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Original article

Testing T cell activation-based promoters in driving a dual CAR T cell by utilizing different transcription factor binding sites

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Abstract

Background: Despite significant advancements in treating hematological malignancies using chimeric antigen receptor (CAR) T cell therapy, several challenges hinder its efficacy. To address the risk of disease relapse caused by antigen loss, researchers have explored approaches such as utilizing dual antigen-targeted CAR constructs. However, achieving optimal dual CAR activity necessitates ensuring the ideal expression of both CARs on the cell surface. Thus, employing T cell activation-based promoters could offer an alternative strategy, where the activation of one CAR triggers the expression of another.
Objective: This study aimed to assess the efficiency of different activation-based promoters in driving the expression of dual CAR.
Methods: Multiple iterations of T cell activation-based transcription factors such as the nuclear factor of (NFAT) activated T cells and nuclear receptor subfamily 4A (NR4A), were employed to induce the expression of dual CARs. The process involved designing a system where the activation of one CAR triggered the expression of another through T cell based transcription factors. This was achieved by integrating specific transcription factor binding sites into the promoter regions of the CAR genes. Upon activation of the initial CAR, the associated transcription factors were activated, leading to the expression of the secondary CAR. As a proof of concept, single chain variable fragments of CD19 and CD20 were utilized to create CD19/CD20 dual CAR T cells. The objective was to express the second CAR (CD20 CAR) upon activation of the first CAR (CD19 CAR) following binding to its corresponding antigen. In this approach, the expression of the CD19 CAR was driven by a constitutive promoter, while the expression of the CD20 CAR was regulated by a T cell activation-based transcription factor activated by the CD19 CAR.
Results: This study showed that the use of eight repeats of the Nuclear receptor subfamily 4A (NR4A) promoter effectively stimulated the expression of CD20 CAR upon the detection of the antigen by the CD19 CAR after 72 h of activation.
Conclusion: This study suggested the possibility of using endogenous CAR T cell-based activation-induced motifs to promote the expression of CAR T cells in a dual antigen-targeted CAR T cell platform.

Keywords: CD19, CD20, chimeric antigen receptor T cells, dual CARs, transcription factors.

Despite notable progress in chimeric antigen receptor (CAR) T cell therapy, challenges persist in ensuring its efficacy, prompting the emergence of strategies such as dual antigen-targeted CAR constructs to combat disease relapse resulting from antigen loss.[1 - 3] However, effective dual CAR T cells require robust promoters for adequate CAR expression, often achieved through the use of strong constitutive promoters or distinct promoters for each CAR.[4 - 6] The prolonged signaling from strong promoters may cause autonomous activation, called tonic signaling, resulting in transgene overexpression, proliferation, cytokine secretion, and eventual CAR T cell exhaustion.[6, 7] Previous studies have investigated the

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use of transcription factors such as the nuclear factor of activated T cells (NFAT) and nuclear receptor subfamily 4A (NR4A) fragments to enhance CAR T cell activation by releasing cytokines conditionally upon antigen encounter; however, their suitability for driving dual CAR expression has not been tested.[8] Therefore, this study aimed to utilize NFAT and NR4A fragments to express dual CARs utilizing single chain variable fragments (scFv) of CD19 (FMC63) and CD20 (1F5) to generate CD19 and CD20 dual CAR T cells as a model. Herein, the CD19 CAR was expressed via a constitutive promoter, whereas CD20 CAR expression was regulated by sequestering NFAT and NR4A upon receiving activation signals from the CD19 CAR upon antigen recognition. T cell activation-based dual CAR offers an alternative platform for simultaneous expression of dual antigen-targeted CARs in the tumor vicinity. This platform also offers advantages in reducing exhaustion and enhancing the specificity of CAR T cells by tightly regulating CAR expression, thus ensuring precise antigen targeting.

Materials and methods

Human peripheral blood mononuclear cells were derived from 1 - 2 healthy donors (all sexes) aged 16 - 60 years with written consent and approval from the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB no. 0203/66, COA no. 0613/2023).

Primary cells and cell lines

Acute lymphoblastic leukemia cell line positive for CD19 marker (NALM6) and human immortalized myelogenous leukemia cell line (K562) cells were previously obtained from American type culture collection and stored in liquid nitrogen. Cells were maintained in RPMI 1640 medium supplemented with Glutamax (Thermo Fisher Scientific, Waltham, MA, USA), penicillin (100 U/mL), streptomycin (100 µg/mL), and 10.0% heat inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific) at 37°C with 5.0% CO_2.

Construction of dual CD19/CD20 chimeric antigen receptors (CARs)

To construct dual CD19/CD20 CAR with human phosphoglycerate kinase (hPGK) promoter, the following steps were taken. First, the hPGK promoter (527 bp) was amplified from the pCCLsin plasmid [9] and was cloned into a pUC19 plasmid.[10] Then, CD19 CAR (1582 bp), consisting of FMC63 anti-CD19-scFv, human CD8 hinge domain, human CD8 transmembrane (TM), 4 - 1BB intracellular costimulatory domain, and CD3ζ activation domain were cloned into the pUC19-hPGK plasmid. Subsequently, the restriction enzymes were used to digest the pUC19-hPGK-CD19CAR along with a sleeping beauty vector backbone (pSBbi-Rp) [11], followed by ligation using T4 DNA ligase to obtain SB-hPGK-CD19CAR. Repetitive fragments of transcription binding motifs, namely 8xNFAT, 8xNR4A, and 4xNFAT + 4xNR4A, were commercially acquired from GeneScript. The SB-hPGK-CD19 CAR backbone and these transcription factor motifs were digested with restriction enzymes. Three independent ligations were performed to construct SB-8xNFAT-hPGK-CD19CAR, SB-8xNR4A-hPGK-CD19CAR, and SB-4xNFAT + 4xNR4A-hPGK-CD19CAR.

Then, green fluorescent protein (GFP) (717 bp) and CD20 CAR (1675 bp) 1F5 anti-CD20-scFv, with the human CD8 hinge domain, human CD28 TM, CD28 intracellular costimulatory domain, and CD3ζ activation domain, were cloned adjacent to the inducible promoters. The GFP and CD20 CAR were separated by split peptide (P2A). Signal sequence was also cloned upstream of both CD19 and CD20 CAR to successfully express CD19 and CD20 scFvs on the T cell surface. After the cloning, three CAR constructs were yielded: SB-8xNFAT-GFP-CD20CAR-hPGK-CD19CAR, SB-8xNR4A-GFP-CD20CAR-hPGK-CD19CAR, and SB-4xNFAT + 4xNR4A-GFP-CD20CAR-hPGK-CD19CAR **(Figure 1A).**

To construct dual CD19/CD20 CAR with the elongation factor 1 (EF1) promoter, PCR amplification of the 1.2 kb long eukaryotic EF1 promoter from the existing pSBbi-RP [11] plasmid was performed. Then, replaced the hPGK promoter was replaced with the EF1 promoter to drive CD19 CAR. All sequences in this construct were similar to the previously mentioned CAR, except for the EF1 promoter **(Figure 1B).**

Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from the healthy donors using Ficol-Paque™ Premium (GE Healthcare, Bio-Sciences-AB, Sweden) and density gradient centrifugation by following the

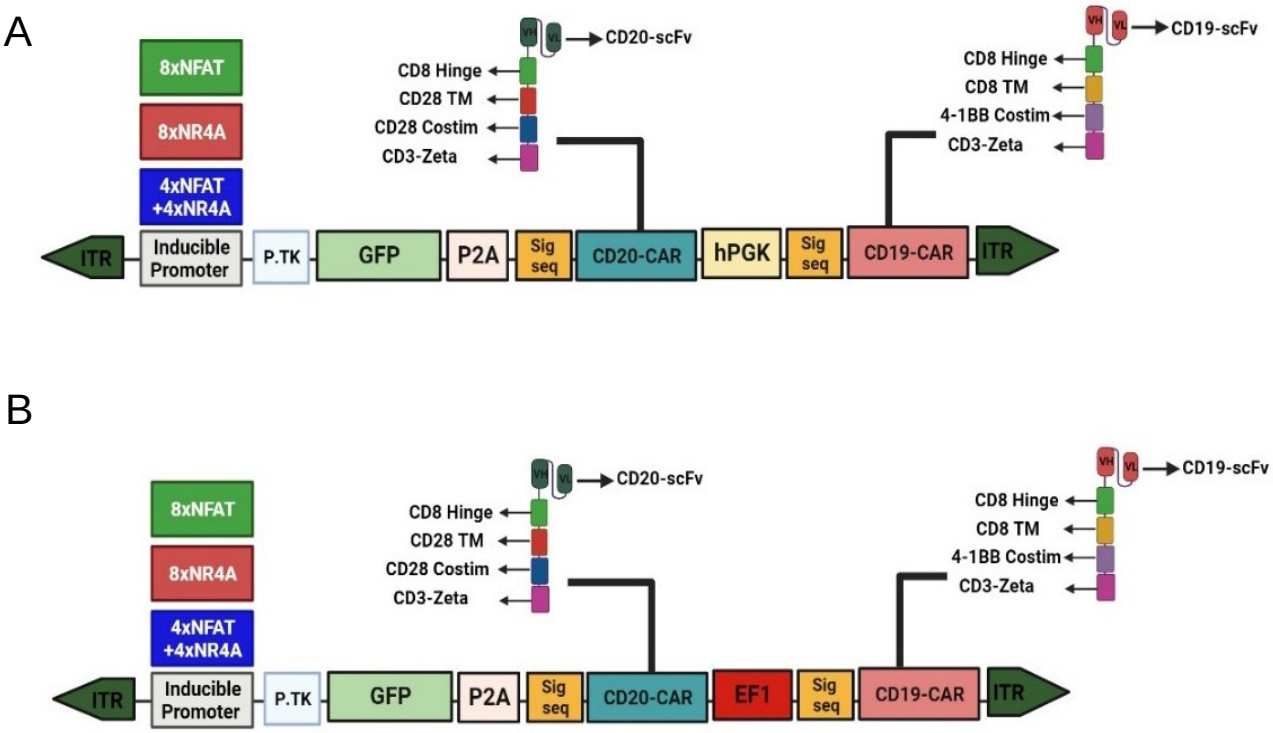

Figure 1. Dual CAR construct in SB backbone. **(A)** Dual CAR construct expressing the first CAR (CD19 CAR) via the hPGK promoter; **(B)** Dual CAR construct expressing the first CAR (CD19 CAR) via the EF1 promoter.

manufacturer's guidelines. Briefly, blood from a healthy donor was diluted in a 1 : 1 ratio with 1X PBS. Then, 20 mL of the diluted blood was overlaid on 15 mL of Ficol-Paque in a 50 mL falcon tube. Subsequently, it was centrifuged at 400 × g for 30 min without a break at room temperature. The monolayer cells from the interphase were carefully transferred to a new 50 mL falcon tube without interrupting the aqueous phase. The cells were then washed two times with 1X PBS at 400 × g for 10 min. The supernatant was discarded carefully, and PBMCs were resuspended with the respective cell media and counted using a Neubauer chamber.

Generation of chimeric antigen receptor (CAR) T cell

Fresh PBMCs were isolated from 1 - 2 healthy donor blood samples using Ficol-Paque™ Premium (GE Healthcare) and density gradient centrifugation. Plasmids were isolated from DH5-α using endotoxin-free Qiagen plasmid maxi kits. PBMCs (4×10^6) were suspended in P3 primary cell 4D-nucleofector X kit reagent (Lonza, Basel, Switzerland) with CAR constructs (SB transposon; 2 µg) and pCMV-CAT (SB transposase; 0.4 µg) at a 5 : 1 ratio in a total volume of 20 µL.

Electroporation was performed using 4D-Nucleofector, followed by incubation in TexMACS medium supplemented with 5.0% FBS, 10 ng/mL IL-7, and 5 ng/mL IL-15 at 37°C with 5.0% CO_2 for 10 min. In parallel, PBMC feeder cells ($10–15 \times 10^6$) were irradiated at 25 gray and mixed with TexMACS medium supplemented with 5.0% FBS, 10 ng/mL IL-7, and 5 ng/mL IL-15. The feeder cells are necessary for the transposon-based CAR to maintain its proliferation and expansion.

The feeder cells and electroporated cells were combined in 48-well plates and further incubated. The cells were cultured for 14 days, with media changes on days 4 and 7 using fresh TexMACS medium supplemented with 5.0% FBS and 10 ng/mL IL-7 and 5 ng/mL IL-15. On day 14, the efficiency of CD19 and CD20 CAR was analyzed by incubating the cells with specific ligands, and flow cytometry was used to detect the expression levels

K562 cell line transfection

To generate CD19 antigen-bearing K562 cell lines, SB transposons [11] (pSBbi-RP: a gift from Kowarz E.) bearing CD19 antigen (pSBbi-RP-CD19) and transposase [12] (pCMV-CAT; a gift from Izsvak Z.) were transfected using the Maxcyte OC-25 electroporation protocol. Electroporation was performed in 2×10^6 K562 cells in a total volume of 25 µL that consisted of 1 µg of pCMV-CAT and 5 µg

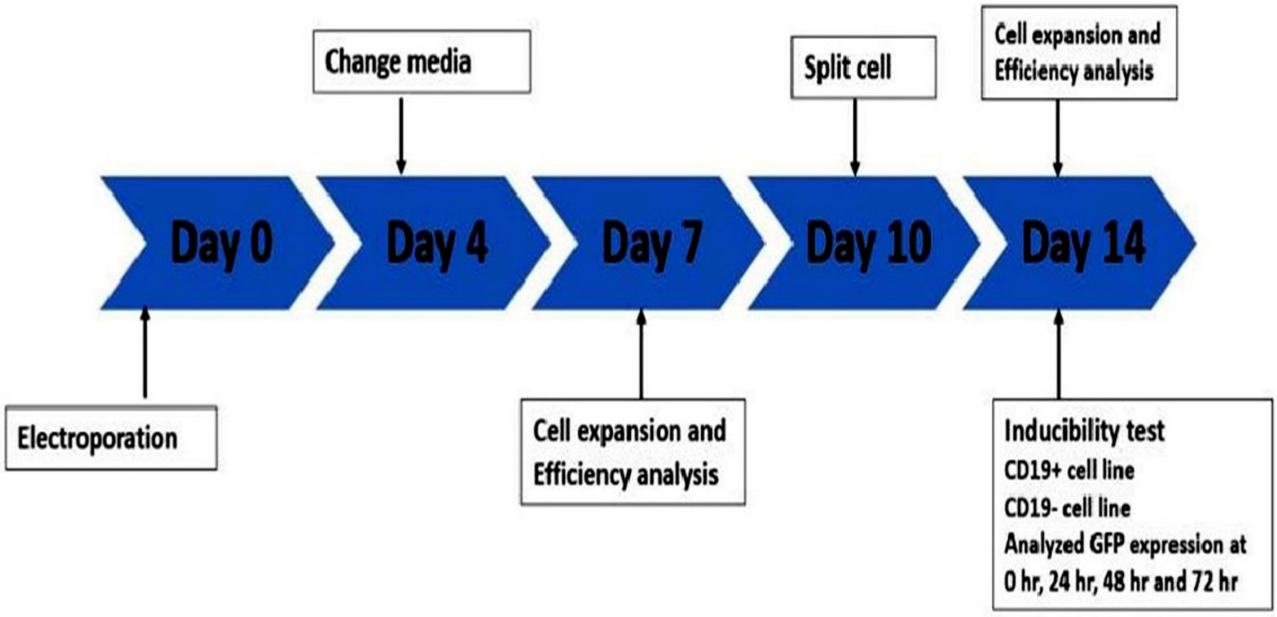

Figure 2. Inducibility test schema for CD20 CAR expression; On day 0, CAR constructs were electroporated in PBMCs, on day 4 the supernatant was removed and replaced with the new media, on day 7 cell viability and efficiency of CAR was checked, on day 10 cells confluency were checked and split, on day 14 the cell expansion was analysed and inducibility test was performed in the presence or absence of $CD19^+$and $CD19^-$ cells at 24 hrs, 48 hrs and 72 hrs.

of pSBbi-RP-CD19. The pSBbi-RP transposon vector carries a red fluorescent marker (dTomato) and a puromycin-resistant gene. Cells were selected with puromycin antibiotics after 24 h of electroporation.

Chimeric antigen receptor (CAR) expression analysis

Cells with their respective antibodies were incubated at 4^oC for 15 min, washed, and analyzed by flow cytometry after resuspending with 400 µL of staining buffer. The flow cytometry results were analyzed using FlowJo. CD19 CAR expression in $CD3^+$ T cells was analyzed using CD19 biotinylated peptide after staining with antibiotin PE antibody (Miltenyi Biotec, Bergisch Gladbach, Germany).

CD20 CAR was measured by the level of GFP expression after CARs co-cultured with $CD19^+$ NALM-6 cells. Then, 10^5 $CD19^+$ NALM-6 cells were co-cultured with 10^5 CAR-transfected $CD3^+$ T cells, and GFP was measured at different time points, i.e., 0, 24, 48, and 72 h **(Figure 2).**

Data analysis

All DNA sequences were analyzed by Geneious Prime software. Schematic illustrations were made using BioRender.

Results

Confirmation of the expression of the second CAR (CD20 CAR) in a construct expressing the first CAR (CD19 CAR) via the human phosphoglycerate kinase (hPGK) promoter

The transfection efficiency of the first CAR, i.e., CD19 CAR was analyzed on day 14 post-transfection. As shown in **Figure 3A**, in donor 1, 7.5%, 8.7%, and 9.0% were CD19 CAR-positive in CAR constructs bearing 8xNFAT, 8xNR4A, and 4xNFAT + 4xNR4A respectively. Similarly, in donor 2, 4.9%, 13.7%, and 14.8% were CD19 CAR-positive in CAR constructs bearing 8xNFAT, 8xNR4A, and 4xNFAT + 4xNR4A, respectively. The mean fluorescent intensity (MFI) values were 107, 114, and 119 molecules per cell in CAR constructs bearing 8xNFAT, 8xNR4A, and 4xNFAT + 4xNR4A from donor 1, respectively. In donor 2, the MFI values were 74.5, 322, and 119 molecules per cell in CAR constructs bearing 8xNFAT, 8xNR4A, and 4xNFAT + 4xNR4A, respectively. A weaker CD19 CAR expression was observed utilizing the hPGK promoter **(Figure 3A - 3B)**. However, to determine the expression of CD20 CAR only after the first antigen interaction, the GFP MFP was examined before and after the antigen stimulation conditions. **Figure 3C** shows that after 48 and 72 h of co-culture with $CD19^+$ NALM6 cells, the MFI steadily reduced in comparison with the no antigen

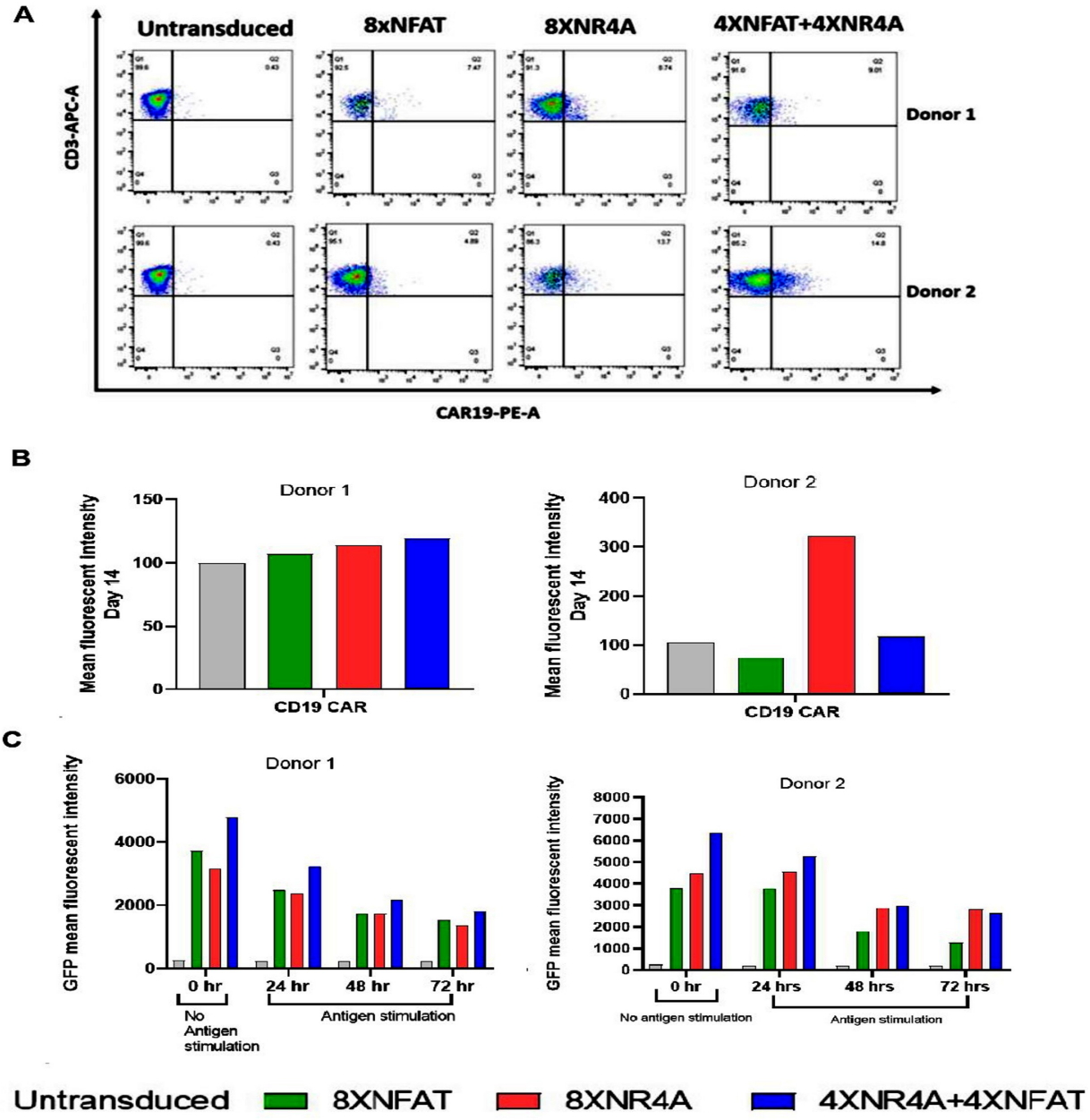

Figure 3. Inducibility test. **(A)** Flow cytometric representation of CD19 CAR expression in two independent healthy blood donors on day 14 post-transfection; **(B)** CD19 CAR mean fluorescence intensity (MFI) on day 14 post-transfection from two independent healthy blood donors; **(C)** GFP mean fluorescence intensity (MFI) at different time points from two independent healthy blood donors. There was no significant GFP induction was observed.

stimulation condition. GFP overexpression was not seen after stimulation with cells expressing the CD19 antigen. Therefore, the hPGK promoter might not be strong enough to drive CD19 CAR and was subjected to promoter interference in this complex construct; thus, owing to the lack of optimal endogenous CAR T cell activation signal, we could not see the increased expression of GFP upon antigen stimulation.

Confirmation of the expression of the second CAR (CD20 CAR) in a construct expressing the first CAR (CD19 CAR) via the EF-1á promoter

The expression of CD19 CAR on day 14 post-transfection was measured. As expected, the expression of CD19 CAR was remarkably improved, where 19.7%, 88.5%, and 37.4% of the cells were CD19 CAR-positive in a construct bearing 8xNFAT, 8xNR4A, and 4xNFAT + 4xNR4A, respectively.

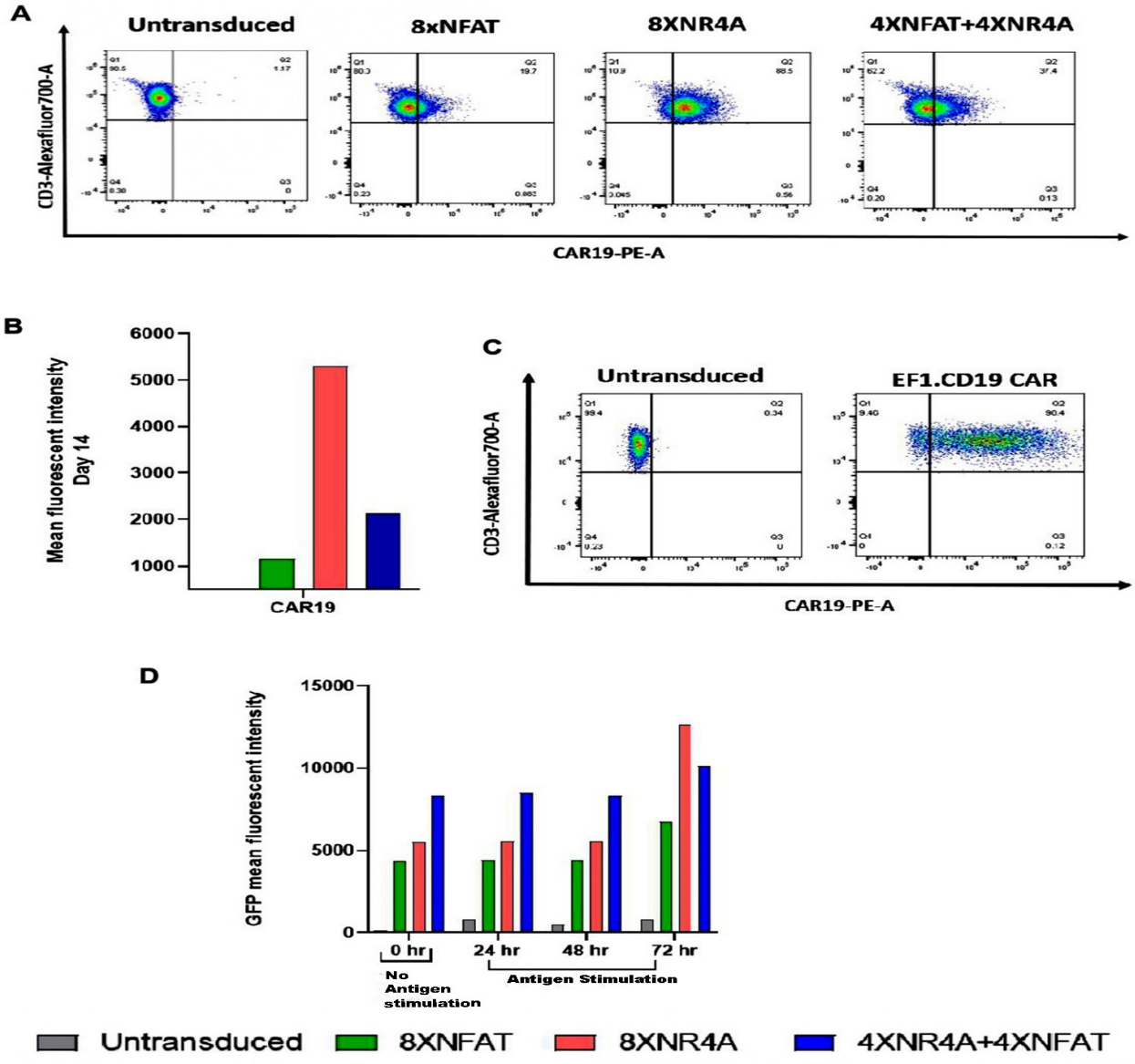

Figure 4. Inducibility test of CAR constructs bearing EF1 promoter. **(A)** Flow cytometric representation of CD19 CAR expression on day 14 post-transfection; **(B)** CD19 CAR mean fluorescence intensity (MFI) on day 14 post-transfection; **(C)** Flow cytometric representation of CD19 CAR expression bearing only the EF1 promoter, on day 14, post-transfection; **(D)** GFP mean fluorescence intensity (MFI) after 24 hrs, 48 hrs and 72 hrs of co-culture with presence of CD19 antigen. This is data from only one healthy blood donor

Interestingly, the CAR construct bearing the 8xNR4A promoter showed a strong CD19 CAR expression **(Figure 4A)**. As shown in **Figure 4B**, the MFI of CD19 CAR was greatly improved in all CAR constructs bearing 8xNFAT, 8xNR4A, and 4xNFAT + 4xNR4A when utilizing the EF-1α promoter. However, the construct bearing 8xNR4A, which showed a strong CD19 CAR expression, also has a high MFI when compared with the constructs bearing 8xNFAT and 4xNFAT + 4xNR4A (5294 *vs* 1150 and 2141) (**Figure 4B**). Although the expression of CD19 CAR was improved in these three CAR constructs even though EF-1α is a strong promoter that efficiently drives CAR in most of the cases, in this study, CAR constructs bearing 8xNFAT and 4xNFAT + 4xNR4A exhibited comparatively weak CD19 CAR expression. Therefore, to determine the transfection efficiency of the EF-1α promoter in a single CAR-bearing construct, a CAR construct with only CD19 CAR was made under the control of the EF-1α promoter (EF1.CD19 CAR), and the CAR expression on day

14 post-transposition was analyzed. As shown in **Figure 4C**, 90.4% of the cells were CD19 CAR-positive, and no interference in function was observed while driving a single CAR.

Furthermore, the MFI of the GFP expression was also measured before $CD19^+$ K562 antigen stimulation at 0 h and after $CD19^+$ K562 antigen stimulation at 24, 48, and 72 h. Interestingly, the GFP expression was also upregulated in all three constructs particularly 72 h post-antigen stimulation compared with 0 h (8x NFAT, 6,688 *vs* 4,368; 8xNR4A, 12,667 *vs* 5,468; and 4xNFAT + 4xNR4A, 10,148 *vs* 8,315) **(Figure 4D).** Out of these three promoters bearing CAR constructs, the 8xNR4A promoter showed the highest expression of GFP, which also showed significant CD19 CAR expression, suggesting that CD20 CAR expression was mediated by CD19 CAR activation.

Discussion

Immunotherapies, such as CAR T cell therapy, have greatly transformed the survival rate of various cancers. However, a substantial number of patients still relapsed with antigen loss after receiving CAR T cell treatment.[3, 13, 14] As a result, two CARs with distinct antigen-binding domains have been expressed simultaneously as part of dual antigen-specific CAR methods.[15 - 17] However, dual CAR T cell therapy has a notable limitation, specifically in terms of simultaneous expression of both CARs, that is, the continuous activation of both CARs may subject the T cells to prolonged stimulation via tonic signaling leading to exhaustion.[18 - 20] Therefore, employing a system where the expression of one CAR is triggered under a specific condition may enhance the precision of CAR T cell response.[21 - 22] As a result, rather than co-expressing two CARs targeting different antigens simultaneously, our strategy emphasizes the conditional expression of the second CAR. To achieve this hypothesis, the repetitive fragments of binding motifs of NFAT, NR4A, and a combination of NFAT and NR4A transcription factors [8] were utilized, which control the expression of the second CAR upon getting the activation signal from the first CAR.

As proof of this concept, the CD19 CAR was utilized as the first CAR and the CD20 CAR as a second CAR. The CD19 CAR is constitutively expressed, albeit at a low density, to serve as the primary receptor. The hPGK promoter was selected for driving the expression of CD19 CAR because it demonstrated effective cytotoxic activity even with lower CAR expression in vitro.[5, 6] A lower density of CAR molecules can reduce the risk of excessive activation and associated toxicities while still effectively targeting CD19-positive cells for immune-mediated destruction. The expression of the second CAR, targeting CD20, is then induced by the activation signal from the CD19 CAR upon its engagement with the antigen.

However, this conditional strategy does not address the challenge of antigen loss variants, where tumor cells may downregulate or lose the expression of the targeted antigens, thereby evading CAR T cell detection and destruction. To monitor CD20 CAR expression, GFP upstream of CD20 CAR was inserted, separated by a P2A site. However, the CAR constructs utilizing the hPGK promoter demonstrated minimal CD19 CAR expression but interestingly exhibited high GFP expression, which is supposed to dictate CD20 CAR expression. We hypothesized a high GFP expression might be caused by the endogenous T cell receptor (TCR) signaling pathways as the release of transcription factors such as NFAT and NR4A is not a rare event.[23] The TCR molecular processes might disrupt the CAR-mediated signaling pathway, potentially causing an increase in baseline GFP expression. To address the issue of insufficient CD19 CAR expression, we opted for the robust constitutive EF1 promoter, known for its strong activity in driving large RNA transcripts. Consequently, the hPGK promoter was replaced with the EF1 promoter. This switch resulted in an improvement in the expression of CD19 CAR across all three constructs. However, constructs incorporating 8xNFAT and 4xNFAT + 4xNR4A exhibited lower CD19 CAR expression than those with 8xNR4A alone. This observation is intriguing because the expression of CD19 remained unaffected when the EF1 promoter was utilized in a single CD19 CAR construct. Therefore, the inclusion of certain transcription factor binding sites in our construct might lead to reduced expression of an adjacent gene (in this case, CD19 CAR) because of competition for transcription regulators.[24, 25]

Although the EF1 promoter has demonstrated effectiveness when positioned alongside other inducible promoters in lentiviral systems, a potential reduction in CD19 CAR expression may be caused by the positional effects of the promoter.[25, 26] Placing a promoter in specific positions within a vector,

particularly in a transposon-based system like ours, might lead to transcriptional interference, where the activity of one promoter may hinder the transcription of another nearby promoter, thus lowering the expression of downstream genes. Consequently, to determine whether the positional effect influences the strength of their gene expression, more investigations into swapping the positions of the EF1 promoter and the inducible promoter may be needed.

Despite the high GFP expression before antigen exposure, the construct with the 8xNR4A promoter, which exhibited the strongest CD19 CAR expression, demonstrated the highest GFP expression after 72 h of antigen stimulation. This observation may imply a potential dose–response relationship in initiating the inducible activity of CAR T cells. In this context, further exploration of different effector-to-target ratios is necessary to better understand the dynamic regulation of inducible CAR T cells and observe how varying ratios may affect their responsiveness. In addition, functional tests may be required to assert the expression of CD20 CAR in our system by co-culturing them to the mixture of only $CD19^+$ and $CD20^+$ cells.

Conclusion

This study established a proof of concept, demonstrating the feasibility of using naturally occurring binding motifs of T cell transcription factors to conditionally express CAR in the design of a dual CAR T cell-targeted therapy. Our study's overall findings suggest that activation-induced CAR is possible; however, the optimization of transcription factor binding sites is crucial. In addition, this study aimed to demonstrate the feasibility or initial efficacy of this approach; thus, we included fewer donors. However, for future studies, to explore more aspects of this platform, at least three donors will be used.

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Conflicts of interest statement

The authors have no commercial, proprietary, or financial interest in the products or companies described in this article. The Ratchadaphiseksomphot Matching Fund from the Faculty of Medicine, Chulalongkorn University.

Data sharing statement

All data generated or analyzed during the present study are included in this published article. Further details are available for noncommercial purposes from the corresponding author on reasonable request.

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