doi.org/10.1016/j.it.2014.03.001 PMID: 24703587

30. Escors D, Breckpot K. Lentiviral vectors in gene therapy: their current status and future potential. Archivum immunologiae et therapiae experimentalis. 2010; 58(2):107–19. https://doi.org/10.1007/s00005-010-0063-4 PMID: 20143172

31. Frigault MJ, Lee J, Basil MC, Carpenito C, Motohashi S, Scholler J, et al. Identification of chimeric antigen receptors that mediate constitutive or inducible proliferation of T cells. Cancer immunology research. 2015; 3(4):356–67. https://doi.org/10.1158/2326-6066.CIR-14-0186 PMID: 25600436

32. Urso K, Alfranca A, Martinez-Martinez S, Escolano A, Ortega I, Rodriguez A, et al. NFATc3 regulates the transcription of genes involved in T-cell activation and angiogenesis. Blood. 2011; 118(3):795–803. https://doi.org/10.1182/blood-2010-12-322701 PMID: 21642596

33. Lynn RC, Weber EW, Sotillo E, Gennert D, Xu P, Good Z, et al. c-Jun overexpression in CAR T cells induces exhaustion resistance. Nature. 2019; 576(7786):293–300. https://doi.org/10.1038/s41586-019-1805-z PMID: 31802004

34. Hong S, Hwang DY, Yoon S, Isacson O, Ramezani A, Hawley RG, et al. Functional analysis of various promoters in lentiviral vectors at different stages of in vitro differentiation of mouse embryonic stem cells. Molecular therapy: the journal of the American Society of Gene Therapy. 2007; 15(9):1630–9.

35. Wang X, Xu Z, Tian Z, Zhang X, Xu D, Li Q, et al. The EF-1alpha promoter maintains high-level transgene expression from episomal vectors in transfected CHO-K1 cells. J Cell Mol Med. 2017; 21 (11):3044–54. https://doi.org/10.1111/jcmm.13216 PMID: 28557288

36. Chang ZL, Silver PA, Chen YY. Identification and selective expansion of functionally superior T cells expressing chimeric antigen receptors. Journal of translational medicine. 2015; 13:161. https://doi.org/10.1186/s12967-015-0519-8 PMID: 25990251

37. Chmielewski M, Hombach A, Heuser C, Adams GP, Abken H. T cell activation by antibody-like immunoreceptors: increase in affinity of the single-chain fragment domain above threshold does not increase T cell activation against antigen-positive target cells but decreases selectivity. Journal of immunology (Baltimore, Md: 1950). 2004; 173(12):7647–53.

38. Han C, Sim SJ, Kim SH, Singh R, Hwang S, Kim YI, et al. Desensitized chimeric antigen receptor T cells selectively recognize target cells with enhanced antigen expression. Nature communications. 2018; 9(1):468. https://doi.org/10.1038/s41467-018-02912-x PMID: 29391449

39. Sakemura R, Terakura S, Watanabe K, Julamanee J, Takagi E, Miyao K, et al. A Tet-On Inducible System for Controlling CD19-Chimeric Antigen Receptor Expression upon Drug Administration. Cancer immunology research. 2016; 4(8):658–68. https://doi.org/10.1158/2326-6066.CIR-16-0043 PMID: 27329987

40. Brooks AR, Harkins RN, Wang P, Qian HS, Liu P, Rubanyi GM. Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. The journal of gene medicine. 2004; 6(4):395–404. https://doi.org/10.1002/jgm.516 PMID: 15079814

41. Burns WR, Zheng Z, Rosenberg SA, Morgan RA. Lack of specific gamma-retroviral vector long terminal repeat promoter silencing in patients receiving genetically engineered lymphocytes and activation upon lymphocyte restimulation. Blood. 2009; 114(14):2888–99. https://doi.org/10.1182/blood-2009-01-199216 PMID: 19589923

42. Gallegos AM, Xiong H, Leiner IM, Susac B, Glickman MS, Pamer EG, et al. Control of T cell antigen reactivity via programmed TCR downregulation. Nature immunology. 2016; 17(4):379–86. https://doi.org/10.1038/ni.3386 PMID: 26901151