Master of Science

Optimal Chemokine Receptors for Enhancing Immune Cell Trafficking for Adoptive Cell Therapy

The Graduate School

of the University of Ulsan of Medicine

Department of Medical Science

DoYeon Han

Optimal Chemokine Receptors for Enhancing Immune Cell Trafficking for Adoptive Cell Therapy

Supervisor: Hee Jin Lee

A Dissertation

submitted to

the Graduate School of the University of Ulsan

In partial Fulfillment of the Requirements

for the Degree of

Master of Science

by

DoYeon Han

Department of Medical Science

University of Ulsan College of Medicine, Korea

August 2024

Optimal Chemokine Receptors for Enhancing Immune Cell Trafficking for Adoptive Cell Therapy

This certifies that the master's thesis of

DoYeon Han is approved.

_____

Committee Chair Dr. Gyungyub Gong

_____

Committee Member Dr. Hee Jin Lee

_____

Committee Member Dr. Byung-Kwan Jeong

Department of Medical Science

University of Ulsan College of Medicine, Korea

August 2024

Abstract

To enhance the effectiveness of adoptive cell therapy (ACT) for solid tumors, the engineering of chemokine receptors on immune cells has been developed. Given the variability in chemokine secretion among different tumor types, it is crucial to identify and modulate the appropriate chemokine receptors. In this study, I utilized extensive RNA sequencing data from both tumor tissues from The Cancer Genome Atlas (TCGA) and normal tissues from Genotype-Tissue Expression (GTEx) to investigate the expression profiles of chemokines. According to analysis I identified eight chemokine receptors that can be paired to chemokines increased in tumor tissues compared to normal, as promising candidates for enhancing ACT. Further examination of tumor-infiltrating lymphocytes (TILs) and chimeric antigen receptor (CAR) T cells revealed that five out of eight candidate chemokine receptors did not exhibit elevated expression in T cells. Among six candidates, I demonstrated that CXCR5 was a particularly promising candidate for enhancing cell migration without compromising cell viability or cytotoxicity, as confirmed by in vitro experiments. In conclusion, my study underscores the potential of five chemokine receptors (CCR6, CCR9, CXCR1, CXCR5, XCR1) as valuable targets for modulation in ACT to enhance cell trafficking and potentially improve cancer therapy outcomes.

Keywords: Adoptive cell therapy (ACT), chemokine, chemokine receptor, CXCR5 receptors

Contents

List of figures and tables

Table

Figure

Introduction

Adoptive cell therapy (ACT) is a treatment using patients' own immune cells, and it involves engineering them to enhance their ability to recognize and attack their tumors. ACT usually uses immune cells like CD8+ T cells (cytotoxicity T cells) which target cancer cells and induce the immune responses.[1, 2] However, their effectiveness for the treatment of solid tumors still remains poor .[3, 4, 5, 6]

One of the causes that challenges ACT for solid tumors is inefficient trafficking and infiltration of T cells.[7] Chemokines and their receptors play an important role in the trafficking and infiltration of immune cells, making them promising candidates to be engineered in ACT.[8] Immune cells with chemokine receptors can migrate to cells with matched chemokine expression. For instance, CXCR5/CXCL13 especially play a role as B cell homing ability in follicles of lymphoid tissues.[9, 10]

One of the methods to enhance ACT, the research has studied expressed chemokine receptors in the immune cells.[11] CAR-T cells that target CD70 are expressed with additional CXCR1/2 receptors to treat aggressive tumors like glioblastoma, ovarian and pancreatic cancer. As a result, enhanced trafficking ability was checked in vitro and in vivo experiments.[12] Another research showed that Mesothelin-targeted CAR-T cells using CCR2b and CCR4 enhanced trafficking ability to treat non-small-cell lung carcinoma.[13] These chemokine receptors have been studied using CAR-T cell therapy.

CAR-T cell therapy can treat the cancer using T cells which target surface antigens. It can be used by patients who expressed common antigens. For this reason, CAR-T cells can be produced in large quantities. However, because of the targeting surface antigens of the tumor, it has low efficiency in the solid tumors.[14] On the other hand, T cell receptor engineered T cells (TCR-T) use naturally occuring T cell receptors (TCR) which can target both cell surface and intracellular antigens presented by MHC class molecules, resulting in higher sensitivity in tumor recognition important for solid tumor treatment.[15] 1G4 TCR is well-established receptor in the TCR-T therapy. 1G4 TCR is known as targeting NY-ESO-1 antigens in the tumor. NY-ESO-1 expressions have been revealed in variable tumor types including breast cancer, non-small cell lung cancers, and myeloma.[16, 17]

In my study, I aim to find the optimal chemokine receptors to use for the ACT, and I analyzed the public databases. One of them, CXCR5, was engineered to be coexpressed in 1G4 TCR-T to prove that the T cell trafficking increased.

Material and Methods

Patients' samples and healthy blood donors

I obtained healthy donor blood samples in the Asan Medical Center. Through peripheral blood mononuclear cell (PBMC) isolation using the Sepmate tube (#85450, STEMCELL, Vancouver, Canada) and lymphoprep (#07801/07811, STEMCELL), I obtained the PBMC cells and stored in CyroStor CS10 buffer (#07930, STEMCELL) in the LN2 tank. Before using the PBMC cells, I rested 1 day in the RPMI 1640 (#22400-089, Gibco, NY, USA) with 10% FBS (#16000-044, Gibco) and 1% penicillin-streptomycin (#15140122, Gibco). 14 patients samples were selected from breast cancer samples that were successfully cultured for both TILs and cancer cells, of which samples were collected previously.[18, 19] This study was approved by the institutional review board of Asan Medical Center (approval no. 2015–0438 and 2017-0784), and written informed consent was obtained from the patients and donors.

Cell lines and cell culture

The human cancer cells Jurkat, A375, MDA-MB-231, MCF-7 and SK-HEP-1 were obtained in ATCC. Jurkat, MDA-MB-231, MCF-7 cells were cultured in RPMI 1640 medium (#A10491-01, Gibco) with 10% FBS and 1% penicillin-streptomycin. A375 and SK-HEP-1 were cultured in DMEM (#11995-065, Gibco) with 10% FBS and 1% penicillin-streptomycin. All cancer cell lines were negative results about mycoplasma using e-MycoTM Mycoplasma PCR Detection Kit (#25235, LiliF).

Databases and analysis

Gene expression RNAseq about tumor tissues in TCGA and normal tissues in GTEx was obtained in UCSC Xena. https://buly.kr/5JJcniQ (accessed on 07 February 2022) The dataset unit was log2 (FPKM+0.001). I obtained a total of 9,807 sample data from TCGA and a total of 7,858 sample data from GTEx. In TCGA, 212 of the 9,807 samples were normal tissue samples, so 9,595 samples were used for analysis excluding them.

Before analysis, I first performed T-test and P-test to determine whether there was a significant difference in the average expression amount in tumor tissues compared to normal tissues. The T-test was used to determine whether this was a dispersion group, such as a dispersion group (two groups with this variance assumption: F-ratio >F rejection, and two groups with such variance assumptions: F-ratio = <F rejection). Based on this, when the P-test was performed (T< = t), only chemokine indicating a value less than 0.001 was selected.

Based on the calculated fold change values, I selected chemokines which expressed more than twice in the tumor tissues than normal tissues. The Fold change value of the selected chemokine was calculated in the following order.

1. Calculating the average amount of expression of normal tissue or tumor tissue of each Chemokine (Unit: log2(FPKM+0.001))
2. (FPFM+0.001) = 2^ (The average amount of expression of normal tissues or tumor tissues of each chemokine)
3. Fold Change = (FPFM+0.001) in tumor tissues/ (FPFM+0.001) in normal tissues

I re-checked the average expression in the normal tissue and tumor tissue of each ligand of the selected chemokine receptors. Through this, the ligand and binding receptors that are highly expressed in tumor tissue were identified. After listing selected chemokines' receptors, I analyzed the expression of chemokines for each sample of TCGA by specifying the upper quartile values of GTEx samples as cut-off values.

Chemokine receptors' expression in CAR-T cells were analyzed using Gene Expression Omnibus (GEO) database. Samples available in six GEO databases (GSE140107, GSE178570, GSE178998, GSE189932, GSE220927, and GSE218791) were selected and analyzed. If the gene value is NA in selected samples, it was treated as 0 and analyzed. For comparison with the sixth GEO databases, I conducted a batch correction using the combat of sva packing in the R program.

Expansion of the TILs and RNA sequencing

TILs were isolated from tumor tissues, expanded and cultured as previously described.[18, 19] The prepared samples were submitted to a Macrogen (Seoul, Republic of Korea) for bulk RNA-sequencing analysis. The library was created by Macrogen using the TruSeq Stranded mRNA Sample Prep Kit. Raw data was processed in the order of QC (fastq trimming), alignment (STAR v2.7.10a), and quantification (RSEM v1.3.3). In the alignment process, reads mapping to the reference genome (GRCh38) was used in. Output data converted to FPKM and used for analysis. Following this, biological and statistical analyses were conducted using the gene expression data, leading to the identification of significant findings and the assessment of statistical significance.

Lentiviral vector constructions

CXCR5 and CXCL13's coding DNA sequence (CDS) source in National Center for Biotechnology Information (NCBI). I isolated RNA in PBMCs using RNeasy Mini kit (#74104, QIAGEN). Extracted RNA was synthesized into CXCR5 gene using Bioneer Pfu pre-mix kit (#K-2301, Bioneer, Daejeon, Republic of Korea) and primers. CXCR5 and 1G4 genes cloned in FUGW lentiviral vector (#14883, Addgene, Watertown, MA, USA). CXCL13 gene was synthesized in the Bionics company and then cloned in pCDH vector (#72266, Addgene). I used primer information that was used for cloning (table 1). I used restriction enzymes Bmt1-HF (R3658, New England Biolabs, Ipswich, MA, USA), Kpn1-HF (#R3142, New England Biolabs) and rCutSmart buffer (#B6004S, New England Biolabs) in digestion processes. DNA purification used Gel extraction kit (#28704, QIAGEN) and PCR

purification kit (#K310001, Invitrogen, Waltham, MA, USA). In ligation processes, I used Gibson Assembly® Master Mix (#E2611, New England Biolabs). MINI prep kit (#CMP0112, LaboPass™, Seoul, Republic of Korea) and MIDI prep kit (#740420.50, MACHEREY-NAGEL, Dueren, Germany) used for obtaining plasmids after MINI and MIDI culture. All elution buffer was using Water-RNase & DNase free (#BW012, BioSolutions, Rockville, USA). I performed electrophoresis to check that it was going correctly during the cloning process. For this, I used a dilution buffer from 50x TAE buffer (#CBT3020, Dyne bio, Seongnam, Republic of Korea) to 0.5x TAE buffer. I used Dyne Agarose star (#DE100, Dyne bio) to make 1% agarose gel. And for electrophoresis, Dyne Loading STAR (#A750, Dyne bio), and Dyne 1 kb Plus DNA ladder (#A738, Dyne bio) were used. The kits described were used according to the manufacturer's experimental protocol.

Table 1. information of primer sequences

GENE		DESCRIPTION	5'-3' SEQUENCE
EF1α-1G4-WPRE		forward	TCCAGTTTGGTTAATTAGCTAGCCGTGAGGCTCCGGTG
		reverse	AGTCATTGGTCTTAAAGGTACCCAGGCGGGGAGG
EF1α-cxcr5-WPRE		forward	TCCAGTTTGGTTAATTAGCTAGCCGTGAGGCTCCGGTG
		reverse	AGTCATTGGTCTTAAAGGTACCCAGGCGGGGAGG
EF1α-1G4-cxcr5-WPRE	EF1A-1G4-T2A	forward	TCCAGTTTGGTTAATTAGCTAGCCGTGAGGCTCCG
		reverse	GTAGTTCATTGGGCCAGGATTCTC
	CXCR5-WPRE	forward	CTGGCCCAATGAACTACCCGC
		reverse	AGTCATTGGTCTTAAAGGTACCCAGGCGGGGAGG
CXCL13-EM7-BleoR	CXCL13	forward	TCTAGAGCCACCATGAAGTTCATCTCGACATCT
		reverse	ACGCGTCGACTCAATGGTGATGGTGATGATG
	EM7-BleoR	forward	ACGCGTCGACGTTGACAATTAATCATCGGCA
		reverse	GGGGTACCTCAGTCCTGCTCCTCG

Creation of Lentivirus

Lenti-X 293T cells cultured in DMEM with 10% FBS, 1% penicillin-streptomycin in T175 flask before doing transfection experiments. For making virus particles, I used lentiviral plasmid samples, packing vectors (gag-pol, ENV, REV) and Lipofectamine™ 3000 Transfection Reagent (#L3000-075, Invitrogen). After 2 days, the supernatant in the 175 T flask was recovered and filtered by Millex® 33 mm PES .45 μm (#SLHPR33RS, Millipore, Burlington, MA, USA) and 50 cc syringe. filtered samples were treated with the Lenti-X™ Concentrator (#631232, Takara, Kusatsu, Japan) in 4 days. All viruses were suspended in RPMI 1640 (#22400-089, Gibco) and aliquoted. To find the virus concentration, I tested p24 titration using Lenti-X™ qRT-PCR Titration Kit (#631235, Takara) according to the manufacturer's protocol.

Production of effector and target cells to transduction

$2X10^7$ PBMCs were cultured in RPMI 1640 (#22400-089, Gibco) supplemented with 5% FBS, 1% penicillin-streptomycin, 20 IU/ml IL-2 (#130-097-748, Miltenyi Biotec, Bergisch Gladbach, NRW, Germany) and 100ul of T Cell TransAct™ human (#130-111-160, Miltenyi Biotec) for 2 days. $2X10^6$ activated PBMCs were seeded with each lentivirus in 6 well plate. and I did a centrifuge for 2 hours at 32°C. After centrifuge, I cultured the cells in RPMI 1640 with 5% FBS, 1% penicillin-streptomycin and 400 IU/ml IL-2. I changed the media every two days after transduction.

A375 Luc cells were used for cancer cells to be expressed as CXCL13. After transduction, A375 Luc+/CXCL13+ cells cultured in DMEM with 10% FBS, 1% penicillin-streptomycin, puromycin (#ant-pr-1, Invivogen, San Diego, CA, USA) and zeocin (#R25001, Thermo Fisher Scientific).

Antibiotic kill curve experiment

To proceed with cell selection for the transduced Luc+/CXCL13+ A375 cell, zeocin of an appropriate concentration was set. I co-cultured A375 cells with 9 points of zeocin concentration (0, 50, 100, 200, 400, 600, 800, 1000 ug/ml) using Quanti-Max™ WST-8 Cell Viability Assay Kit (#QM 1000, Biomax, Guri, Republic of Korea). I tested the kit according to the manufacturer's protocols. Cell viability rate was calculated with the described formula using optical density of nano drop devices.

% of Cell viability rate = (OD sample value - OD blank value) / (OD control value - OD blank value) × 100

Flow cytometry

FACS analysis was performed to check the expression of the transduced PBMCs. After Fc blocking using Human TruStain FcX™ (#422302, Biolegend) for 5 min at RT, transduced PBMCs were performed fluorescence-activated cell sorting (FACS) staining with antibodies for 20 min at 4°C in the dark. Antibodies are listed in table 2. PBMCs washed and resuspended with FACS buffer by 3 times. The cells were centrifuged at 4°C for 5 min and resuspended with DAPI (#D3571, Invitrogen). I performed FACS analysis using FACSCanto ™II device (BD Biosciences, Santa Clara, CA, USA). Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Table 2. Antibodies used for flow cytometry

MARKER	FLUORESCENCE	CAT NO.	COMPANY
human CD3	APC/Cyanine7	300318	Biolegend
human CD4	FITC	300506	Biolegend
human CD8a	PerCP/Cyanine5.5	301032	Biolegend
mouse TCR β chain	PE	109208	Biolegend
human CD185 (CXCR5)	APC	356908	Biolegend

Migration ability test

I used 6.5 mm Transwell® with 5.0 μm in the 24-well plate (#3421, Corning, NY, USA). To examine migration ability, I put the effector cells in upper chambers and put chemotaxis buffer (0.1% BSA in the RPMI 1640) with recombinant human CXCL13 protein (#574704, Biolegend, San Diego, California, USA) in downstream chamber. I incubated for 2 hours in the CO2 incubators. After incubation, all the buffer was collected to the FACS tubes (#352052, Falcon London, UK), and mixed with 100ul of precision count beads (#424902, Biolegend). Each ratio was calculated with precision count beads protocol using FACS data.

Western blot

For checking migration signaling, cells that were expressed 1G4 or 1G4-T2A-CXCR5 incubated in the RPMI 1640 with recombinant human CXCL13 protein for 0, 5, 10 and 15 minutes. Then, the washing process was carried out with DPBS. To extract proteins in the cells, I used a RIPA

buffer which was added 1 mM PMSF (#9806, Cell signaling technology, Danvers, MA, USA) and incubated for 5 minutes in the ice. After centrifuge 14,000G for 10 minutes in the 4℃, I obtained a supernatant which contained proteins. I quantified the amount of protein to use via BCA assay using Pierce™ BCA Protein Assay Kits (#23227, Thermo Fisher Scientific, Waltham, MA, USA). To check migration signaling, I used p44/42 MAPK (Erk1/2) Antibody, Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody, Akt Antibody and Phospho-Akt (Ser473) Antibody (#9101, #9102, #9271, #9272, Cell signaling technology).

CXCL13 ELISA assay

The cells were cultured in a 75T flask in DMEM (#11995-065, Gibco) with 10% FBS, 1% penicillin-streptomycin and antibiotics. After 48 hours, the supernatant was collected from the 75T flask and centrifuged at 1500 RPM for 10 minutes at 4 ℃. Subsequently, The recovered supernatant was diluted by 1/100 and used as a sample. The assay was performed following the manufacturer's protocol.

Interleukin-2 (IL-2) and interferon gamma (IFN-γ) production

The cells were co-incubated in a 24-well plate with an Effector-to-Target (E:T) ratio equivalent to the cell-to-media ratio used in the Luciferase assay. After 24 hours, the supernatant was collected from the 24-well plate and centrifuged at 1500 RPM for 10 minutes at 4 ℃. Subsequently, The recovered supernatant was diluted by 1/25 and used as a sample. The ELISA procedure was performed following the manufacturer's protocol.

Luciferase-based cytotoxicity assay

3×10^4 LUC+/CXCL13+ A375 cells (target cells) were cocultured with transduced PBMCs as effector-to-target (E: T) ratios = 1:1, 3:1, 10:1, in white 96 well flat bottom plates for 24 h. The target cells were treated with 0.1% Triton-X-100 to measure maximal cell death relative light units (RLU). After 24 h, 100 μl of BrightGlo Reagent (#E2620, Promega, WI, USA) was added to analyze luciferase assay. Luminescence was measured by Promega device after 5 min of incubation according to the manufacturer's protocol. Percentage Specific lysis was calculated using the following formula.

% specific lysis = 100% × (control PBMCs death data − sample data) / (control PBMCs death data − maximal cell death data).

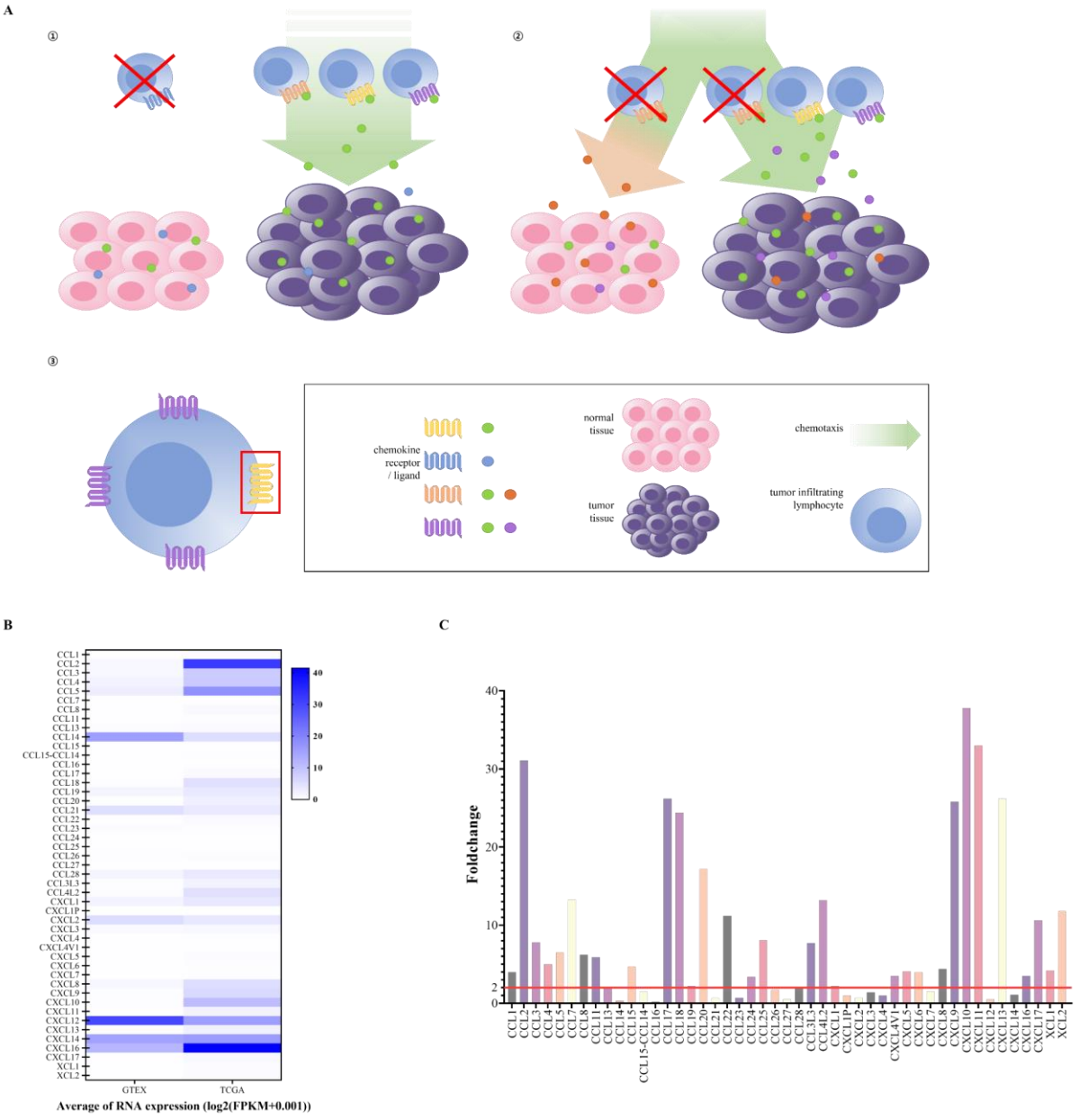
Statistical methods

All experiments were performed in duplicate at least. Statistical analyses were performed using Graphpad Prism (GraphPad Software, La Jolla, CA, USA). Data are shown as mean ± standard deviation (SD). Twoway-ANOVA was used to compare differences between two or three groups. P values less than 0.05 were considered statistical significance (*P < 0.05, **P < 0.01, ***P < 0.001, *****P<0.0001).

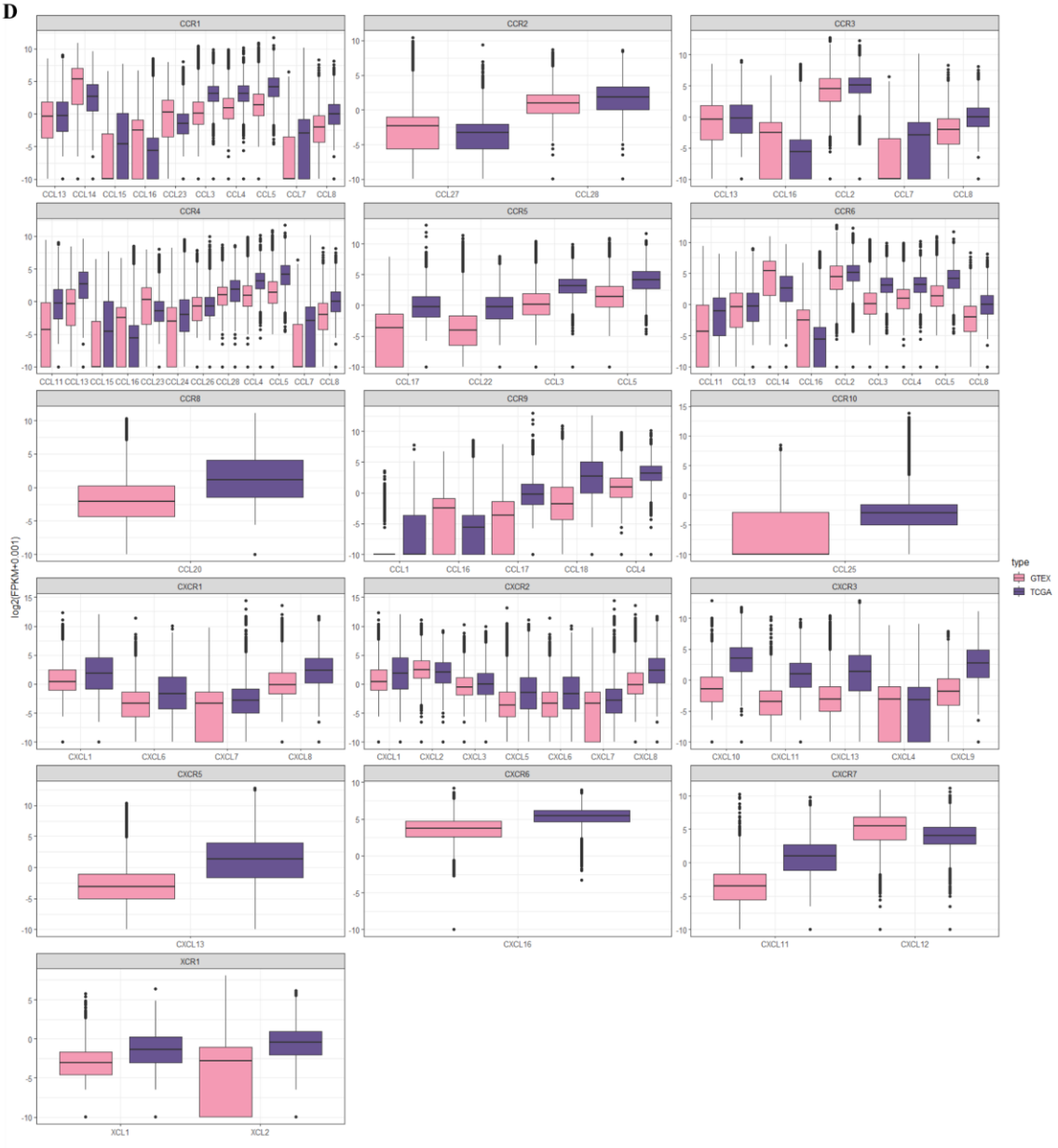
Results

Investigation of chemokines overexpressed in the tumor tissues compared to normal and their matched receptors

To investigate candidate chemokine ligand-receptor pairs for immune cell trafficking, I analyzed chemokine and chemokine receptors' expression in humans according to 3 steps (Figure 1A). When the gene expression levels of chemokines in tumor tissues were compared to normal tissues using TCGA and GTEx data, 31 of 37 chemokines had more than two-fold increase in tumor tissue than normal tissue (Figure 1B-C). Because chemokines are known to correspond to one or more receptors, chemokines and responding receptors were summarized (Table 3). For each 16 chemokine receptors, I compared corresponding chemokine expression in normal tissues and tumor tissues (Figure 1D). From this, I excluded the receptor that binds to the highly expressed chemokines in normal tissues. Based on the calculated cut-off value that determine whether gene expression is increased or not (Table 4), I checked the amount of chemokines' expression in each tumor tissue in TCGA matched for the selected 8 chemokine receptors (CCR4, CCR6, CCR9, CXCR1, CXCR3, CXCR5, CXCR6, XCR1) (Figure 1E). In addition, I calculated the number of samples in which all chemokines matching each chemokine receptor exceed the cut-off value. Corresponding chemokines of CXCR5 were increased in 70% of the collected TCGA samples, making it the most promising candidate receptor regarding pan-cancer chemokines (Figure 1F).



D



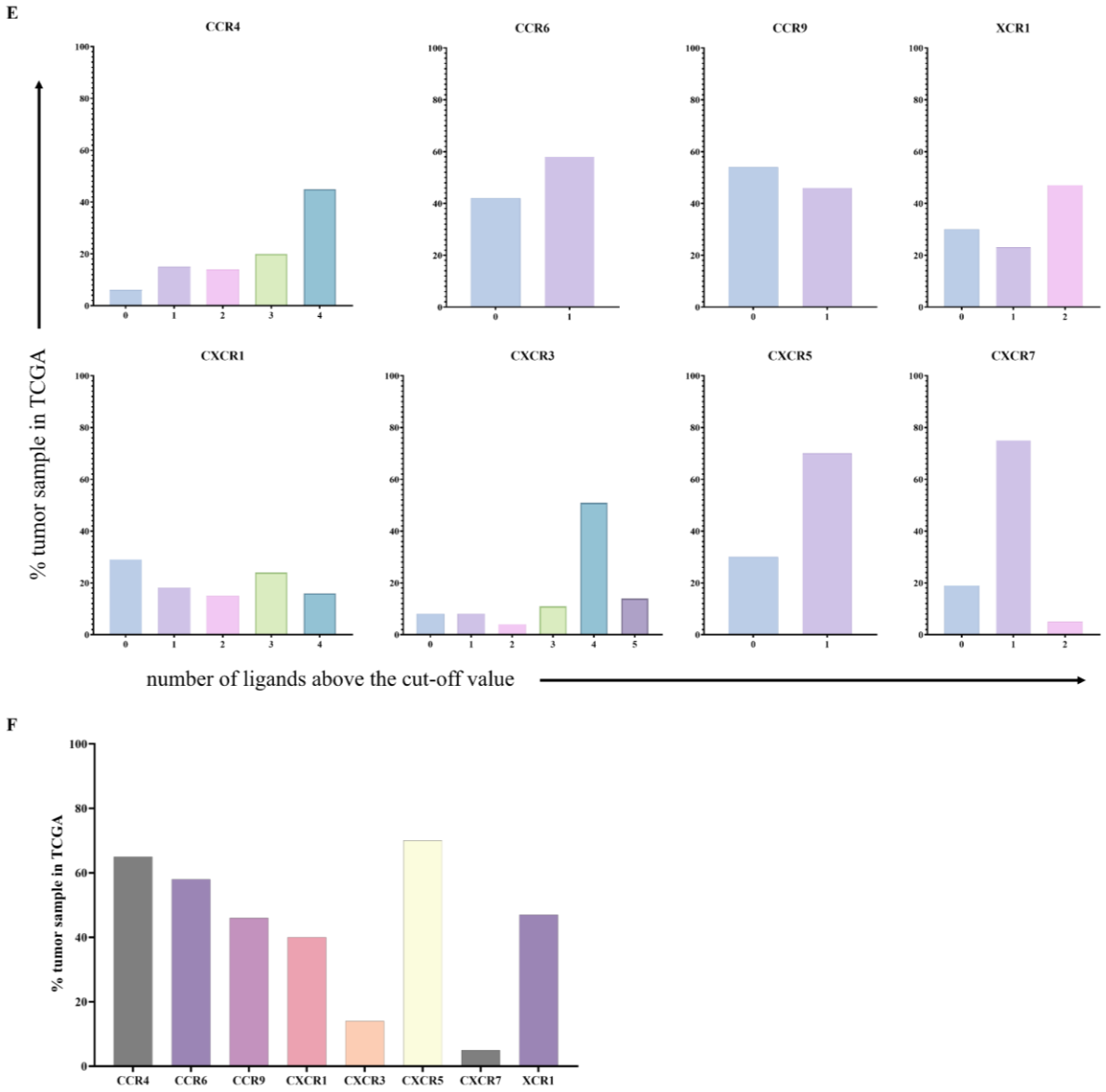
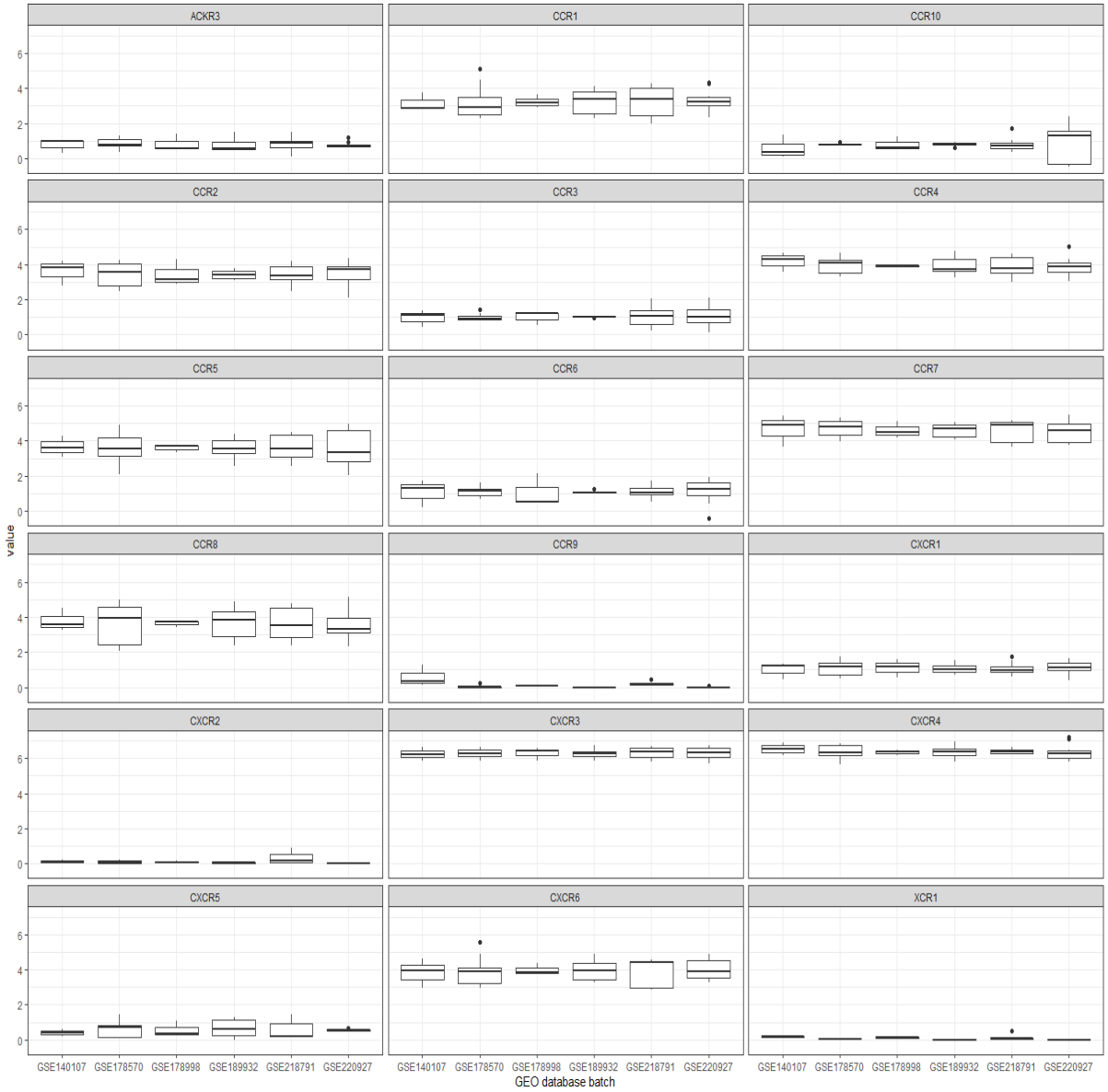


Figure 1. Analysis of chemokines in TCGA and GTEx. (A) The process conducted to select effective chemokine receptors. (B) The chemokines' expression in tumor and normal tissues using the database TCGA and GTEx. (C) Calculated fold changes described in the method (D) Selected chemokine receptors' ligand expression in TCGA and GTEx. (E) Tumor samples' percentage having higher expression compared to each chemokines' cut-off. (F) Percentage of Tumor samples in which all chemokines of chemokine receptors are above cut-off.

Evaluation of expression of candidate chemokine receptors in lymphocytes and engineered T cells

To check chemokine receptors' basal expression in lymphocytes, I analyzed them in CAR-T cells and TILs. I selected six GEO databases of CAR-T cells with chemokine receptors' expression. Among 8 candidate chemokine receptors, CCR6, CCR9, CXCR1, CXCR5 and XCR1 showed very low expression in CAR-T cells (Figure 2A). Additionally, I performed bulk RNA sequencing on the expanded TILs (n=14) to examine the amount of chemokine receptor expression. CCR4, CCR6, CCR9, CXCR1, CXCR5 and XCR1 expression were not increased in expanded TIL compared to average expression of the entire chemokine receptors (Figure 2B). Finally, five chemokine receptors without increase in both CAR-T cells and TILs were selected to be candidate chemokine receptors that can be promising targets to be engineered in TCR-T.



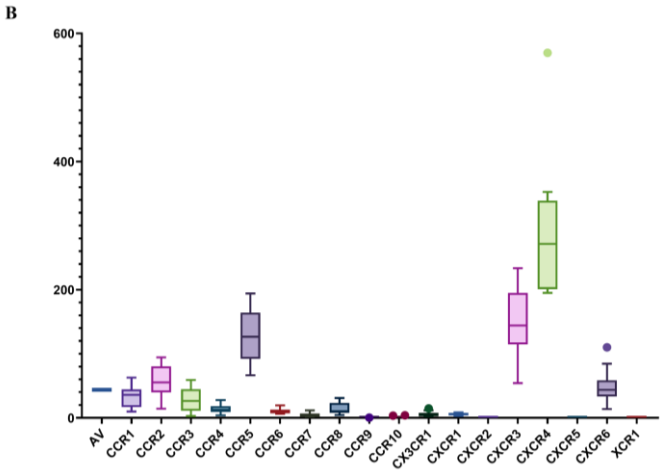


Figure 2. Analysis of chemokine receptors' expression in CAR-T and expanded TILs. (A) Chemokine receptor expression in the CAR-T cells in the GEO database. (B) Chemokine receptor expression in the expanded TIL using RNA sequencing.

Table 3. The list of the chemokine receptors and each corresponding chemokine

chemokine receptor	chemokine
CCR1	CCL3, CCL4, CCL5, CCL7, CCL8, CCL13, CCL14, CCL15, CCL16, CCL23
CCR2	CCL2, CCL7, CCL8, CCL13, CCL16
CCR3	CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL16, CCL23, CCL24, CCL26, CCL28
CCR4	CCL3, CCL5, CCL17, CCL22
CCR5	CCL2, CCL3, CCL4, CCL5, CCL8, CCL11, CCL13, CCL14, CCL16
CCR6	CCL20
CCR8	CCL1, CCL4, CCL16, CCL17, CCL18
CCR9	CCL25
CCR10	CCL27, CCL28
CXCR1	CXCL1, CXCL6, CXCL7, CXCL8
CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8
CXCR3	CXCL4, CXCL9, CXCL10, CXCL11, CXCL13
CXCR5	CXCL13
CXCR6	CXCL16
CXCR7	CXCL11, CXCL12
XCR1	XCL1, XCL2

Table 4. The calculated cut-off value using the upper quartile value using normal tissues' chemokine expression in GTEx

chemokine	upper quartile value (FPKM)
CCL3	1.89
CCL5	3.04
CCL17	-1.43
CCL22	-1.69
CCL20	0.25
CCL25	-2.93
CXCL1	2.46
CXCL6	-1.35
CXCL7	-1.32
CXCL8	1.98
CXCL4	-1.09
CXCL9	0.22
CXCL10	0.46
CXCL11	-1.69
CXCL13	-1.03
XCL1	-1.69
XCL2	-1.09
CXCL16	4.68

Engineering 1G4 TCR-T to express chemokine receptor CXCR5

I engineered vector structures using EF1a promoter to examine the function of TCR-T according to the expression of chemokine receptor CXCR5. Then 1G4, CXCR5, and CXCR5-1G4 coexpressing lentiviral vectors were generated (Figure 3A). PBMCs were transduced using each lentivirus transduction after activation. Transduction-efficiency was confirmed at 12 days after transduction through FACS (Figure 3B). Cell viability and growth were not changed in all groups, suggesting that there was no problem with cell function by transduction (Figure 3C-D).

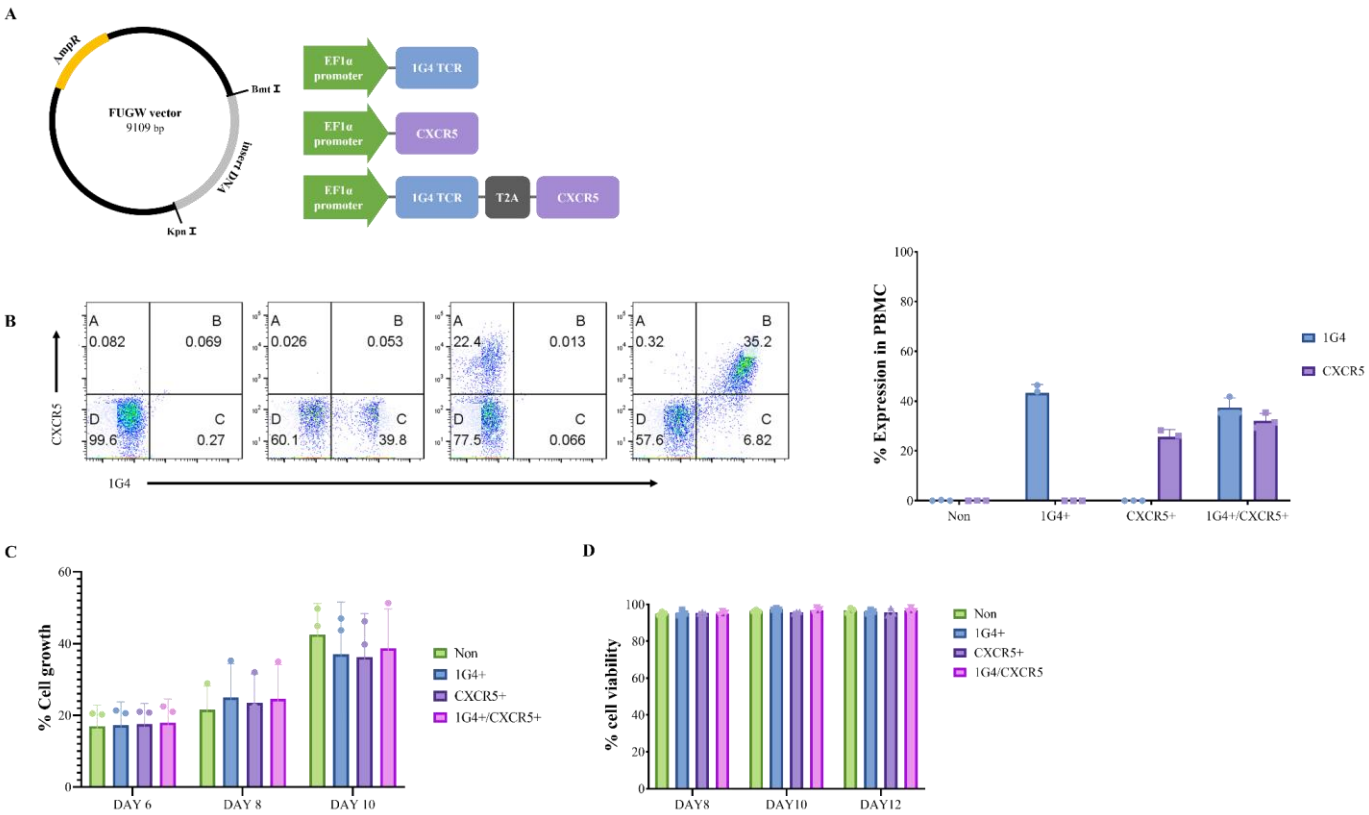


Figure 3. Construction and characteristics of engineered PBMCs. (A) Cloning design scheme. (B) Transduction-efficiency 12 days after transduction. (C) Percentage of cell growth after transduction. (D) Percentage of cell viability after transduction.

CXCR5-1G4 coexpressed TCR-T showed enhanced migration to tumor

Trans-well migration assay was used to examine whether CXCR5 expressing TCR-T migration can be increased according to matched chemokines. Indeed, matched increase of chemokine CXCL13 concentration induced gradual increase of migration in 1G4-CXCR5 coexpressing TCR-T, while migration in TCR-T expressing only 1G4 did not change (Figure 3A-B). In addition, migration-related ERK and AKT pathways were activated in 1G4-CXCR5 coexpressed TCR-T over time by CXCL13 stimulation (Figure 3C).

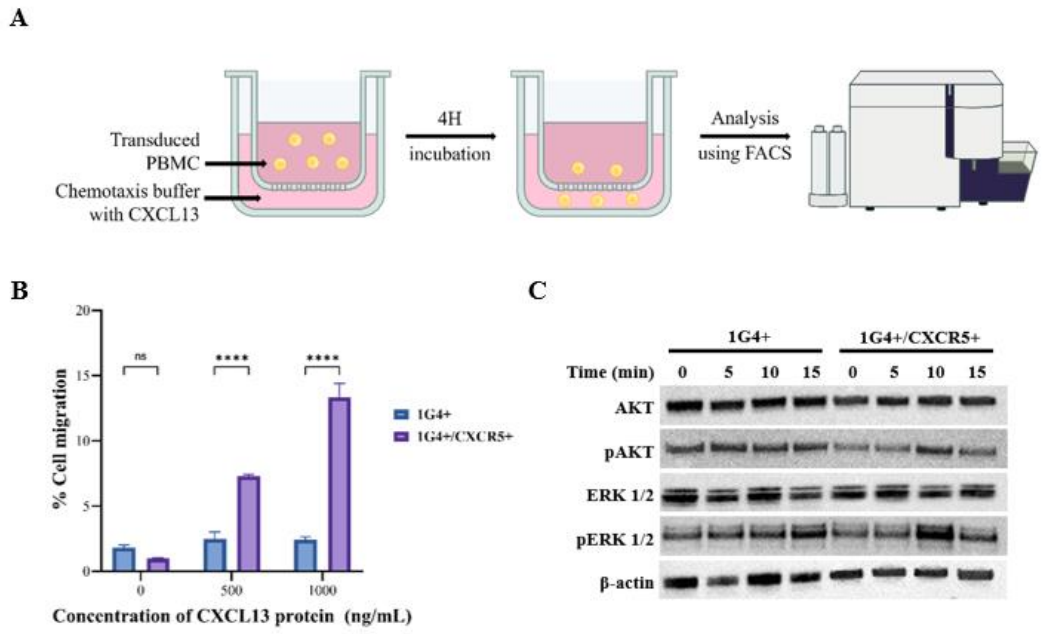


Figure 4. Evaluation of migration ability depending on the presence or absence of CXCR5.
(A) The graphical representation of transwell assay process (B) Percentage of cell migration according to CXCL13 concentration (C) 1G4 TCR-T or CXCR5-1G4 co-expressing TCR-T were stimulated with CXCL13 for described times. Phosphorylation of AKT and Erk1/2 was analyzed by western blot.

CXCR5-1G4 coexpressed TCR-T showed no change in cytotoxicity

To produce the target cancer cell line to examine cytotoxicity, RT-PCR and ELISA were performed to select cell lines with both CXCL13 and NY-ESO-1 expression. RT-PCR demonstrated that all five cell lines had CXCL13 RNA expression, but they were not measured by ELISA (Figure 5A). Since it is well known that NY-ESO-1, the target antigen of the TCR used, is expressed in A375 cells, the target cell line was selected as A375 cells. A375-Luc-CXCL13 cells were transduced with described structure and selected with Zeocin to express CXCL13 in A375 cells enough to induce CXCR5-expressing cell migration (Figure 5B). To establish Zeocin concentration to make a stable cell line, I performed WST-8 assay with Zeocin and A375 cells (Figure 5C). CXCL13 ELISA confirmed CXCL13 expression in A375-Luc-CXCL13 cells (Figure 5D). Finally, differences of cytotoxicity in 1G4, CXCR5, and CXCR5-1G4 co-expressing TCR-T targeting A375-Luc-CXCR13 cells were evaluated. Through the Luciferase assay, it was confirmed that cytotoxicity of 1G4 TCR-T and CXCR5-1G4 co-expressing TCR-T is similar (Figure 5E). In addition, INF-γ and IL-2 concentrations in the cultured media of effecter TCR-T with target A375-Luc-CXCR13 cells were not changed by CXCR5 expression of T cells (Figure 5F).

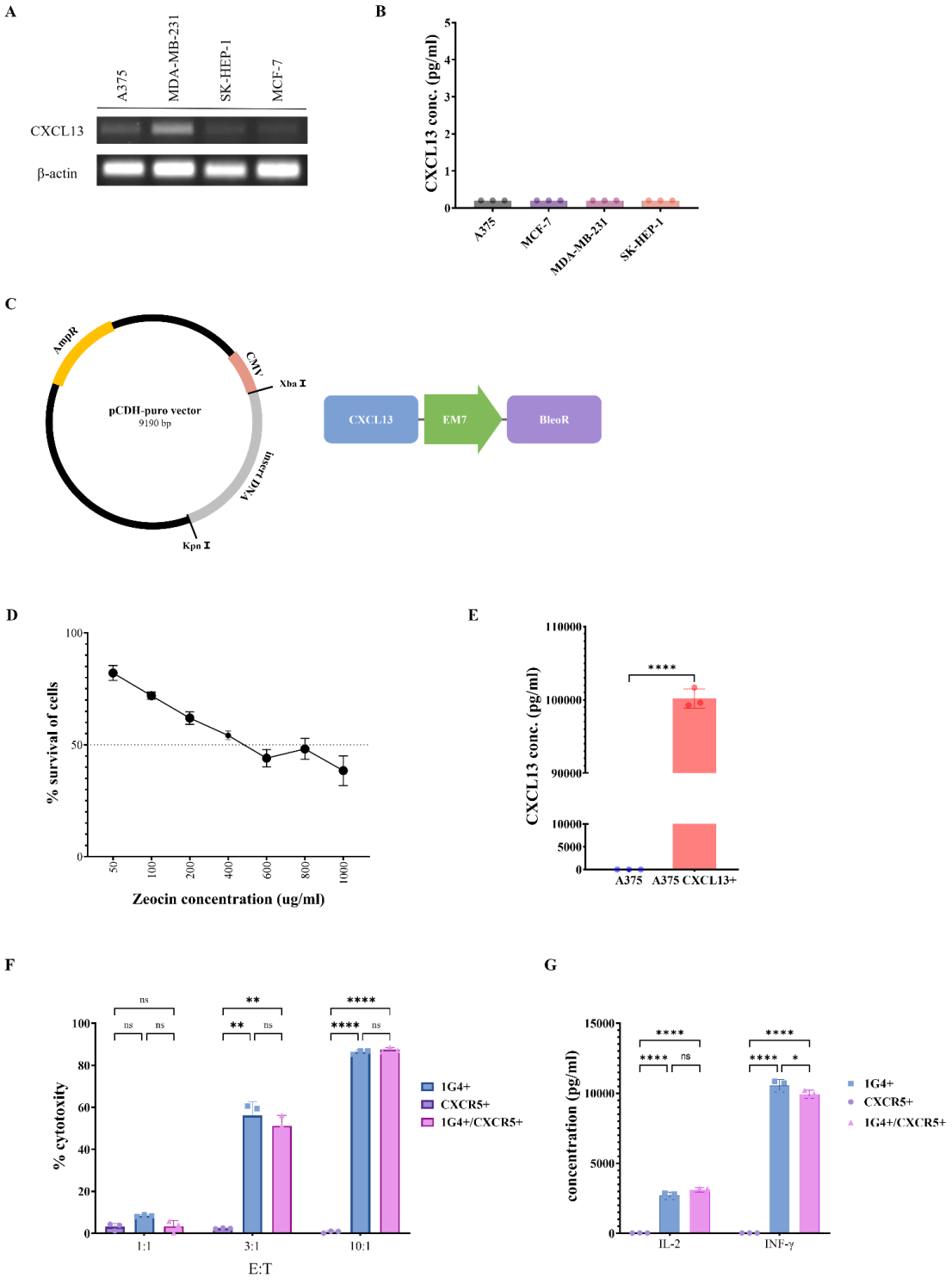


Figure 5. CXCR5 overexpression in TCR-T did not change cytotoxicity targeting CXCL13 expressing A375 cells. (A) CXCL13 RNA expression in cancer cell lines (B) Cloning design for target cells (C) Setting zeocin antibiotic concentration for stable cell lines (D) CXCL13 protein expression in transduced A375 cells (E) Luciferase assay based cytotoxicity (F) IL-2 and IFN-γ production after co-culture with transduced PBMC and target cell (1:10) for 24h

Discussion

This study aimed to improve the efficacy of ACT for solid tumors, where it has shown limited effectiveness. As a way to improve ACT, chemokine receptors have been studied in some cancers, but there was no attempt to identify candidate receptors able to treat pan-cancer.[20, 21, 22, 23, 24, 25] I systematically identified chemokine receptors capable of facilitating efficient migration through chemotaxis in pan-cancer, which might be more helpful when exact origin of tumor can not be determined. My approach involved comparing the expression of chemokines in normal and tumor tissues using the database. I compared chemokine expression between normal and tumor tissues, utilizing fold change values and selected 16 potential receptors. I considered the ligands associated with each chemokine receptor and excluded those receptors that, if utilized, might lead to migration into normal tissue due to higher expression of the corresponding chemokines in normal tissue among the 16 receptors. In this analysis, I utilized upper quartile values of each chemokine from GTEx data as a considered approach for evaluating the impact of the chosen chemokine receptors in the TCGA data.[26, 27, 28] This process led to the identification of specific receptors (CCR4, CCR6, CCR9, CXCR1, CXCR3, CXCR5, CXCR6 and XCR1) that hold promise for effective application in pan-cancer studies.

Furthermore, through the analysis of RNA sequencing data from TILs and a GEO database on CAR-T cells, I observed that certain chemokine receptors were highly expressed in native lymphocytes or engineered T cells. In addition, some studies have shown high expression of some chemokine receptors in PBMCs.[29, 30] The chemokine receptor, which is already highly expressed in immune cells, does not need to be further expressed. Consequently, I excluded chemokine receptors with well-established trafficking functions (CCR4, CXCR3 and CXCR6) from my considerations.

This screening process led us to anticipate that CXCR5, with the highest expected trafficking efficiency, could prove particularly effective for mediating tumor migration in pan-cancer. The selected CXCR5 receptor binds to CXCL13, which also has binding affinity for CXCR3. While CXCR3 exhibits high expression levels in both resting and activated T cells, it's worth noting that CXCL13 competes with CXCL10 for binding and may attenuate CXCL10-induced calcium flux.[31] Calcium flux represents a crucial signaling pathway associated with chemotaxis, cell migration, and other biological functions. In contrast, the CXCL13/CXCR5 signaling pathway has been shown to augment calcium flux and may thus enhance cell migration functions.[32] Consequently, engineering CXCR5 to interact with CXCL13, with its potential to mitigate the migration function of CXCR3, is anticipated to further enhance cell migration capabilities.

Through in vitro experiments, I have confirmed that the additional expression of CXCR5 can induce cell migration. CXCL13, which binds to CXCR5, is naturally expressed in greater quantities

within tumor tissues compared to normal tissues. My experimental data demonstrate that the additional expression of CXCR5 does not impact cell growth and viability but does show an increase in migration, dependent on CXCL13 chemotaxis and concentration. I have also observed that there is no significant difference in cytotoxicity function and activation between T cells expressing the NY-ESO-1 targeting 1G4 TCR alone and those co-expressing 1G4 TCR and CXCR5. This suggests that CXCR5 does not interfere with the functionality of TCR but may enhance trafficking functions through CXCL13 chemotaxis within tumor cells. One previous study showed CXCR5 is able to enhance trafficking in ACT of patients with non-small cell lung cancer similar to my results.[30] Furthermore, my study demonstrated that CXCR5 is a potential candidate to enhance trafficking in ACT of patients with wide-ranging cancer types.

However, there are certain limitations to my current study, including the absence of in vivo experimentation and the need for further exploration regarding the impact of each chemokines on biological functions in various diseases including cancers.[33, 34, 35] Additionally, it is crucial to consider factors such as the affinity for overlapping ligands by multiple chemokine receptors.[36]

Conclusion

My research represents a significant step forward to improve ACT strategies. By identifying and confirming the T cell trafficking abilities of specific chemokine receptors through a systematic approach, I have laid the groundwork for further research in this area. My future endeavors will involve expanding my investigation to encompass a broader range of chemokine receptors and conducting in vivo experiments to further validate my findings. Ultimately, I aim to progress to clinical studies, with the overarching goal of enhancing the effectiveness of ACT that can be adopted in the treatment for the vast majority of solid tumors.

Reference

1. Zhang, P., Zhang, G. & Wan, X. Challenges and new technologies in adoptive cell therapy. *J Hematol Oncol* **16**, 97 (2023).
2. Rohaan, M.W., Wilgenhof, S. & Haanen, J. Adoptive cellular therapies: the current landscape. *Virchows Arch* **474**, 449-461 (2019).
3. Klebanoff, C.A., Rosenberg, S.A. & Restifo, N.P. Prospects for gene-engineered T cell immunotherapy for solid cancers. *Nat Med* **22**, 26-36 (2016).
4. Ivica, N.A. & Young, C.M. Tracking the CAR-T Revolution: Analysis of Clinical Trials of CAR-T and TCR-T Therapies for the Treatment of Cancer (1997-2020). *Healthcare (Basel)* **9** (2021).
5. Li, H. *et al.* Antitumor activity of EGFR-specific CAR T cells against non-small-cell lung cancer cells in vitro and in mice. *Cell Death Dis* **9**, 177 (2018).
6. Kershaw, M.H. *et al.* A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res* **12**, 6106-6115 (2006).
7. Anandappa, A.J., Wu, C.J. & Ott, P.A. Directing Traffic: How to Effectively Drive T Cells into Tumors. *Cancer Discov* **10**, 185-197 (2020).
8. Foeng, J., Comerford, I. & McColl, S.R. Harnessing the chemokine system to home CAR-T cells into solid tumors. *Cell Rep Med* **3**, 100543 (2022).
9. Gunn, M.D. *et al.* A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. *Nature* **391**, 799-803 (1998).
10. Legler, D.F. *et al.* B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. *J Exp Med* **187**, 655-660 (1998).
11. Chen, C., Liu, X., Chang, C.Y., Wang, H.L.Y. & Wang, R.F. The Interplay between T Cells and Cancer: The Basis of Immunotherapy. *Genes-Basel* **14** (2023).
12. Jin, L. *et al.* CXCR1- or CXCR2-modified CAR T cells co-opt IL-8 for maximal antitumor efficacy in solid tumors. *Nat Commun* **10**, 4016 (2019).
13. Wang, Y. *et al.* Chemokine Receptor CCR2b Enhanced Anti-tumor Function of Chimeric Antigen Receptor T Cells Targeting Mesothelin in a Non-small-cell Lung Carcinoma Model. *Front Immunol* **12**, 628906 (2021).
14. Marofi, F. *et al.* CAR T cells in solid tumors: challenges and opportunities. *Stem Cell Res Ther* **12**, 81 (2021).
15. Yun, K., Siegler, E.L. & Kenderian, S.S. Who wins the combat, CAR or TCR? *Leukemia* **37**, 1953-1962 (2023).
16. Thomas, R. *et al.* NY-ESO-1 Based Immunotherapy of Cancer: Current Perspectives. *Front Immunol* **9**, 947 (2018).

17. Robbins, P.F. *et al.* Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* **29**, 917-924 (2011).
18. Lee, H. *et al.* Clinicopathological factors associated with tumor-infiltrating lymphocyte reactivity in breast cancer. *Cancer Immunol Immunother* **69**, 2381-2391 (2020).
19. Lee, H.J. *et al.* Expansion of tumor-infiltrating lymphocytes and their potential for application as adoptive cell transfer therapy in human breast cancer. *Oncotarget* **8**, 113345-113359 (2017).
20. Ruixin, S. *et al.* Expressing IL-15/IL-18 and CXCR2 improve infiltration and survival of EGFRvIII-targeting CAR-T cells in breast cancer. *Biochem Pharmacol* **212**, 115536 (2023).
21. Ng, Y.Y., Du, Z., Zhang, X., Chng, W.J. & Wang, S. CXCR4 and anti-BCMA CAR co-modified natural killer cells suppress multiple myeloma progression in a xenograft mouse model. *Cancer Gene Ther* **29**, 475-483 (2022).
22. Zhong, C. & Chen, J. CAR-T cell engineering with CCR6 exhibits superior anti-solid tumor efficacy. *Sci Bull (Beijing)* **66**, 755-756 (2021).
23. Ng, Y.Y., Tay, J.C.K. & Wang, S. CXCR1 Expression to Improve Anti-Cancer Efficacy of Intravenously Injected CAR-NK Cells in Mice with Peritoneal Xenografts. *Mol Ther Oncolytics* **16**, 75-85 (2020).
24. Liu, G. *et al.* CXCR2-modified CAR-T cells have enhanced trafficking ability that improves treatment of hepatocellular carcinoma. *Eur J Immunol* **50**, 712-724 (2020).
25. Idorn, M. *et al.* Chemokine receptor engineering of T cells with CXCR2 improves homing towards subcutaneous human melanomas in xenograft mouse model. *Oncoimmunology* **7**, e1450715 (2018).
26. Bartha, A. & Gyorffy, B. TNMplot.com: A Web Tool for the Comparison of Gene Expression in Normal, Tumor and Metastatic Tissues. *Int J Mol Sci* **22** (2021).
27. Hu, M. *et al.* Identification of Differently Expressed Genes Associated With Prognosis and Growth in Colon Adenocarcinoma Based on Integrated Bioinformatics Analysis. *Front Genet* **10**, 1245 (2019).
28. Li, X. *et al.* A comparison of per sample global scaling and per gene normalization methods for differential expression analysis of RNA-seq data. *PLoS One* **12**, e0176185 (2017).
29. Gorchs, L., Oosthoek, M., Yucel-Lindberg, T., Moro, C.F. & Kaipe, H. Chemokine Receptor Expression on T Cells Is Modulated by CAFs and Chemokines Affect the Spatial Distribution of T Cells in Pancreatic Tumors. *Cancers (Basel)* **14** (2022).
30. Li, G. *et al.* CXCR5 guides migration and tumor eradication of anti-EGFR chimeric antigen receptor T cells. *Mol Ther Oncolytics* **22**, 507-517 (2021).

31. Jenh, C.H. *et al.* Human B cell-attracting chemokine 1 (BCA-1; CXCL13) is an agonist for the human CXCR3 receptor. *Cytokine* **15**, 113-121 (2001).
32. Kazanietz, M.G., Durando, M. & Cooke, M. CXCL13 and Its Receptor CXCR5 in Cancer: Inflammation, Immune Response, and Beyond. *Front Endocrinol (Lausanne)* **10**, 471 (2019).
33. Bule, P., Aguiar, S.I., Aires-Da-Silva, F. & Dias, J.N.R. Chemokine-Directed Tumor Microenvironment Modulation in Cancer Immunotherapy. *Int J Mol Sci* **22** (2021).
34. Korbecki, J. *et al.* CC Chemokines in a Tumor: A Review of Pro-Cancer and Anti-Cancer Properties of the Ligands of Receptors CCR1, CCR2, CCR3, and CCR4. *Int J Mol Sci* **21** (2020).
35. Spaks, A. Role of CXC group chemokines in lung cancer development and progression. *J Thorac Dis* **9**, S164-S171 (2017).
36. Rajagopalan, L. & Rajarathnam, K. Ligand selectivity and affinity of chemokine receptor CXCR1. Role of N-terminal domain. *J Biol Chem* **279**, 30000-30008 (2004).

Abstract (in Korean)

고형암에 대한 입양 면역 세포 치료법 (ACT)의 기능을 향상시키기 위해 면역 세포에 대한 케모카인 수용체가 연구되어지고 있습니다. 여러 암 종에서 케모카인 발현에 대한 다양성이 보이기 때문에, 적절한 케모카인 수용체를 확인하고 이를 이용하는 것은 중요합니다. 본 연구에서는 케모카인 발현 경향성을 확인하기 위해 공용 데이터 베이스인 암 게놈 아틀라스 (TCGA)의 종양 조직 데이터과 유전자형-조직 발현 (GTEx)의 정상 조직 데이터를 활용했습니다. 분석을 통해 ACT 를 강화할 유망한 후보로 종양 조직에서 정상에 비해 증가한 케모카인 수용체 8 개를 확인했습니다. 종양 침투 림프구 (TIL)와 키메라 항원 수용체 (CAR) T 세포의 발현을 분석했을 때, 선택된 케모카인 수용체 8 개 중 5 개가 T 세포에서 높은 발현을 나타내지 않았다는 것이 확인되었습니다. 5 개의 후보 중 CXCR5 가 시험관 내 실험에서 확인된 바와 같이 세포 생존율이나 세포 독성에 영향을 주지 않고 세포 이동을 강화할 수 있음을 보여주었습니다. 결론적으로, 우리의 연구는 5 개의 케모카인 수용체 (CCR6, CCR9, CXCR1, CXCR5, XCR1)가 세포 이동를 개선하고 잠재적으로 암 치료 결과를 개선하기 위한 ACT 의 작용 방법으로서의 가능성을 보여줍니다.

핵심어 : 입양 면역 세포 치료법 (ACT), 케모카인, 케모카인 수용체, CXCR5 수용체