# Effects of different serum supplements on PiggyBac CD19 Chimeric Antigen Receptor T cells

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ผลของสารเสริมซีรั่มชนิดต่างๆต่อพิกกี้แบคซีดีสิบเก้าไคเมอริกแอนติเจนรีเซพเตอร์ทีเซลล์

น.ส.มุลิตา แสนยานุสิน



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มุลิตา แสนยานุสิน : ผลของสารเสริมซีรั่มชนิดต่างๆต่อพิกกี้แบคซีดีสิบเก้าไคเมอริกแอนติเจนรีเซพเตอร์ทีเซลล์. ( Effects of different serum supplements on PiggyBac CD19 Chimeric Antigen Receptor T cells) อ.ที่ปรึกษาหลัก : อ. นพ.กรมิษฐ์ ศุภพิพัฒน์, อ.ที่ปรึกษาร่วม : ศ. พญ.ณัฏฐิยา หิรัญกาญจน์

ไคเมอริกแอนติเจนรีเซพเตอร์ทีเซลล์เป็นวิธีการรักษาแนวใหม่สำหรับผู้ป่วยโรคมะเร็งเม็ดเลือดขาวที่มีโรคกลับมาเป็นซ้ำหรือไม่ตอบสนองต่อการรักษา คุณลักษณะของคาร์ทีเซลล์ที่ได้จะมีความเกี่ยวข้องกับประสิทธิภาพและผลข้างเคียงของการรักษาทางคลินิก ชนิดของสารเสริมซีรั่มที่ใช้ในขั้นตอนเพาะเลี้ยงเพื่อเพิ่มจำนวนเซลล์ในห้องปฏิบัติการมีผลต่อคุณลักษณะและคุณสมบัติของคาร์ทีเซลล์ที่ใช้วิธีไวรัสในการผลิต ในงานวิจัยชิ้นนี้จะศึกษาผลกระทบของการใช้สารเสริมซีรั่มชนิดต่างๆในการผลิตพิกกี้แบคคาร์ทีเซลล์ ซึ่งเป็นวิธีการผลิตที่ไม่ใช้ไวรัส พิกกี้แบคซีดีสิบเก้าคาร์ทีเซลล์จะถูกเพาะเลี้ยงในห้องปฏิบัติการด้วยสารเสริมซีรั่มที่แตกต่างกัน ได้แก่ fetal bovine serum, human AB serum, human platelet lysate และ xeno-free serum replacement จากนั้นทำการประเมินผลของการใช้สารเสริมซีรั่มที่แตกต่างกัน ต่อการเพิ่มจำนวนของเซลล์, transfection efficiency, ลักษณะและส่วนประกอบของเซลล์ที่ได้ รวมถึงประสิทธิภาพในการฆ่าเซลล์มะเร็งเป้าหมายในหลอดทดลอง ผลการทดลองพบว่าการใช้ xeno-free serum replacement มีการแสดงของคาร์โมเลกุลบนทีเซลล์,อัตราการเจริญเติบโตของเซลล์ และประสิทธิภาพในการฆ่าเซลล์มะเร็งเป้าหมายในหลอดทดลองเมื่อเพาะเลี้ยงร่วมกันเป็นเวลา 24 ชั่วโมงที่ไม่แตกต่างจากการใช้สารเสริมซีรั่มชนิดอื่นๆ อย่างไรก็ตามพบว่า ในกลุ่มที่มีการใช้ xeno-free serum replacement มีจำนวนของ naïve T cell ที่เพิ่มขึ้น และ มีอัตราการเพิ่มจำนวนของคาร์ทีเซลล์หลังจากมีการกระตุ้นเซลล์ด้วยเซลล์มะเร็งที่มีการแสดงออกของซีดีสิบเก้าเป็นระยะเวลานานและต่อเนื่องที่สูงกว่าสารเสริมซีรั่มชนิดอื่น ดังนั้นงานวิจัยนี้แสดงผลสนับสนุนการใช้ xeno-free serum replacement เป็นสารเสริมซีรั่มทางเลือกสำหรับการเพาะเลี้ยงเพื่อเพิ่มจำนวนคาร์ทีเซลล์ด้วยวิธีพิกกี้แบค



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Mulita Sanyanusin : Effects of different serum supplements on PiggyBac CD19 Chimeric Antigen Receptor T cells. Advisor: KORAMIT SUPPIPAT, M.D. Co-advisor: Prof. NATTIYA HIRANKARN, M.D.

Chimeric Antigen Receptor (CAR) T cell is a novel therapy for relapse and refractory hematologic malignancy. Characteristics of CAR T cells is associated with clinical efficacy and toxicity. Type of serum supplements used during cultivation affect the immunophenotype and function of viral-based CAR T cells. This study explores the effect of serum supplements on non-viral piggyBac transposon CAR T cell production. PiggyBac CD19 CAR T cells were expanded in cultured conditions containing either fetal bovine serum, human AB serum, human platelet lysate or xeno-free serum replacement. Then, the effect of different serum supplements on cell expansion, transfection efficiency, immunophenotypes and anti-tumor activity were evaluated. Xeno-free serum replacement exhibited comparable CAR surface expression, cell expansion, and short-term anti-tumor activity comparing with conventional serum supplements. However, CAR T cell cultivated with xeno-free and serum-free cultured condition exhibited increase naïve T cell population and better T cell expansion after long term co-culture as well as during tumor rechallenge assay. This study supports the usage of xeno-free serum replacement as an alternative source of serum supplements for PiggyBac based CAR T cell expansion.



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# ABBREVIATIONS

| | |
|---|---|
| CAR: | Chimeric Antigen Receptor |
| CAR T Cell: | Chimeric Antigen Receptor T Cells |
| CD19: | Cluster of Differentiation 19 |
| FBS: | Fetal Bovine Serum |
| HS: | Human AB Serum |
| HPL: | Human Platelet Lysate |
| SR: | Serum Replacement Reagent |
| PB: | PiggyBac Transposon System |
| FDA: | Food and Drug Administration |
| EMA: | European Medicines Agency |
| GMP: | Good Manufacturing Practice |
| PBMCs: | Peripheral Blood Mononuclear Cells |
| NALM-6: | B Cell Precursor Leukemia Cell Line |
| IL-7: | Recombinant Human Interleukin 7 |
| IL-15: | Recombinant Human Interleukin 15 |
| G-REX: | Gas-Permeable Rapid Expansion Culture Device |
| IL-2: | Interleukin 2 |
| IL-4: | Interleukin 4 |
| IL-6: | Interleukin 6 |

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|---|---|
| IL-10: | Interleukin 10 |
| IFN-g: | Interferon Gamma |
| TNF-a: | Tumor Necrosis Factor Alpha |
| PD-1: | Programmed Cell Death Protein 1 |
| TIM3: | T-cell Immunoglobulin and Mucin Domain 3 |
| LAG3: | Lymphocyte Activation Gene 3 |
| TIGIT: | T-cell Immunoglobulin and ITIM Domain |

# CHAPTER 1

# INTRODUCTION

## BACKGROUND AND RATIONALE

### Chimeric antigen receptor (CAR) T cell

Chimeric antigen receptors (CAR) T- cell therapy has delivered a major progression in the treatment of hematologic malignancies [1]. CARs are engineered receptors to redirect and reprogram T cells after that infused back into the patient to specifically target and destroy cancer cells [2]. Genetically engineering T cells to express CAR targeting CD19 have significantly improved overall clinical response and survival against relapsed and refractory B cell hematologic malignancy leading to FDA and EMA approval of commercial CD19 CAR T cell products [3-5].



### CD19-targeted CAR T cells

CAR T Cells targeting the B cell molecule, CD19, are designed to transform patient's autologous T-cells into series killers against their B-Cell lymphoma [3]. The CD-19 was selected to use as scFv not only its frequent and high expression in B cell malignancies, but also its restricted expression in normal tissue [6]. Moreover, CD19 CAR T Cell demonstrated impressive outcomes such as high response rate and durable remission in patients with refractory B Cell malignancies [4].

In 2017, two CD-19-directed CAR-T cell immunotherapy received FDA approval consist of Kymriah from Novartis which used for pediatric patients and young adults with relapsed or refractory B cell acute lymphoblastic leukemia (ALL). Another is Yescarta from Kite Pharma which used for adults with R/R B cell lymphoma, including DLBCL and other aggressive NHL subtypes [5].

## CAR T Cell structure

The basic structure of CAR is comprising of an extracellular domain, a transmembrane domain and a signaling domain. These domains can have a significant effect on the CAR expression , CAR function and safety for using in clinical setting [3, 7].

### Extracellular domain

The extracellular domain is the domain of a membrane protein that is outside the cytoplasm and exposed to the extracellular space. The extracellular domain consists of signal peptide, antigen recognition region and spacer. The function of signaling peptide is to direct nascent protein into the ER (endoplasmic reticulum). The single chain variable fragment (scFv) of antibodies, can theoretically recognize any type of surface antigen expressed on a target cell, including proteins and glycolipids. The interconnection between antigen binding domain and transmembrane domain depends on the spacer. The simpler form of spacer is the hinge region of IgG1 and is adequate for scFv based constructs [3, 7, 8].

## Transmembrane domain

The transmembrane domain is composed of a hydrophobic alpha helix that extend the membrane. The consistency of the receptor is relied on the transmembrane domain. CD28 transmembrane domain is one of the most stable receptors [7]. The main function of transmembrane domain is to attach the CAR in T cell membrane nevertheless, there is evidence supports that the function of CAR T cell is related to transmembrane domain [8].

## Signaling domain

The most common component is CD3 zeta included three immunoreceptor tyrosine-based activation motifs (ITAMs). After antigen recognition, the receptors cluster and signal were activated, then the signal is transmitted to the T cell [3].

## Costimulatory domain



CAR intracellular domains are usually derived from costimulatory molecules from CD28 family (including CD28 and ICOS) or the tumor necrosis factor receptor family of genes (including 4-1BB, OX40 or CD27) CD28 and 4-1 BB are the most widely used costimulatory intracellular domains in CARs. CARs that combining CD28 or 4-1 BB intracellular domains revealed similar response rates in patients with hematologic malignancies [8].

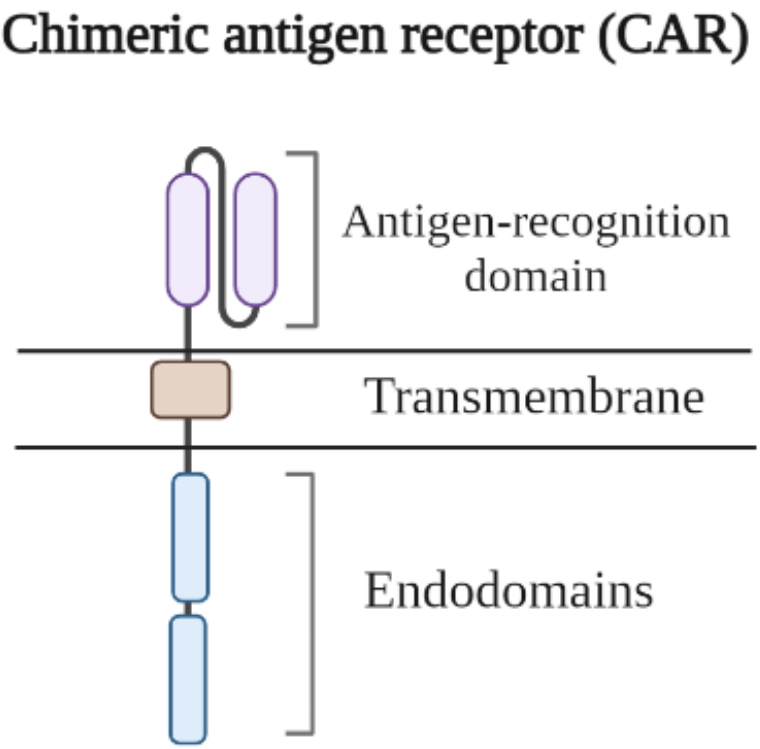



*Figure 1 Structure of Chimeric antigen receptor (CAR)*



## Generation of CAR T Cell

Since the initial development of CARs in 1989, CAR-T cells can be divided into fifth generation according to the structure of the Intracellular domain [7]. when the CAR binds to the tumor antigen, T cell becomes activated, proliferates, and destroys the tumor cells, apart from MHC or HLA expression. This makes CAR-T cells suitable for any patient, regardless of their HLA type [9].

## First generation

Single structure from the CD3 ζ- chain or FcεRIγ from the intracellular domain for the first generation of CARs is the typical characteristics, which is the primary transmitter of signals from endogenous T cell receptor (TCR) [7].

## Second generation

The Second-generation CARs are made by the dual signaling domain that used for T cell activation. Three different receptor types such as the T-cell antigen receptor, cytokine receptors and co-stimulatory receptors are included. First signal which is the special signal that activated by the TCR will recognizes the antigenic peptide-MHC complex on the surface of antigen-presenting cells. Second signal is the co-stimulatory signal that created by co-stimulatory molecule such as CD28/B7 which encourage the IL-2 synthesis to accomplished T cells activation and prevent apoptosis [7].



## Third generation

The third-generation CARs were generated by combining multiple signaling domains to increase efficacy with powerful cytokine production and killing ability. The selection of co-stimulatory molecule is also significant [7].

## Fourth generation

The fourth generation CARs were developed from second generation of CARs by adding IL-12 to the base of constructs. This generation are known as T cell redirected for universal cytokine-mediated killing (TRUCKs) which increase the T cell activation, activate and attract innate cells to eradicate antigen-negative cancer cells in targeted lesion [7]. Further, IL-12 armored CAR-T cells can enhance the proliferation and antitumor efficacy compared to conventional CARs [9].

## Fifth generation

The fifth generation CARs aim to activate all three signals including TCR (CD3ζ domain), co-stimulatory (CD28 domain) and cytokine (via JAK/STAT pathway) which are required to optimal T cell activation, in an antigen dependent. The publication revealed that engineering CAR-T cells with cytokine-encoding genes enhanced the proliferation, survival and antitumor activity, compared to conventional CARs [9].

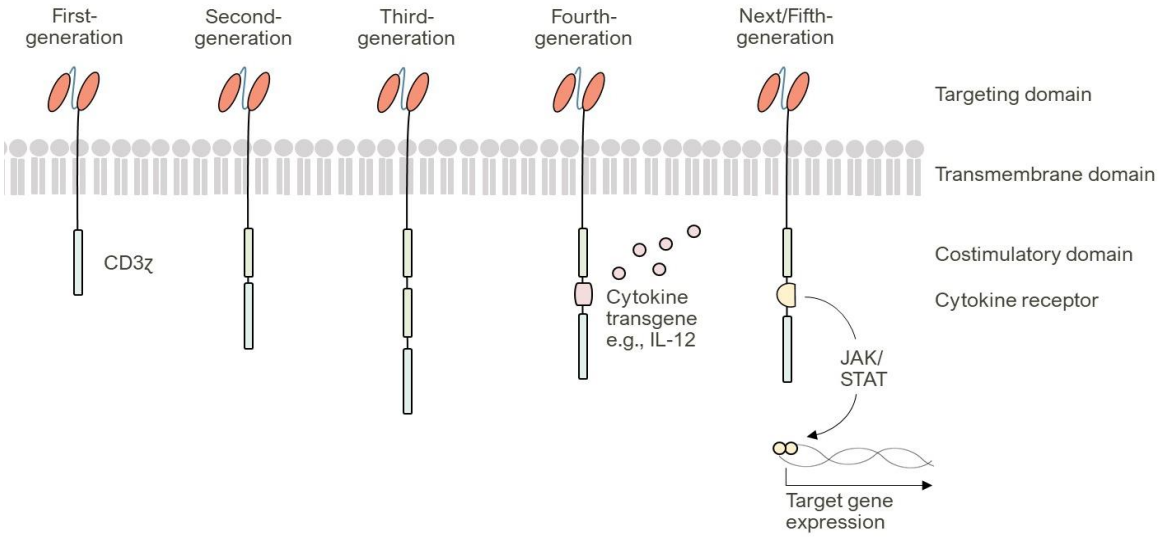



*Figure 2 Schematic of the CAR generations*

## Universal CAR T cells

Universal CAR T cells generated from healthy donors that potential to overcome many immune defects associated with cancer treatment. The universal CAR T cell therapies might give an opportunity to modify the manufacturing of engineered cells and decrease the cost of treatment when compared with autologous patient-specific T cells [10].



## CAR T cell manufacturing

CARs are engineered fusion protein consist of an antigen recognition domain derived from a monoclonal antibody, T cells signaling and co-stimulatory domain. CAR T cells product demands several cautiously performed steps, and quality control testing is performed during the manufacturing [2].

## Apheresis

CAR T cells Therapy starts with separating the patient's WBCs from whole blood by leukapheresis [11]. The blood components are normally separated by density with continuous or intermittent centrifugation methods using density gradient media. During the apheresis process, red blood cells and platelets are commonly removed in a washing step due to the red blood cells can influence the efficacy of product and platelets can lead to clumping of the cells [12].

## Activation

Antigen presenting cells will stimulate naïve T cells for proliferation and differentiation. T cells are activated by interactions between the T cell receptor (TCR) and the major histocompatibility complex which located on the surface of dendritic cell and through costimulatory molecules such as CD28, 4-1BB and OX40. To avoid the burdensome process of co-culture with DCs, several methods that simulate the natural stimulation of T cells have been developed and implemented [12].



## Transduction

CARs transduced by using the gene delivery that can be divided into viral and non-viral methods. For gene delivery, electroporation of naked DNA, plasmid-based transposon/ transposase systems and viral vectors have been applied. However, there

are some technical problems when trying to scale up lentiviral manufacturing, including lot to lot variability but these issues have been resolved by using newer production methods. Also, the use of the transposon/transposase system manufacturing have some problems in 12% of cases included inability to grow the cells and contamination during manufacturing [11, 12].

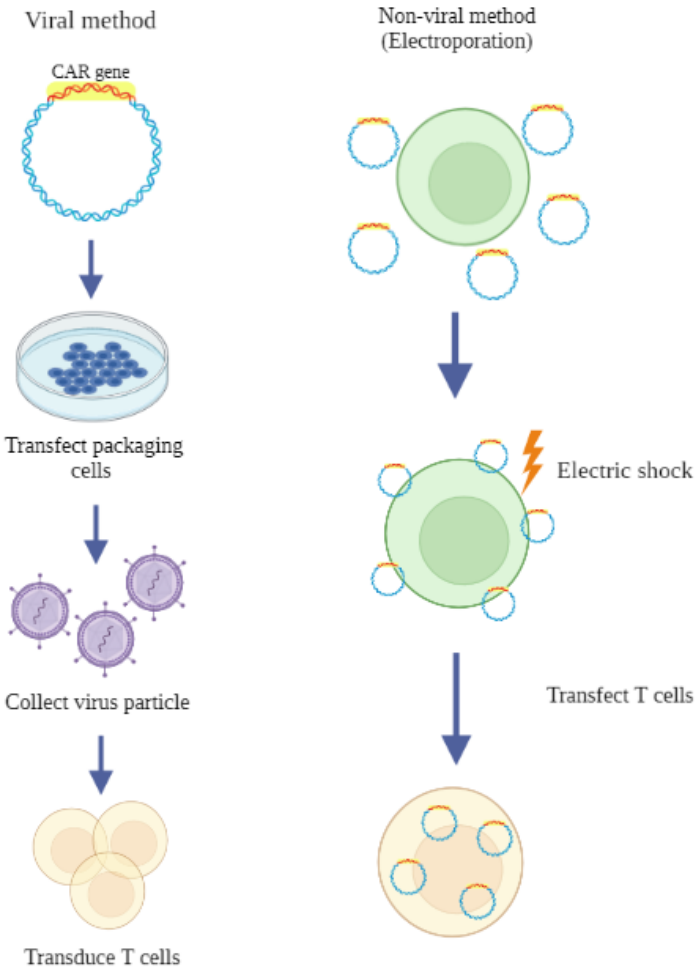



*Figure 3 Transduction method (Viral, Nonviral)*

## Expansion

To reach a cell numbers that needed for clinical application, the functionally closed systems was recommended to use for expanding T cells after gene transferring due to they can decrease the risk of contamination [11]. A variety of methods can be used including T-flasks, plates or culture bags and bioreactors such as the G-Rex flask which is a cell culture flask with a gas-permeable membrane at the base allows cells to grow to a high density without compromising gas exchange [13].

Even though, there are many choices of culture medium but still need a move toward to use of serum free formulations such as X-VIVO 10 and AIM-V. Interleukin 2 (IL-2) or interleukin7 (IL-7) plus interleukin-15 (IL-15) plays as a center-dependent. From a practical approach, it is crucial for CAR T-cell manufacturing to ensure their supply chain and to be able to verify other GMP-compliant reagents and expendables to protect their process [14].

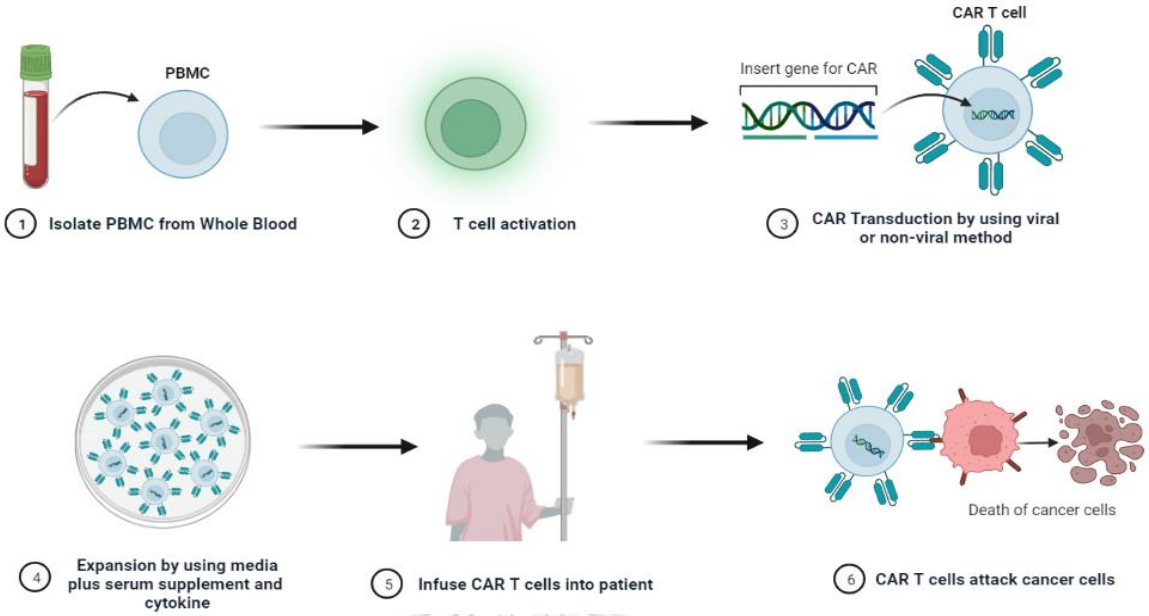



*Figure 4 Schematic for generating CAR T cell*

## Viral based CAR T cell versus Non-viral CAR T cell

An important barrier of CARs is developing a proper CAR expression vector capable of genetically modifying a wide population of T cells. Both viral and non-viral vectors are used to produce CAR T cells [15].



### Viral based CAR T cell

Using viral gene transfer, retroviral or lentiviral, which required for T cell activation and can lead to high efficiencies of transduction. Basic viral vectors that required for viral survival and function are the gag, pol and env genes, gag encodes structural proteins, pol encodes enzymes which needed for reverse transcription and

integration into the genome of host cell, and env encodes the viral envelope glycoprotein [16].

The limitation of viral vectors consists of the safety issues caused by the high limited capacity for multigene insertion, co-transduction, large manufacturing cost and produce genotoxicity [17, 18]. To solve these problems, the non-viral gene transfer was used to achieve the forced expression of the introduced ectopic CAR [19].

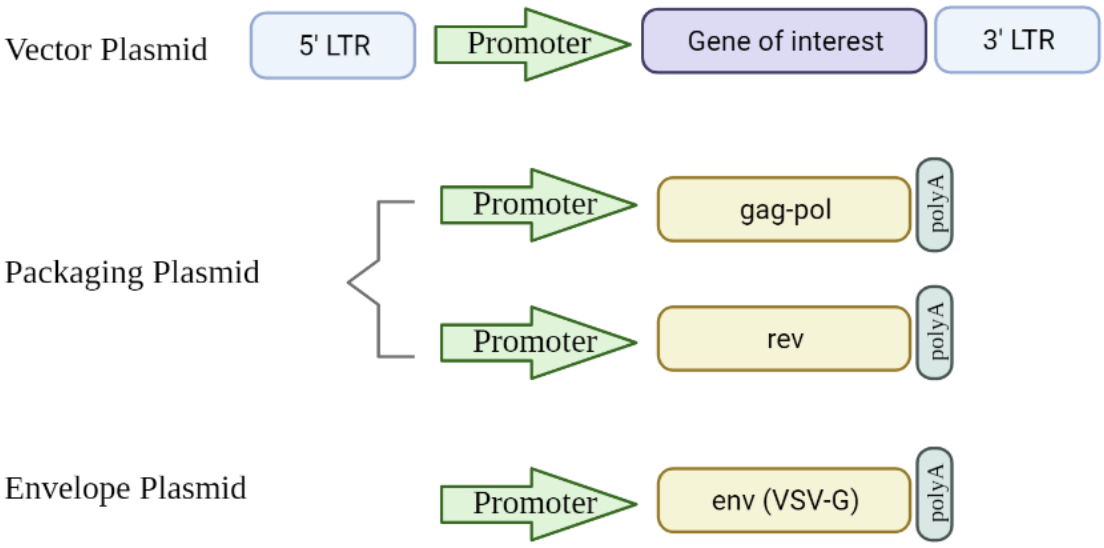



*Figure 5 Schematic of lentiviral vector*

## Non-Viral based CAR T cell

Genetic modification of T cells using transposon system as merging plasmids is an appealing replacement [20]. A nonviral vector for highly efficient site-specific integration would be useful for numerous applications in transgenesis, as well as gene

therapy [21]. Transposon system is a hopeful non-viral technique for stable genetic modification [22].

## PiggyBac transposon system

PiggyBac (PB) transposon system is composed of PiggyBac transposase, and a separate transfer plasmid carrying transgene of interest [23]. The PiggyBac element inserts a transgene between the inverted repeat elements and the transposition activity is enabled by supplying the PiggyBac transposase enzyme from a separate transgene into the genome at TTAA nucleotide elements [11, 17]. Previous works have demonstrated that PiggyBac based CD19 CAR T cell achieved satisfactory transduction efficiency and prominent anti-tumor activity [24] with similar integration profile comparing with viral vector [17]. Currently, PiggyBac transposon system provides alternative strategy to avoid viral vector related issues leading to more flexible and cost-effective CAR T cell manufacturing.

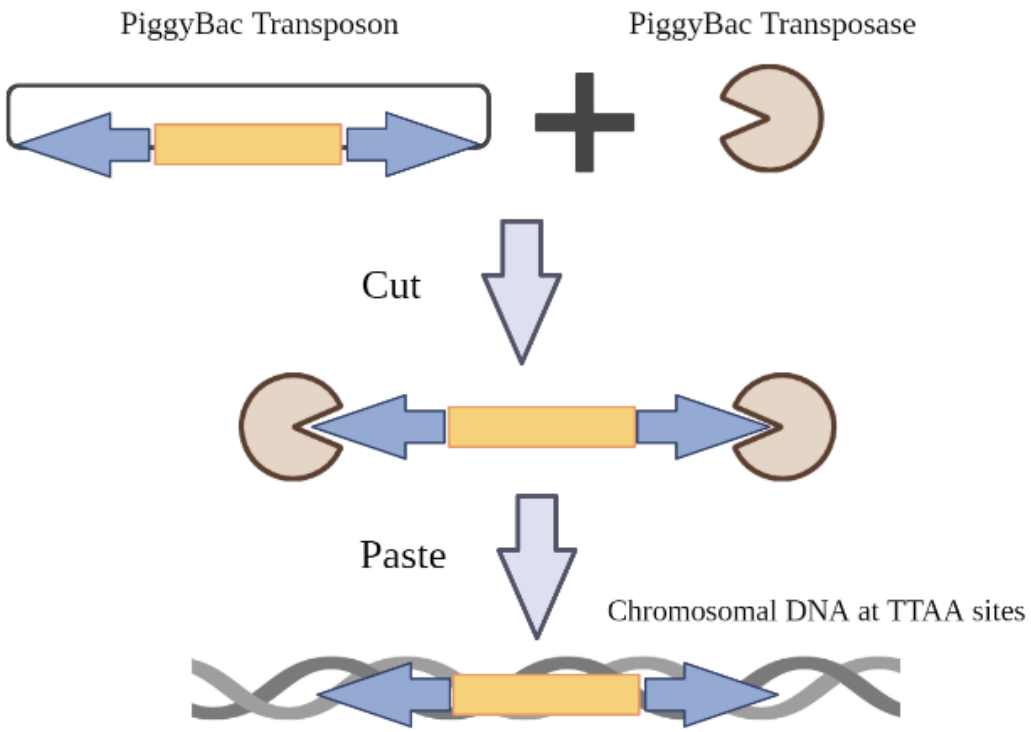



*Figure 6 Schematic of PiggyBac Systems*

## Role of serum in cell culture

Serum is one of the most important components of cell culture media and serves as a source of crucial substances which promote, sustain cell growth and cell functions. In mammalian cell culture, serum is often used as supplement to culture media (added in the range of 5 – 15 % (v/v)) to promote and maintenance cell growth. Serum can be obtained commercially from manufacturers, and they are sourced from a variety of species including bovine, chicken, caprine (goat), human, ovine, porcine and rabbit [25].

## Fetal Bovine serum in cell culture and limitation

Fetal bovine sera (FBS) are most commonly used as a standard supplement for T cell and CAR T cells expansion [26]. However, FBS has raised several concerns, including the risk of contamination from bovine spongiform encephalopathy, increased immunogenicity, and ethical issues [27-29]. Previous studies have reported the use of FBS for cell cultivation causing immune reactions and anaphylaxis in patients that can affect the therapeutic outcome [28, 30]. Thus, alternative serum supplements have been developed to overcome the limitation of FBS [29, 31].

## Alternatives serum supplement in T cell/CAR T cell culture

**Human AB serum (ABS or HS)** generated from human serum from type AB donors that lacks antibodies against the A and B blood-type antigens. In a clinical setting ABS is the most common media supplement for T cell culture and ABS also supply limitations and may not be sufficient to meet the expected demand for immunotherapies [32]. Moreover, human serum is expensive, requires additional agent testing and could potentially contain emerging infectious agents [33].



**Human platelet lysate (HPL)** generated from common platelet units by a simple freeze-thawing procedure. For the production of clinical cell therapies, HPL is widely recognized as a valuable replacement to both FBS and human AB serum [32].

Therefore, HPL can support the cell growth and proliferation, as platelets plays a crucial function in tissue renewal and wound healing [31]. The disadvantage includes danger of transmission of human disease by known or unknown human viruses [28].

**Serum-free medium** does not include any serum either from animal or human but in replacement of serum or plasma, supplements are sometimes included [28]. Additional, serum-free medium reduces operating costs and process variability and removes a potential source of infectious agents [34]. The use of serum-free medium is beneficial because they have defined formula, less lot-to-lot variability and offer a more consistent production of CD19 CAR T Cells [35].

However, there are still limited evidence on the effect of differential serum supplements on non-viral based CAR T cells, in which the gene transferring process depends on electroporation that can heavily impact cell viability and healthiness. Therefore, in this study evaluated the effect of different serum supplements including Fetal bovine serum (FBS), Human AB serum (HS)(Xeno-free serum),Human platelet lysate(HPL)(Xeno-free serum) and Serum Replacement (SR)(Xeno-free and serum-free chemically defined reagent) on PiggyBac CD-19 Chimeric Antigen Receptor T cells (PB CD-19 CAR T cells) characteristics and function.

## RATIONALE

FBS have several limitations on GMP compliance production of CAR T cell. Alternative serum supplements are needed to be explored for replacing FBS in GMP compliance manufacturing of CAR T cell. There are several evidence indicated that alternative source of serum supplement can influence the CAR T Cell characteristics in viral based CAR T cell transduction. Currently, there is no evidence on the effect of alternative serum source on non-viral based CAR T cell. This study will compare the effect of different serum supplements (FBS, HS, HPL and Serum replacement) on PiggyBac CD-19 CAR T cell characteristic and function.

## RESEARCH QUESTIONS

Research questions of this study are:

1. Does different type of serum supplement affect the characteristics of CD-19 CAR T cell generated by PiggyBac Transposon systems
2. Can serum replacement (SR) be used for PiggyBac transposon based in CD-19 CAR T cell manufacturing

## HYPOTHESIS

We hypothesize that Serum replacement can be used for PiggyBac CD-19 CAR T Cells manufacturing with good expansion, phenotype and function compared to FBS.

## OBJECTIVES

The objectives of this study are:

1. To compare the effects of different serum supplement on PiggyBac CD-19 CAR T cell expansion
2. To compare the effects of different serum supplement on PiggyBac CD-19 CAR T cell phenotype
3. To compare the effects of different serum supplement on PiggyBac CD-19 CAR T cell function

# CHAPTER 2

# LITERATURE REVIEW

## Chimeric antigen receptor T cells (CAR T Cells)

The important advantages of CAR T cells therapy are the capability of T cell to expand, proliferation and persistence through the memory T cells. CARs are synthetic molecule including signal domain and antigen binding domain that derived from monoclonal antibody from either murine or humanized [36].

### First generation versus Second generation of CARs

To reveal the importance of costimulatory domain, clinical studies utilization the first-generation CAR T cell and found the limited efficacy and persistent of CAR T cells. In the same way, Baylor College of Medicine treated relapse/refractory B-NHL patient with first-generation and second- generation CD19 CAR T cell. They noticed that the second generation that containing CD28 costimulatory domain had a superior expansion and persistence of CAR T cell products, highlighting the crucial of CAR T-cell costimulatory domain [37].

41BB versus CD28 costimulatory domains

Most widely co-stimulatory domain in second generation of CARs is CD28 or 4-1 BB. Several studies revealed that CAR T cell incorporate with 4-1BB domain showed higher persistence of CAR T cell in recipient. In pediatric patients that treated with CD19 CAR T cell incorporate 4-1BB revealed a greater rate of minimal residual disease negative and complete response [37].

Consistency with Xiangyu zhao et al. that investigated the efficacy and safety concern of CD28- or 4-1BB based CD19 CAR T cells in r/r B-ALL therapy. In B-ALL-bearing mice model found that when gave the CAR T dose approximately 1 x $10^6$ cells, 4-1BB group are more great eradicated tumor cells and prolong the persistence than CD28 leading to superior antitumor efficacy and less severe adverse events [38].



**Limitation of CAR T cell therapy in term of CAR persistence**

Long-term persistence is one of major important factor for durable remission in hematologic malignancies. Several documentations from pre-clinical and clinical studies reveals that to manufacturing CAR T cell, the final product which was enriched of less differentiated T cell, naïve T cell or central memory T cell, will provide the superior tumor clearance than the CAR T Cell which was enrichment of effector T cell or effector memory T cell. Also, The CAR T cells that maintained the less differentiated T cells

resulting in prolong immune surveillance after antigen clearance and can predictive the successful expansion and sustained remission in clinical application [39].

Clinical evidence reported that for adoptive transfer therapy the less differentiated T cells is important for antitumor efficacy and patient outcome due to this phenotype increased the expansion, persistence, and ability to differentiate into effector progeny [40].

To enhance CAR T cell persistence from the perspective of control the T cell differentiation, the choice of cytokine and media play a crucial role. Normally, *In vitro* culture system frequently used IL-2 for T cells expansion and differentiation. However, the use of IL-2 will support the formation of terminally T cells and development of regulatory T cells. To solve this issues, other cytokine that support the less differentiated T cells will use as a replacement of IL-2 such as IL-7 and IL-15. Also, the choice of the culture media plays an importance role in the CAR T cells persistence and durable control disease. [40]



## Side effect of CAR T Cells

The progress of CAR T cell therapy is obstructed by several serious adverse events as an example cytokine released syndrome, neurotoxicity, and on-target off tumor toxicity [41].

For cytokine release syndrome (CRS) is an important side effect of CAR T cell therapy. CRS is an immune over-activation caused by multiple cytokines produced by CAR T cell. The structure of co-stimulatory domain of CARs can induce cell activation, proliferation and increases effector function when the receptor molecules bind to target antigen led to overproduction of cytokines. Accordance with the data, a level of CRS is required in immune response due to cytokine production illustrate the CAR T cells were activated and function after infusing to patients [41].

## CD19 CAR T Cell

Kathryn M. Cappell and colleagues investigate the long-term outcomes of anti-CD19 CAR T cell therapy in relapsed B cell malignancies patients that participated in phase 1 clinical trial. They found that the durable ongoing remission lasting up to 113 months for follicular lymphoma, 99 months for CLL and 97 months for DLBCL/PMBCL . Also, the long-term adverse effects were rare [42].



In the same way, Nirali N Shah and colleagues conducted a phase 1 of CD19.28z CAR T cell in children and young adults with relapsed or refractory B-cell acute lymphoblastic leukemia (B-ALL), They found that 62% achieved a complete remission and 90.3% were minimal residual disease. With 4.8 years follow up, median overall survival rate is equal 10.5 months. For those underwent the allogeneic

hematopoietic stem-cell transplant (alloHSCT), median overall survival rate is equal 70.2 months, the rate of relapse after alloHSCT was 9.5% at 24 months and 5 years disease free survival following alloHSCT was 61.9% [43].

## Viral versus Non-viral CAR T cell

Up to the present time, the frequently common method to generate CAR T cell is use a viral- based vectors or non-viral vectors. The use of viral vector may provide a higher efficiency of CAR T cells unfortunately, viral vectors carry the barrier in CAR T cells manufacturing not only the insert site of transported DNA limited but also difficulty in producing high titers of stable vector particles. According to current Good Manufacturing Practice (cGMP) the use of non-viral vectors become more feasible for clinical grade manufacturing. A non-viral vector represented by transposons-based system and this system provide safe and reliable DNA transfers. The sleeping beauty (SB) transposon system can be used as a replacement for viral based vectors. However, the integration profile of SB transposon quite random due to their integration at TA sites. The outstanding competition of SB transposon systems is PiggyBac (PB) transposon system that uses a simple cut and paste mechanism. The integration site of PB system is not random because PB usually prefers TTAA sites. The use of PB system exhibited higher transposition activity in mammalian cells [44, 45].

Zhicai Lin and colleague investigate the effect of piggyBac transposons system and Lentivirus vector on CD19 CAR T cells *in vitro* and *in vivo* experiments. Both piggyBac and Lentivirus CD19 CAR T cells were not significant different in percentage of CAR positive cell , anti-tumor activity and level of IFN-g secretion. Interestingly, PiggyBac CD19 CAR T cells can maintain the level of central memory T cell higher than lentivirus CD19 CAR T cells resulting in superior persistence. In xenograft mouse model, both piggyBac and lentivirus CD19 CAR T cells strongly inhibited the tumors cells however more mice treated with piggyBac can survive longer than mice treated with lentivirus. These results support that piggyBac system have specific activities and function, making it an effective tool for generating CAR T cell that can be used in adoptive transfer therapy [46].



## PiggyBac transposon and PB CAR T cell

Daniel L. Galvan and colleague investigate piggyBac integrations in a primary human T lymphocyte. They found that piggyBac have a higher ability to integrate as a stably transfection and offered an efficiency approximately 40 % in primary human T cells [18].

Similarly, Yozo Nakazawa et al. studied the potential of piggyBac system for adoptive transfer therapy in human T lymphocyte. The results revealed that without

selection the piggyBac system mediated a stable expression approximately 20 %. During culturing period, T cell that cultivated with IL-15 can increased the level of expression to 40 % and the expression were sustained over 9 weeks in culture [20].

Then, Daisuke Morita et al. try to improve the manufacturing of piggyBac-modified CD19 by adding the autologous activated T cells as a feeder cells , stimulated T cells via viral antigen and using a CH2CH3free in CD19 CAR construct to provide a better persistence and function. They found that more than 30% of T cell express the CAR gene via adding autologous activated T cells as a feeder cells and viral antigen. CAR expression increases to 51.4 % $\pm$ 14 % by using CH2CH3free in CD19 CAR construct. Moreover, when starting as 10 x $10^6$ cells of PBMCs after 14 days culturing CAR T cell were expanded to 27.8 $\pm$ 28.0 x $10^6$ cells. In summary, piggyBac system can be used as an alternative to viral gene transfer for CAR T cell therapy [24].



Kayoko Nakamura and colleague attempt to improve the quality and efficacy of CAR T cell therapy that characterized by memory-rich phenotype and lower expression of exhaustion by using piggyBac HER2 CAR T cells models. They found that excess stimulation may cause cell death , the use of tumor cell-based feeder or PBMC will enhance the efficacy of transposon mediate CAR manufacturing. However, the use of tumor cell as a feeder cell may require longer expansion period, enrich of effector phenotype, and induce the T cell exhaustion. The antigen presenting feeder cell offer

physiologic interaction that mimic the immune system leading to optimal activation and expansion of memory-rich CAR. In this study, piggyBac HER2 CAR T cells can maintain less differentiated T cell and with minimal PD1 expression resulting to sustained tumor control *in vitro* and *in vivo* [47].

Correspondingly, the quality of CAR T cells therapy are related to memory phenotype and exhaustion levels which was affect the long-term persistence and function of CAR T cell product. To solve with these issue, Masaya Suematsu and colleague evaluate the advantage of CD45RA positive subpopulation as a starting material for generating piggyBac CD19 CAR T cells. They generate piggyBac CD19 CAR T cells from CD45RA positive and CD45RA negative PBMC subpopulation then compare the phenotype and function. The data indicated that piggyBac CD45RA positive CAR T cells have a higher efficiency after 24 hours transduction and better expansion capacity after 14 days of culturing periods. Also, piggyBac CD45RA positive CAR T cells can decrease the expression of exhaustion markers, senescent markers, and enrichment of less differentiated T cells. *In vivo* data demonstrate that piggyBac CD45RA positive CAR T cells can prolong tumor control and support the expansion of CAR T cells even after 50 days of treatment. In this study suggest that piggyBac CD45RA positive CAR T cells can maintain memory-rich phenotype and provide superior antitumor function than CD45RA negative [48].

## Effect of alternative serum supplements on T cell and CAR T cell characteristic and function

### The effect of Human AB serum (ABS or HS)

Andrew R. Medvec and colleagues develop a fully defined medium that could expand all human T cell subsets in the absence of human serum. Then they investigate the ability of serum- free media to expand T cells for adoptive T cell therapy when compared with human serum, they found that human serum may include the agents that harmful for T cell expansion and survival. Moreover, human serum decreased T cell functionality in vitro and in vivo [33].

In the same way, Saba Ghassemi and colleagues investigate the effect of concentrated growth factor extract, purified from human transfusion grade blood fractions, can supports in CAR T cell expansion, and function they found that human serum provides additional stimuli for cell proliferation and survival without any xenogenic components. However, higher concentrations of human serum can suppress T cell growth. [49]

## The effect of Human Platelet Lysate (HPL)

Alejandro Torres Chaves and colleagues evaluate the effect of different sera containing Fetal bovine serum (FBS), Human AB serum (ABS) and Human platelet lysate (HPL) on CAR T cell function *in vitro* and *in vivo* experiments. They found that *in vitro* experiment HPL-exposed CAR T cells showed higher level of Naïve T cell and Memory T cell with superior proliferation and anti-tumor effects in long term. In mouse xenograft model, HPL-exposed CAR T cells can enhance *in vivo* anti-tumor effects and percent of survival. For *ex vivo* experiment, HPL exhibited enhanced anti-tumor response with higher proliferation of CAR T cells. Also, HPL-exposed CAR T cells generated from patient's PBMCs reveal superior anti-tumor effect in long term *in vitro* coculture experiments [50].

Emanuele Canestrari and colleagues explore the use of human platelet lysate (HPL) compared to standard serum supplement including fetal bovine serum (FBS) and human AB serum (ABS) for modified T cell production. They discover that the transduction efficiency with three different promoters were similar in all 3 conditions but a moderate increase in transgene product (MFI fold change) was observed in HPL group when compared with ABS. For T cell expansion in HPL was comparable when compared with ABS but slightly less than the expansion in FBS group. Interestingly, HPL showed a higher percentage of central memory T cell phenotype when compared with

FBS and ABS. These results suggest that the use of HPL as a serum supplement during the production of modified T cells is a reasonable alternative to ABS [32].

## The effect of Xeno-free serum replacement (SR)

Andrew R. Medvec and colleagues develop a fully defined medium that could expand all human T cell subsets in the absence of human serum. They investigate the ability of serum- free media to expand T cells for adoptive T cell therapy and found that expanding T cell in the serum free medium improves both the functionality and durability of engineered T cell also provide the durable anti-tumor response [33].

Karoline W Schjetne and colleagues develop a xeno-free serum replacement supplement to support ex vivo expansion and lentiviral transduction of polyclonal T cells, they observed that serum replacement showed the similar growth kinetics, total fold expansion and transduction efficiency when compared with pooled human serum. Number of CD4 positive and CD8 positive were comparable. In the restimulation T cells setting, serum replacement showed the similar cytokine profile together with proliferation when compared with human serum [51].

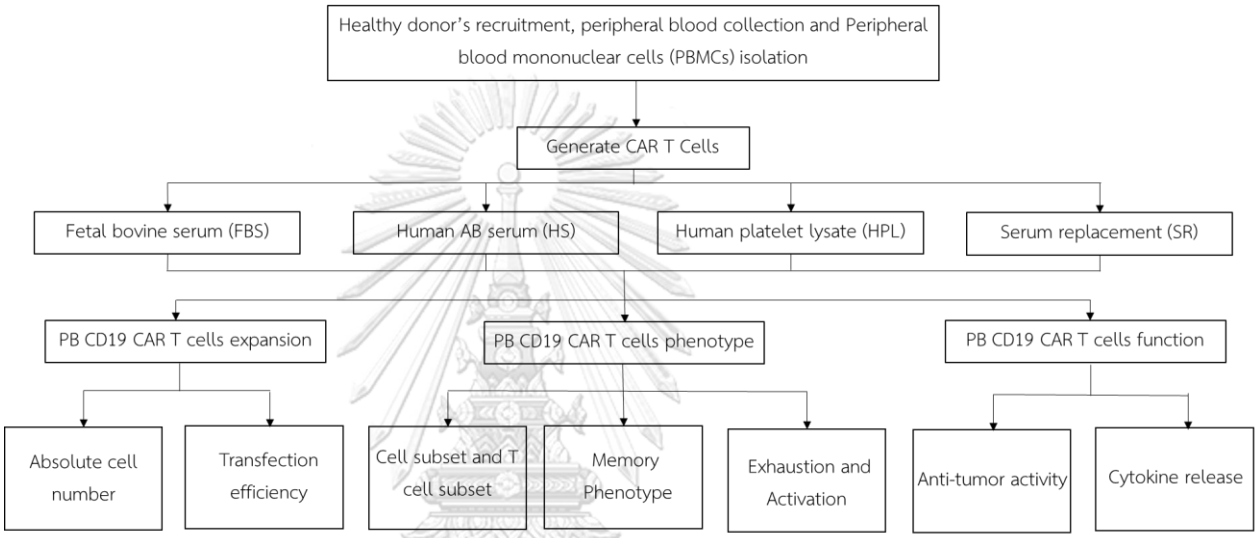
Corey Smith and colleagues investigate the use of xeno-free serum replacement (SR) as an alternative to both HS and FBS in ex vivo expansion of human T cells, they noticed that xeno-free serum replacement (SR) promote the efficient ex vivo expansion

of polyclonally activated T cells similar to HS. However, T cells cultured in SR were predominantly with the central memory T cell phenotype. Additionally, they also investigate the use of G-Rex in culture system with SR and found that SR can be used effectively to optimize the expansion of antigen specific T cells. Then, they summarize that SR provides a novel platform strategy for clinical grade adoptive cellular therapies manufacturing.

# CHAPTER 3

# MATERIALS AND METHODS

## RESEARCH DESIGN




### Inclusion criteria



Volunteers are eligible to be included in the study only if all of the following criteria apply:

1. Capable of giving signed informed consent and provision of signed and dated written ICF prior to any mandatory study specific procedure and analyses
2. Volunteers must be ≥ 18 years of age at the time of signing the informed consent. All genders are permitted.
3. No history of immunodeficiency and hematologic malignancy

## Exclusion criteria

Volunteers are excluded from the study if any of the following criteria apply:

1. Prior or current treatment with immunosuppressive drugs or chemotherapy
2. For women- currently pregnant or breast-feeding
3. Previous allogenic bone marrow transplant

## Sample collection

The samples from healthy volunteers will be obtained after signing of informed consent and procedures will be performed with the approval of Institutional Ethics Committee of Chulalongkorn University.

## Population

Healthy volunteers in Thailand



# METHODOLOGY

## Primary cell and cell lines

NALM-6 cell was maintained in RPMI 1640 medium, supplemented with 1X Glutamax (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.), 1% Penicillin-

Streptomycin, and 10% Heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.).

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers after informed consent on protocols approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB NO.489/64). All experiments were performed in accordance with the relevant guidelines and regulations.

Generation of PB CD-19 CAR T cells Cells with different serum supplements

PBMCs were isolated from peripheral blood samples by density gradient centrifugation using Ficoll-Paque™ PREMIUM (GE Healthcare Bio-Sciences AB, Sweden). To generate CAR T cells, PBMCs 4 x $10^6$ cells were mixed with P3 Primary Cell 4D-Nucleofector X Kit reagent (Lonza, Basel, Switzerland) that contained piggyBac transposon plasmid carrying CD-19 CAR and transposase plasmid. Afterward, the PBMCs were electroporated by using a 4D-Nucleofector machine (Lonza, Basel, Switzerland). Electroporated cells were cultured in 48 well plates that contained TexMACS medium (Miltenyi Biotec, Bergisch Gladbach, German) plus 5% of different serum supplements including Fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, Massachusetts, U.S.), Human AB serum (HS, Sigma Life Science, Saint Louis, USA), Human platelet lysate (HPL, Stemcell Technologies, Vancouver, Canada) and Serum Replacement (SR, Thermo Fisher Scientific, Waltham, Massachusetts, U.S.), and 10 ng/ml of IL-7, 5 ng/ml of IL-15.

Electroporated cells were cultured with irradiated PBMC at a 1:1 ratio. After transfection, cells will be cultured for 14 days during that time culture media plus 5% of different serum supplements were changed on days 4, 7, and 10 after transfection with 10 ng/ml of IL-7 and 5 ng/ml of IL-15. Cell count was done to determine cell number and fold expansion.

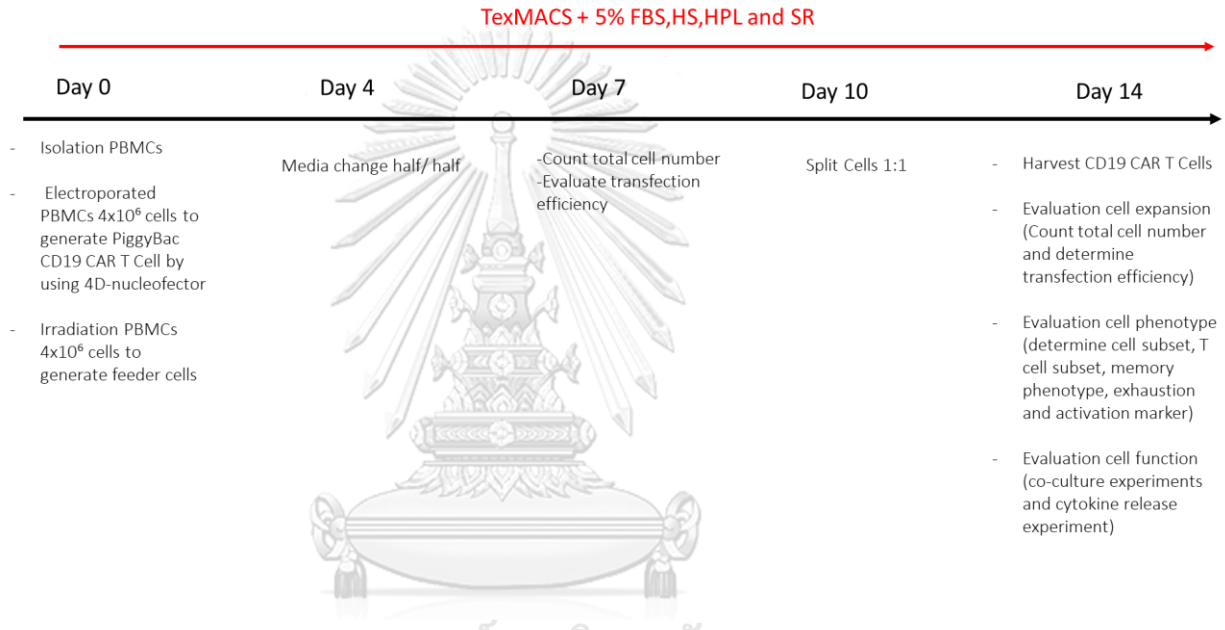



*Figure 7 Methodology schema*



## Comparing the expansion of PiggyBac CD-19 CAR T Cells

On day 7 and 14 after transfection, PB CD-19 CAR T cells will count the viability cell using Trypan Blue and PB CD-19 CAR T cells were aliquoted approximately $2x10^5$ cells for measure transfection efficiency by flow cytometry. Percent of cell expansion and fold change were calculated. The formular of total cell = (total live cell ÷ number of

counting corner) x dilution factor x suspension volume x $10^4$(chamber volume) and the formular of fold change = number of cells at the interesting time point ÷ number of cells at the beginning.

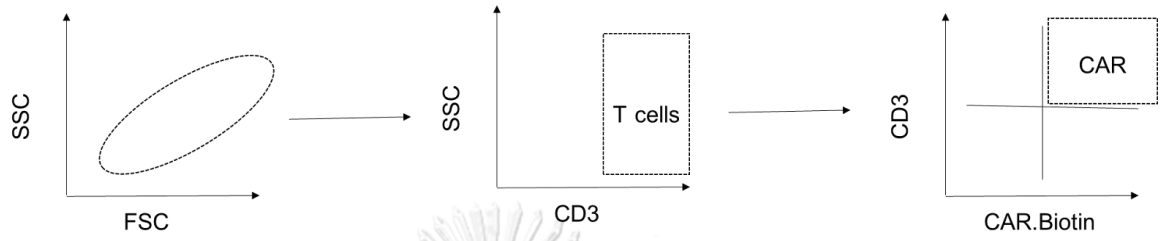



*Figure 8 Gating Strategy to determine transfection efficiency*

## Comparing the phenotype of PiggyBac CD-19 CAR T Cells

On day 14 after transfection, PB CD-19 CAR T cells that culture with different of serum supplement were harvested and aliquoted approximately 2x$10^5$ cells per test to determine cells phenotype including cell subset (CD3,CD56,CD14,CD19),T cell subset (CD3,CD4,CD8), Memory Phenotype (CD45RO , CD62L), Exhaustion marker (PD-1,TIM-3,LAG-3,TIGIT) and Activation marker (CD25, CD69) by using Flow cytometry. Likewise, cells were stained with antibodies for 15 min at 4° C and washed with staining buffer 4 ml after that the supernatant will be discarded. The pellet will be resuspended in 500 ul of staining buffer.

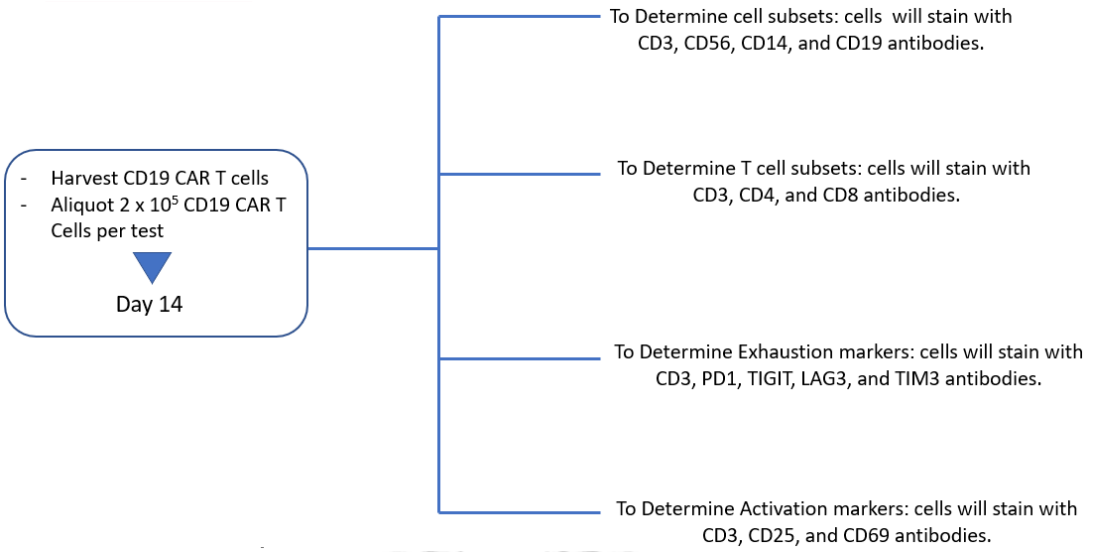



*Figure 9 Schematic of cell phenotype*

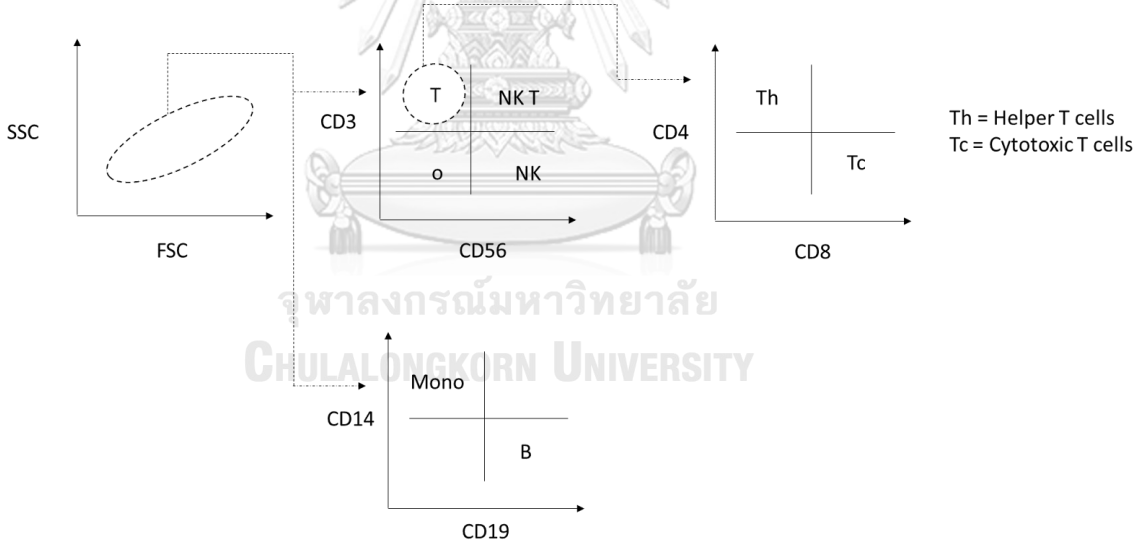





*Figure 10 Gating Strategy to determine cell subset and T cell subset*

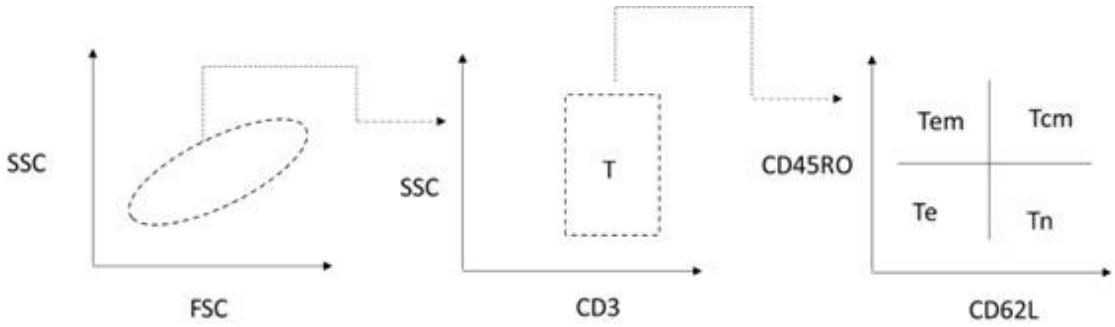



*Figure 11 Gating Strategy to determine memory phenotype*

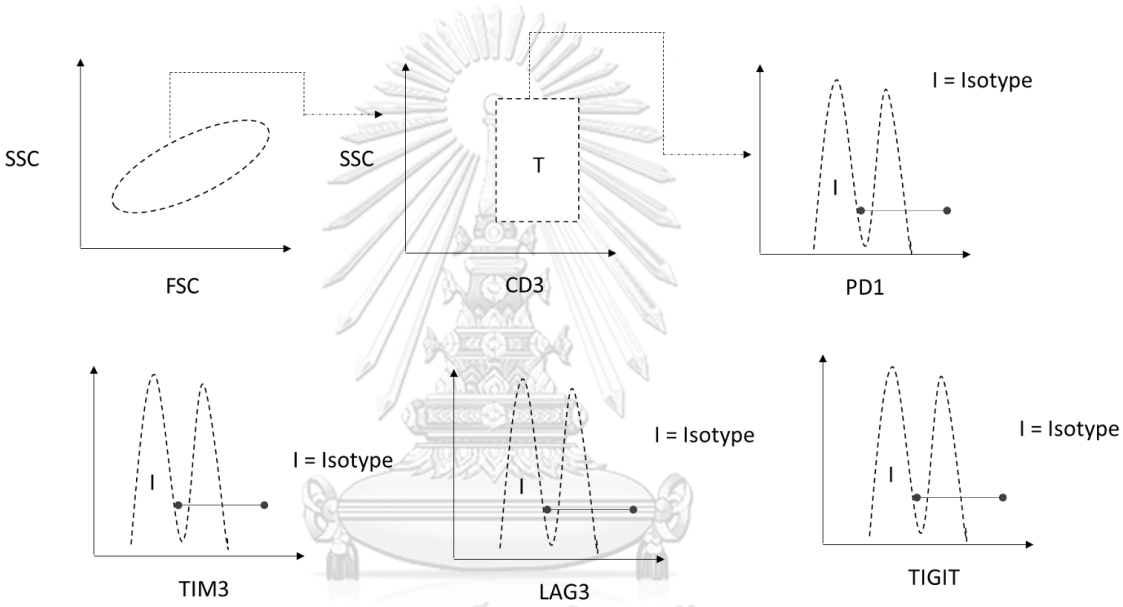





*Figure 12 Gating Strategy to determine exhaustion markers*

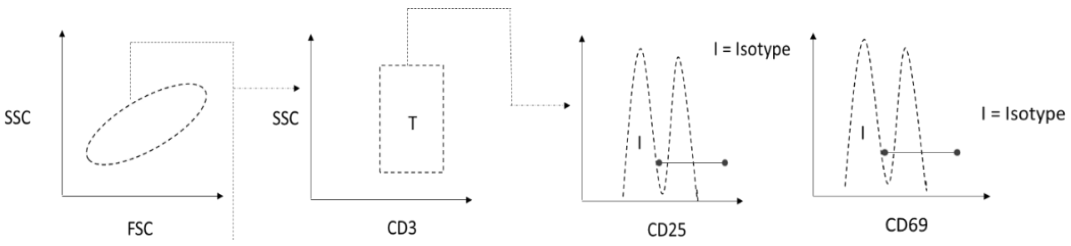



*Figure 13 Gating Strategy to determine activation markers*

## Comparing the function of PiggyBac CD-19 CAR T Cells

### Short term co-culture assay

The short-term antitumor activity of PB CD-19 CAR T cells was examined by co-culturing of effector T cells with the NALM6 cell line at effector/target (E: T) ratios of 1:25, 1:5, 1:1 and 5:1 in 96 well plate with TexMACS medium plus 5% FBS without cytokines for 24 hours. At the end of culture, the absolute number of effector and target cell was determined using flow cytometry, and the percentage of inhibition was calculated using the following formula:

$$\text{Inhibition} = 100 - \left(\frac{number\ of\ Target\ (experiment)}{number\ of\ Target\ (Target\ alone)} \times 100\right)$$

T cells were analyzed for the expression of activation and exhaustion markers using flow cytometry.



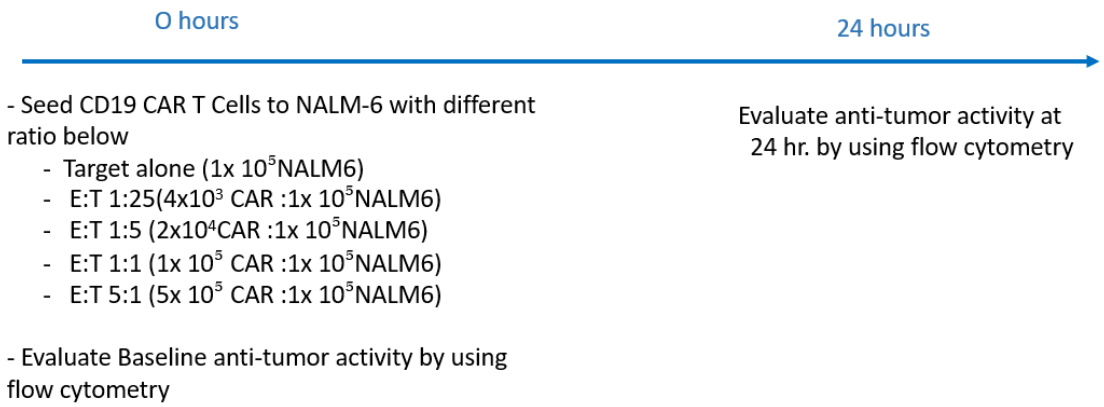



*Figure 14 Schematic of 24 hr. short-term co-culture experiments at different Effector: Target (E: T) ratio.*

## Long term co-culture assay

The antitumor activity and expansion of PB CD-19 CAR T cells was examined by co-culturing of effector T cells with the NALM-6 cell line at E: T ratio of 1:5 in G-REX 24-well plate (Wilson Wolf, USA) with TexMACS medium plus 5% FBS without cytokines for 7 days. At the end of culture, the absolute number of effector and target cell was determined using flow cytometry, and the percentage of inhibition was calculated as mentioned above. T cells were analyzed for expansion and the expression of CAR, activation markers, exhaustion markers using flow cytometry.

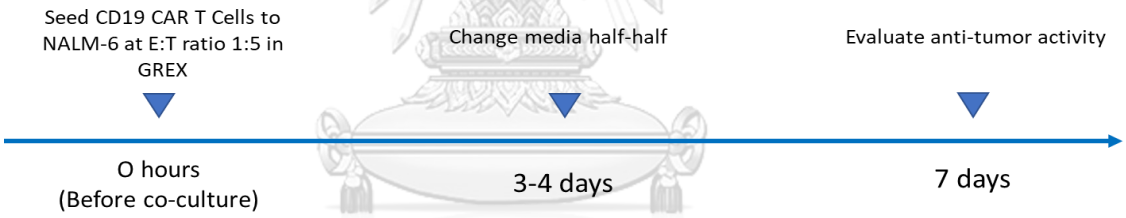





*Figure 15 Schematic of 7 days long term co-culture assays in G-REX system at Effector: Target (E: T) ratio 1:5.*

## Tumors rechallenge assay

The antitumor activity and expansion of the PB CD-19 CAR T cells was examined by co-culturing with NALM-6 cell line at effector/target (E:T) ratios 1:1 for 24 hours. Then cells were harvested and counted for total cell number then absolute T cells number

were calculated from the percentage of T cell determined by flow cytometry. Afterward, T cells were replated and coculture with fresh NALM-6 cell line at a ratio 1:1. Cell counting and replating were repeated every 24 hours for 3 rounds.

After co-culturing for 24 hours in each round, PB CD-19 CAR T cells were analyzed for the percentage of T cell and tumor cell, the expression of activation markers, and exhaustion markers using flow cytometry.

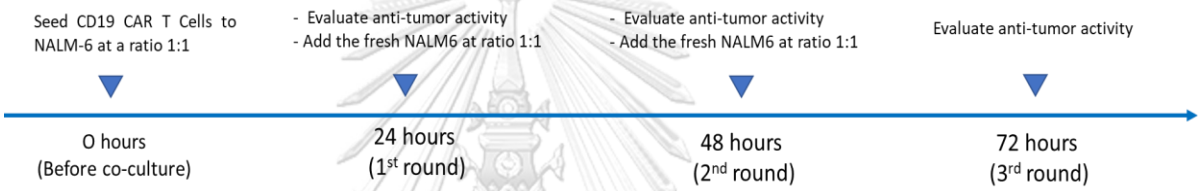



*Figure 16 Schematic of tumors rechallenge assay at Effector: Target (E: T) ratio 1:1.*



## Cytokine releasing assay

PB CD-19 CAR T cells were co-cultured with NALM6 at an E:T ratio of 1:1 for 24 h. Culture supernatants were then collected and stored at −20°C. Quantification of cytokines was analyzed by flow cytometry using the BD™ CBA Human Th1/Th2 Cytokine Kit II (BD Bioscience, USA), and the data were analyzed by FCAP Array version 4 software (BD Bioscience, USA).


*Figure 17 Schematic of Cytokine release assay*

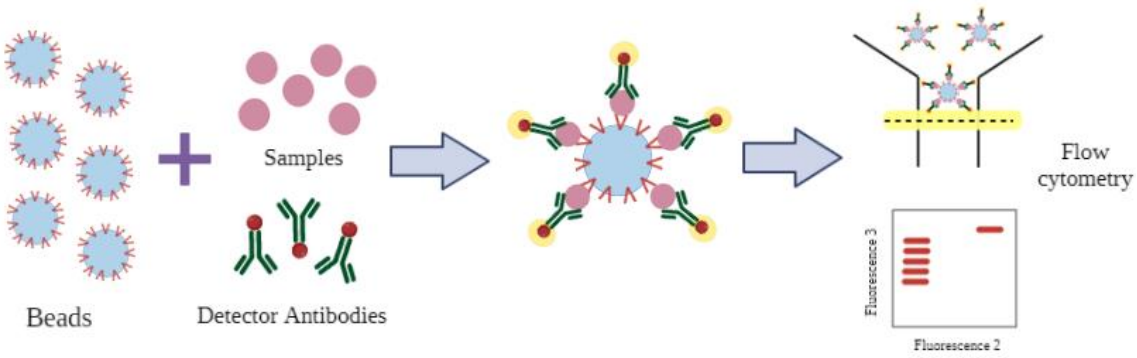



*Figure 18 The principle of Cytometric Bead Array (CBA) assay*



## Statistics

Data are presented as means ± SEM. Statistical analysis was performed using GraphPad Prism version 8 (GraphPad Software). One-way and two-way ANOVA was used to determine statistical significance. P-values less than 0.05 were considered statistically significant.

## Ethics approval

All experimental procedures were approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (approval number: 489/64, date of approval, June 14, 2021. Informed consent was signed by all participants.

# CHAPTER 4

# RESULTS

## All serum supplements similarly support the expansion of PB CD-19 CAR T cells

First, we compared the effects of different serum supplements on PB CD19 CAR T cell expansion. Electroporated cells were cultured in TexMACS plus 5% of FBS, HS, HPL and SR for 14 days and the cells were collected on day 7 and day 14 for further analyses (Figures 19A). There were not statistically significant between total cell number and fold expansion among the 4 sources of serum supplement (Figures 19B and 19C). On day 7 after electroporation, the transduction efficiency was 19.03% ± 3.41, 13.75% ± 3.53, 10.89% ± 3.19, and 13.69% ± 2.70 for FBS, HS, HPL, and SR respectively, which was increased to 52.79% ± 6.42 (FBS), 37.36% ± 6.315 (HS), 35.4% ± 5.89 (HPL), and 41.77% ± 5.40 (SR) on day 14. Although we observed a slightly higher percentage of CAR expression in CD19 PiggyBac CAR T cell cultured with FBS, there were not statistically significant of transfection efficiency and absolute CAR T cell number among four conditions (Figures 19D, 19E, and 19F). As a result, the different of serum supplements didn't affect the cell expansion and transfection efficiency.

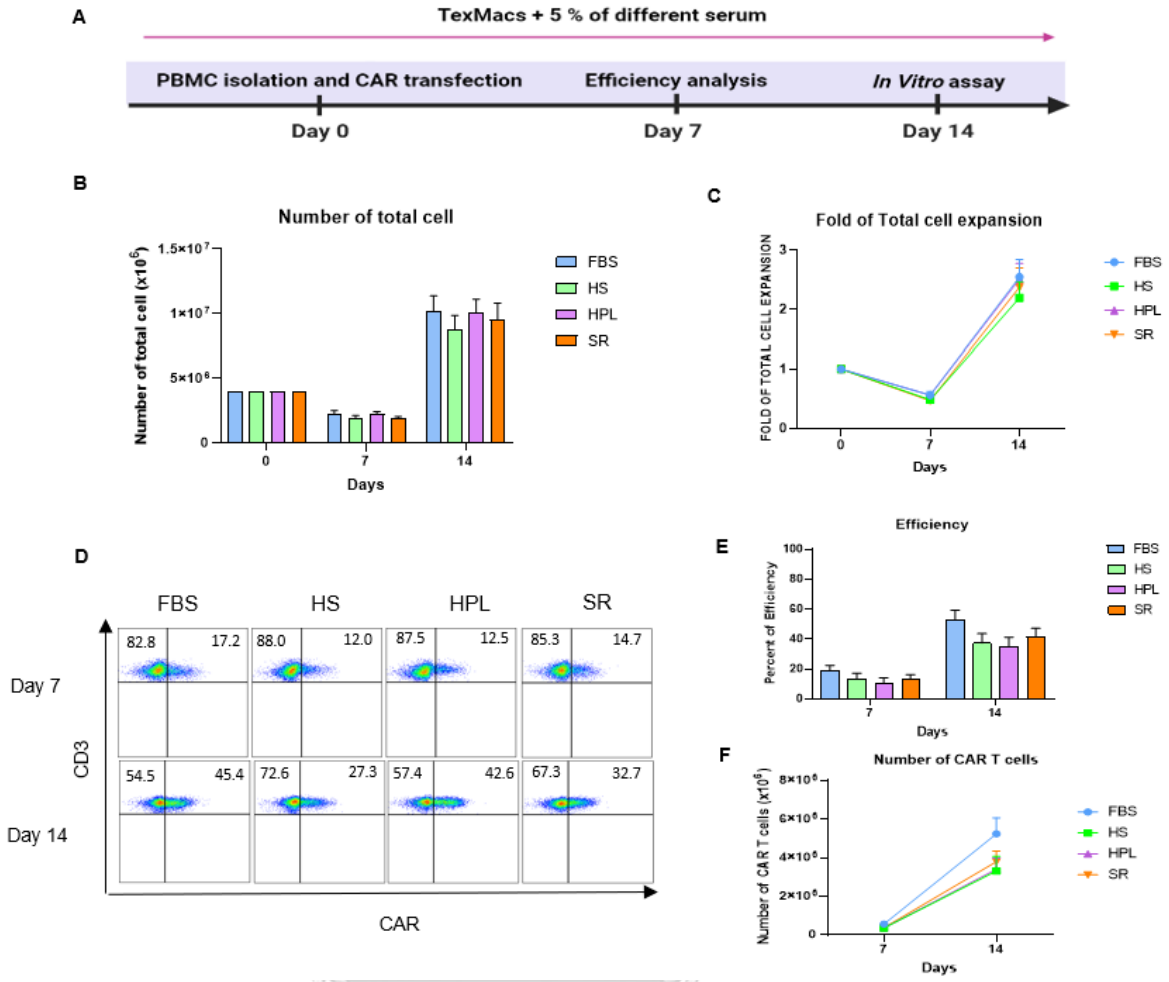



*Figure 19 Electroporated cells expanded in different serum supplements.*

(A) Schematic CAR T cell generation. (B) Expansion of total cells in different serum supplement. (C) Data of total cell expansion represent as fold expansion on 0, 7 and 14. (D) Representative flow cytometry plot demonstrating CAR expression on day 7 and day 14 after transfection. (E) Transfection efficiency of CAR (F) CAR+ T cell numbers were calculated by total cell number and percentage of CD3+CAR+ cells on days 7 and 14 after transfection, The data shown are mean ± S.E.M (n=7). Significance was determined by one-way ANOVA.

## Cultivation of PB CD-19 CAR T cells with SR promoted the maintenance of a naïve memory phenotype

We next evaluated the immune-phenotypic profile of PB CD-19 CAR T cells cultured in different serum supplements and found that there were no difference in percentage of $CD3^+$ T cell and CD4:CD8 ratio of CAR T cells cultured with four different serum supplements (Figures 20A, 20B, 20C and 20D). Memory phenotype was determined by expression of CD45RO and CD62L as depicted by figure 20E. The percentage of terminal effector T cell and central memory T cell were comparable between four groups. However, PB CD-19 CAR T cells cultured with HS exhibited higher percentage of effector memory T cell comparing with those cultured with FBS, HPL and SR and the percentage of naïve T cell was highest in a group of CAR T cells cultivated with SR [37.68%± 2.17 (FBS), 27.13%± 3.4(HS), 17.38% ± 0.921(HPL), and 55.24%± 9.83(SR), p-value =0.0056] (Figures 20F). In addition, we evaluated exhaustion and activation markers of PB CD-19 CAR T cells after 14 day of culture and observed no difference in between each group (Figures 20G and 20H). In summary, cell subset, exhaustion and activation were comparable in all conditions. However, SR-cultivated PB CD19 CAR T cells exhibited the higher level of naïve phenotype.

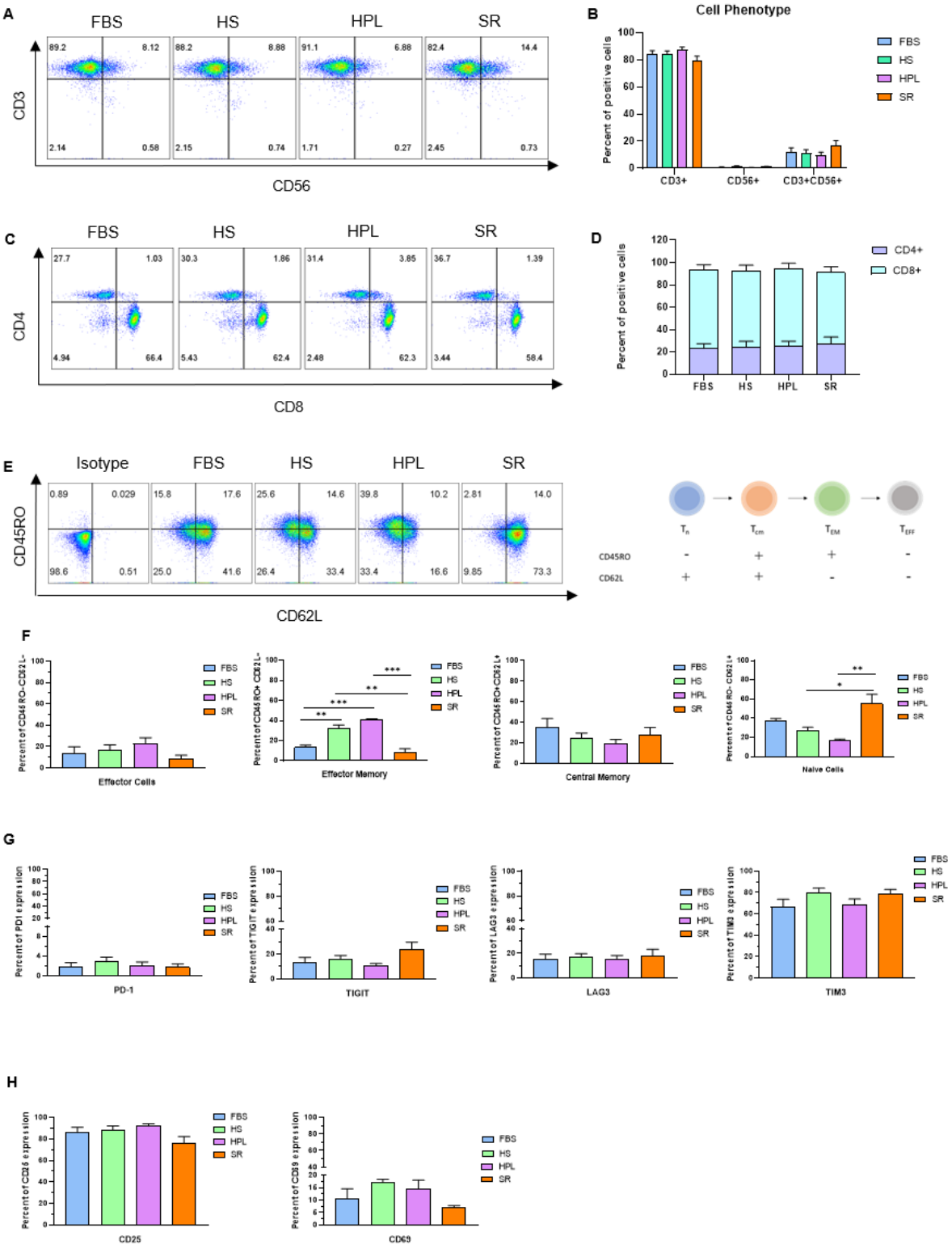

A
FBS
HS
HPL
SR
CD3
CD56
89.2
8.12
2.14
0.58
88.2
8.88
2.15
0.74
91.1
6.88
1.71
0.27
82.4
14.4
2.45
0.73
B
Cell Phenotype
Percent of positive cells
CD3+
CD56+
CD3+CD56+
C
CD4
CD8
27.7
1.03
4.94
66.4
30.3
1.86
5.43
62.4
31.4
3.85
2.48
62.3
36.7
1.39
3.44
58.4
D
CD4+
CD8+
E
Isotype
CD45RO
CD62L
0.89
0.029
98.6
0.51
15.8
17.6
25.0
41.6
25.6
14.6
26.4
33.4
39.8
10.2
33.4
16.6
2.81
14.0
9.85
73.3
F
Effector Cells
Effector Memory
Central Memory
Naive Cells
G
PD-1
TIGIT
LAG3
TIM3
H
CD25
CD69

*Figure 20 phenotypical of PiggyBac CD19 CAR T Cells.*

The phenotypical of CAR T cells were analyzed on day 14 after transfection (A) Representative flow cytometry plot demonstrating the cell subset stained with fluorochrome-conjugated anti-CD3 and anti-CD56 on day 14 after T cell transfection. (B) Mean percentage of CD3+CD56+ are shown. (C) Representative flow cytometry plot demonstrating T cell subset, stained with fluorochrome-conjugated anti-CD4 and anti-CD8 antibodies. (D) Mean percentage of CD3+CD8+: CD3+CD4+ ratio on day 14 after transfection are shown. (E) Representative flow cytometry analysis of memory phenotype. The memory phenotypical of CAR T cells were analyzed on day 14 after transfection, CAR T cells were stained with anti-CD45RO and anti-CD62L antibody (TEM: CD45RO+, CD62L-, TCM: CD45RO+,CD62L+, TN: CD45RO-,CD62L+, TE: CD45RO-,CD62L-). (F) Phenotype of T cells on day 14 after transfection shown the percentage of naïve (TN), central memory (TCM), effector memory (TEM), and terminal effector (TE) T cells. (G) The phenotype of T cells on day 14 after transfection showed the percentage of exhaustion markers including PD-1, TIGIT, LAG3, and TIM3. (H) The phenotype of T cells on day 14 after transfection showed the percentage of activation markers including CD25, and CD69. Data represents as mean ± S.E.M (n = 8) and significance was determined by one-way ANOVA, * for $p<0.05$, ** for $p<0.01$, *** for $P<0.001$.

**SR cultured PB CD-19 CAR T cells increase IL-4 secretion, and HPL cultured PB CD-19 CAR T cells increase IL-2, IL-10 and IL-6 secretion after antigen stimulation**

To determine the CAR T cell cytokine secretion profile, PB CD-19 CAR T cells expanded with either FBS, HS,HPL, or SR were harvested on day 14 and subjected for target cell stimulation. Measurement level of cytokine after antigen stimulation revealed a nonsignificant of IFN-g and TNF-a among 4 conditions (Figures 21A and 21B). However, we found a significant increase of IL-4 level after 24 hours antigen stimulation in SR group [9.793 pg/ml ± 3.309 (FBS), 7.166 pg/ml ± 1.63(HS), 3.70 pg/ml ± 0.885(HPL), and 23.859 pg/ml ± 5.419 (SR), p-value =0.0024]. Whereas the level of IL-2, IL-10, and IL-6 were significant increase in HPL group. For IL-2, 2649.14 pg/ml ± 362.59 (FBS), 4448.18 pg/ml ± 1458.49 (HS), 11715.52 pg/ml ± 2049.76 (HPL), and 3394.64 pg/ml ± 647.21 (SR), p-value =0.0003]. For IL-10, 19.61 pg/ml ± 4.84 (FBS), 27.51 pg/ml ± 4.7 (HS), 51.22 pg/ml ± 7.78 (HPL), and 22.81 pg/ml ± 2.77 (SR), p-value =0.0028]. For IL-6, 35.52 pg/ml ± 23.50 (FBS), 51.33 pg/ml ± 13.85 (HS), 192.996 pg/ml ± 49.55 (HPL), and 41.98 pg/ml ± 17.36 (SR), p-value =0.0074]. (Figures 21C, 21D, 21E and 21F). To sum up, IFN-g and TNF-a were comparable in all conditions, but SR-cultivated PB CD19 CAR T cells exhibited a higher level of IL-4. On the other hand, HPL-cultivated PB CD19 CAR T cells exhibited a higher level of IL-2,IL-6, and IL-10.

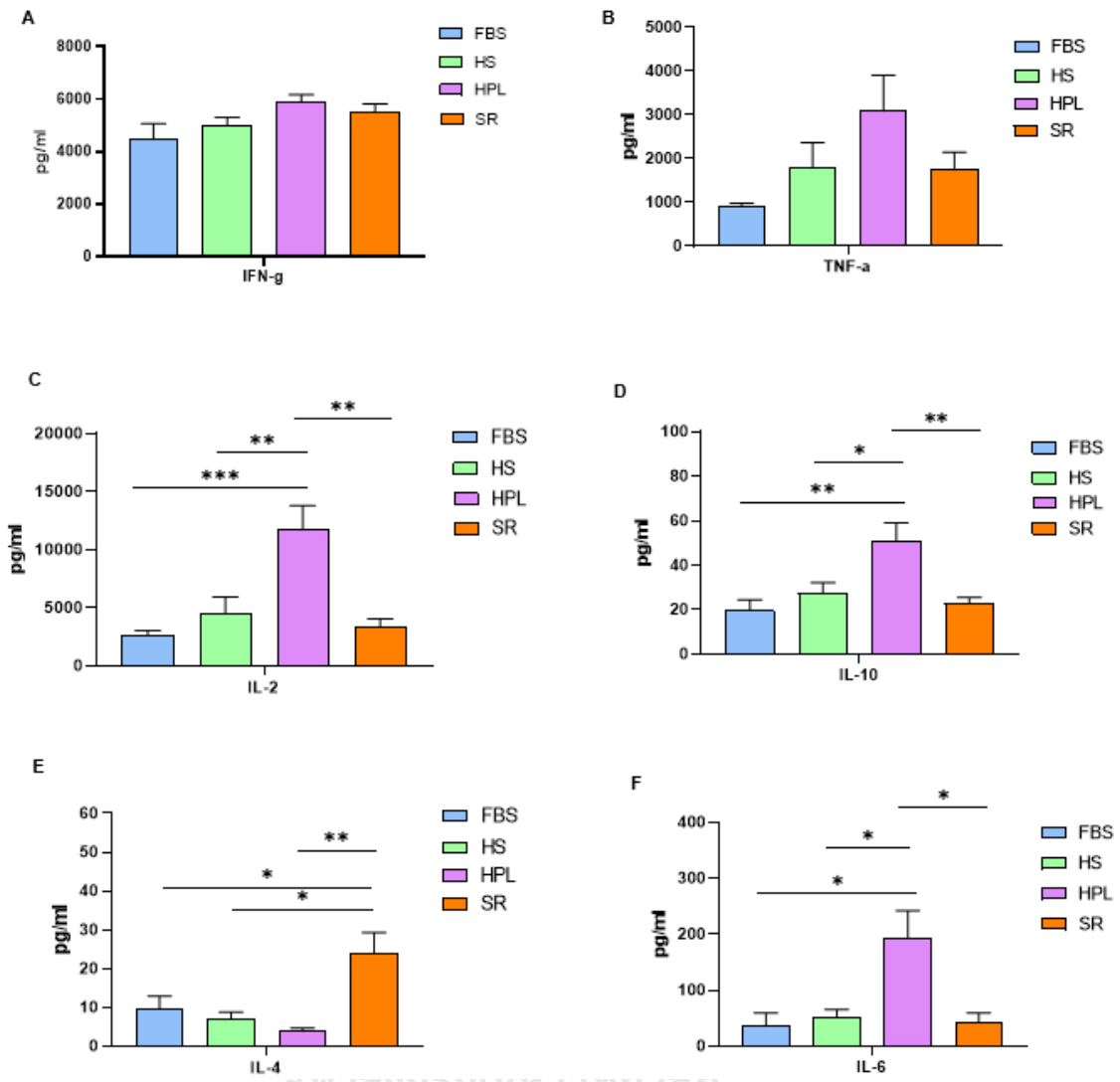





*Figure 21 PiggyBac CD19 CAR T Cells increase cytokine production after stimulations with leukemia cells.*

PiggyBac CD19 CAR T cells were co-cultured with leukemia cell line, NALM6 at Effector: Target (E: T) ratio 1:1 without adding cytokine for 24 hours. Quantification of flow cytometric analysis of cytokine including (A) IFN-g, (B) TNF-a, (C) IL-2, (D) IL-10, (E) IL-4, and (F) IL-6 secretion after co-culture. *P*-value significance is indicated as * for $p<0.05$, ** for $p<0.01$*** for $p<0.001$. Data represents is mean ± SEM (n = 8).

## PB CD19 CAR T cells cultivated with SR showed better proliferation in chronic and repeated antigen exposure models

To evaluate cytolytic function and antigen specificity of CAR T cells maintained in different serum supplements, we performed a 24-h co-culture assay with various E:T ratio and found that PB CD19 CAR T cell cultured with either FBS, HS, HPL, or SR displayed potent cytotoxic function against CD19+ target in a dose dependent manner with the percent inhibition rate was more than 90% at E:T ratio of 1:5. There were no statistically significant of inhibition ratio between each conditions (Figure 22A and 22B). We then performed a long-term co-culture assay to evaluate long-term anti-tumor activity and T cell proliferation (Figures 22C). At the end of 7-day co-culturing, CAR T cells from four differential serum supplements exhibited comparable potent antitumor activity, where the tumor cells were barely detected (Figure 22D and 22E). Interestingly, only CAR T cell expanded with SR has significantly higher T cell expansion after 7 days of co-culturing with target compared with FBS, HS, and HPL (Figure 22F). Additionally, CAR expression after co-culturing with target cells was determined and showed to be comparable among four groups (Figure 22G and 22H).

To imitate the chronic antigen stimulation *in vivo*, we evaluated the anti-tumor and proliferation capacity of CAR T cells after multiple rounds of antigen exposure using a sequential co-culture assay(Figure 23A). We found that PB CD-19 CAR T cells from all

four groups completely eliminated tumor cells during each round of tumor re-challenge, but the SR group exhibited significantly higher T cells proliferation after multiple rounds of tumor re-challenge comparing with FBS group, HS group, and HPL group (Figures 23B, 23C and 23D). This result is consistent with those observed in 7-day co-culturing assay. As a conclusion, anti-tumor activities were comparable in all conditions. Although, a higher T cell proliferation after antigen stimulations were observed in SR group.

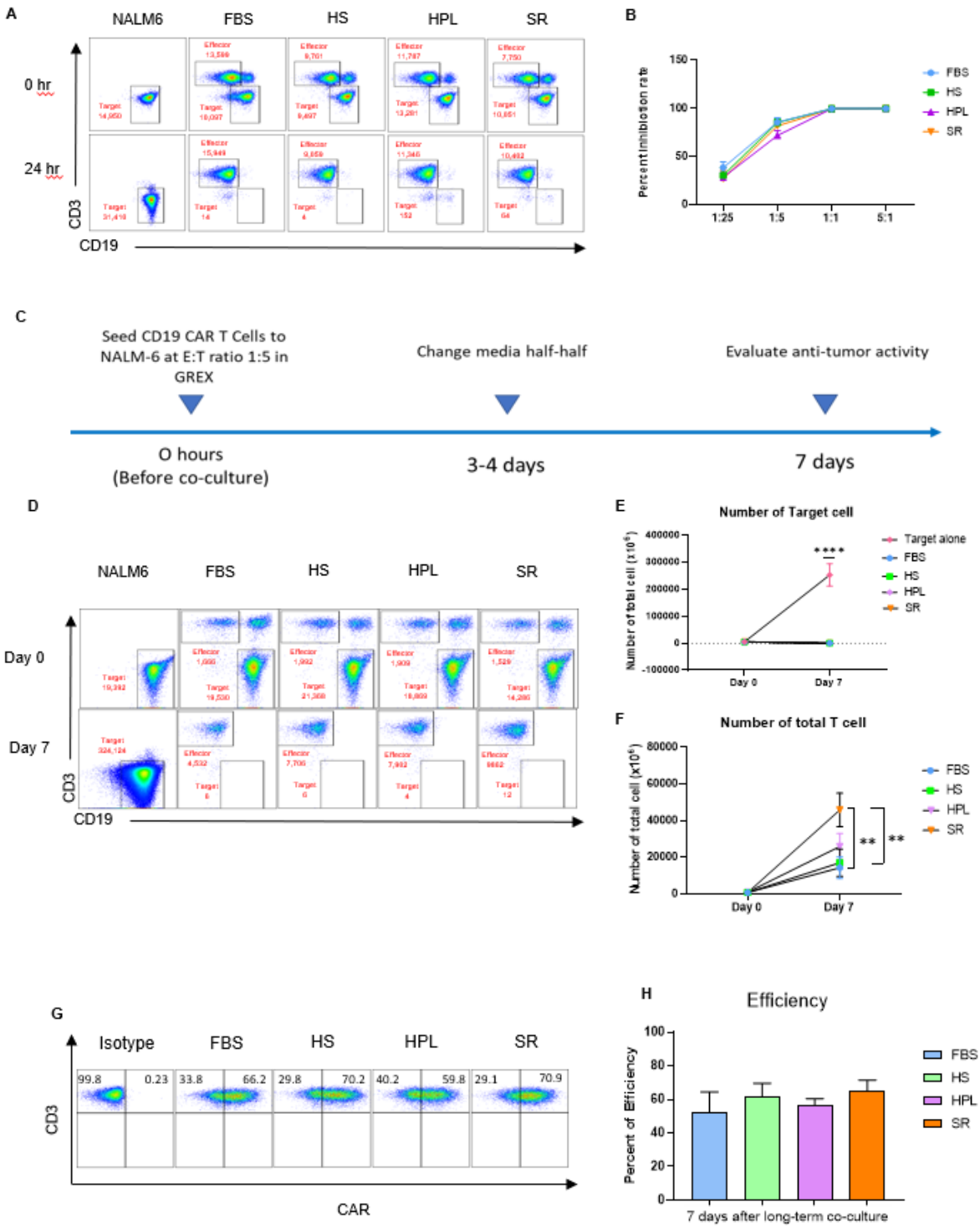



*Figure 22 PiggyBac CD19 CAR T Cells with different serum show potent anti-tumor activity against leukemia cell line (NALM6).*

PiggyBac CD19 CAR T Cells were co-cultured with leukemia cell line, NALM-6 at Effector: Target (E: T) ratio 5:1, 1:1, 1:5 and 1:25 without adding cytokine for short-term co-culture (24 hours), at Effector: Target (E: T) ratio 1:1 for sequential co-culture for 24 hours in each round. At the indicated point, number of tumor cells and T cells were determined by flow cytometry. (A) Representative flow cytometry of short-term co-culture at ratio 1:1 are shown. (B) The Inhibition ratio of short-term co-culture at 24 hours of co-culture is calculated. Data represents is mean ± SEM (n = 5). (C) Schematic of 7days long-term co-culture experiments at Effector: Target (E: T) ratio 1:5. (D) Representative flow cytometry of long-term co-culture at ratio 1:5 are shown. (E) The number of Target cells, after long-term co-culture with NALM-6 for 7 days at ratio E:T of 1:5. (F) The number of Total T cells, after long-term co-culture with NALM-6 for 7 days at ratio E:T of 1:5. (G) Representative flow cytometry plot demonstrating CAR expression on 7days long-term co-culture experiments. (H) Efficiency at days 7 after long-term coculture. *P*-value significance is indicated as * for $P<0.05$, ** for $P<0.01$, *** for $P<0.001$,**** for $P<0.0001$. Data represents is mean ± SEM (n = 4).

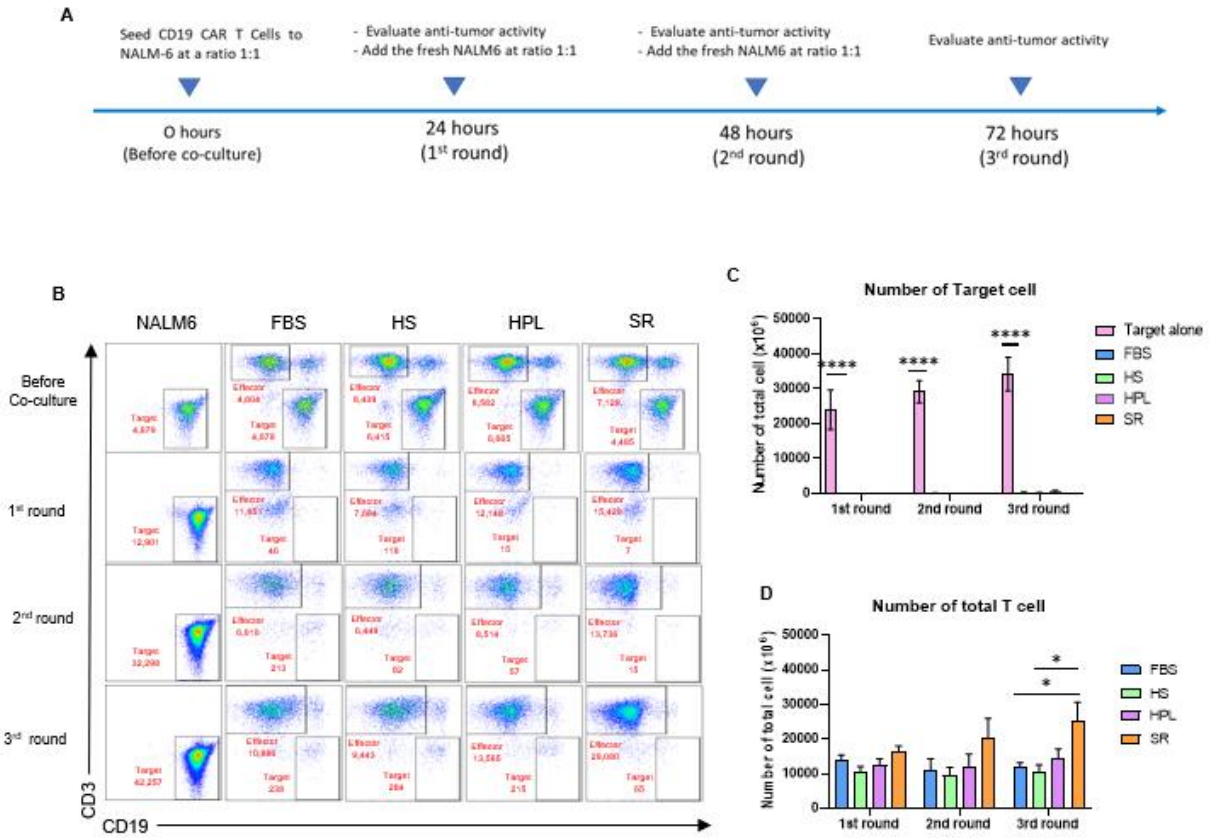



*Figure 23 PiggyBac CD19 CAR T Cells with different serum show potent anti-tumor activity against leukemia cell line (NALM6) at various E:T ratios.*

(A) Schematic of sequential co-culture experiments (B) Representative flow cytometry resulted of sequential co-culture for 24 hours in each round are shown. (C) The number of Target cells, after sequential co-culture with NALM-6 for 24 hours in each round at ratio E:T of 1:1. (D) The number of Total T cells, after sequential co-culture with NALM-6 for 24 hours in each round at ratio E:T of 1:1. *P*-value significance is indicated as * for P<0.05, ** for P<0.01, *** for P<0.001,**** for P<0.0001. Data represents is mean ± SEM (n = 3).

**SR cultured PB CD-19 CAR T cells exhibited lower early activation marker with similar exhaustion profile after multiple rounds of antigen exposure.**

To further investigate the effects of distinct serum supplement on the immunophenotype of PB CAR T cell. We analyzed the expression of exhaustion and activation markers after each round of antigen exposure. We found that the expression level of exhaustion markers wasn't different in all conditions (Figure 24A, 24B, 24C and 24D). This was also observed in the 7-day co-culture experiment where differential serum supplement did not impact CAR T cell exhaustion phenotype after prolong antigen stimulation (supplementary figure 1). However, for activation markers, SR group exhibited lower expression of CD69 but not CD25 after each round of tumor re-challenge comparing with other conditions (Figure 24E and 24F). In summary, exhaustion and CD25 marker were comparable in all conditions but lower expression of CD69 were observed in SR group.

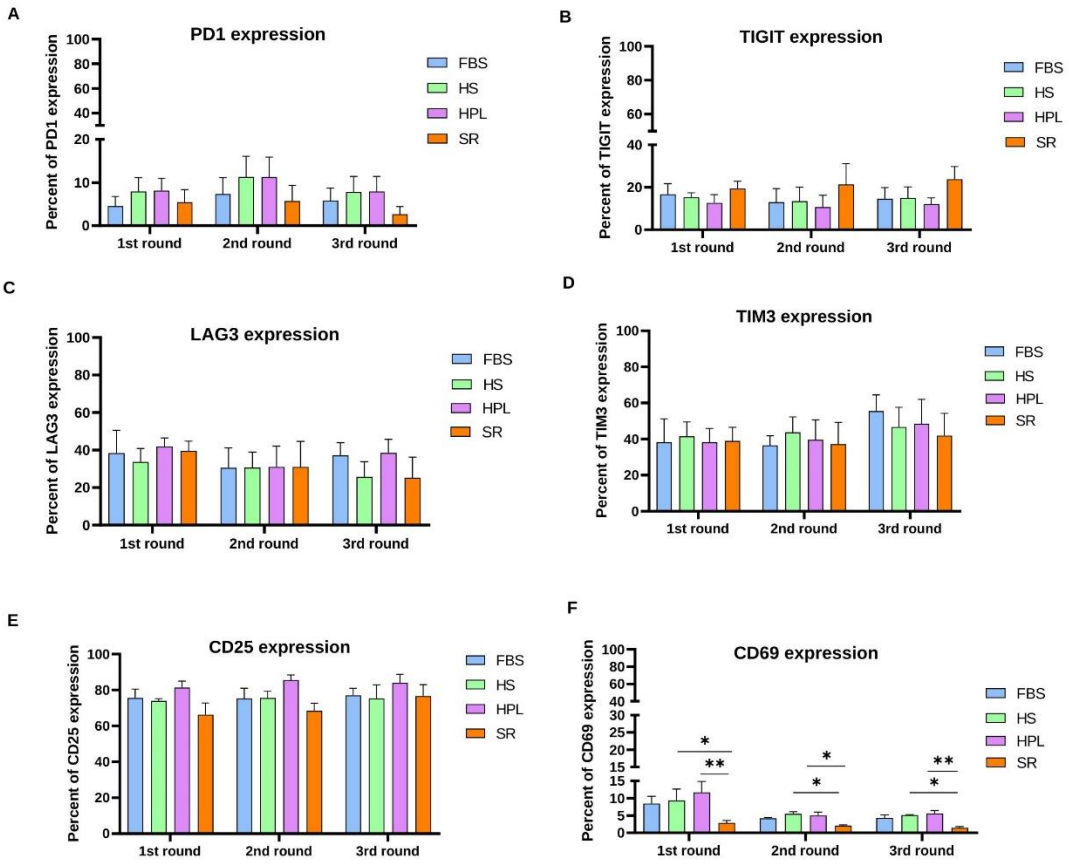



*Figure 24 PiggyBac CD19 CAR T cells expressed inhibitory molecules and activation molecules after sequential co-culture with leukemia cells (NALM6).*

PiggyBac CD19 CAR T cells were sequential co-cultured with leukemia cancer cell line (NALM6) at Effector: Target (E:T) ratio 1:1 without cytokine. (A) The percentage of PD-1 (B) TIGIT (C) LAG3 (D) TIM3 (E) CD25 and (F) CD69 expressing on different serum supplements (FBS, HS, HPL, and SR) at ratio 1:1 for 24 hours in each round. *P*-value significance is indicated as * for P<0.05, ** for P<0.01, *** for P<0.001. Data represents is mean ± SEM (n = 3).

# CHAPTER 5

# DISCUSSION AND CONCLUSION

In the present study, the influence of different serum supplements on immune characteristics and function of PiggyBac based CAR T cell were evaluated. We have reported that different source of serum supplements did not affect cell expansion rate, T cell subset, as well as PB transfection efficiency. However, expansion of PB CD19 CAR T cell using serum-free chemical reagent SR resulted in a higher percentage of naïve T cells; a preferable phenotype for *in vivo* persistence. Although the tumor elimination rate was not altered by different type of serum supplement, CAR T cells cultivated in SR-supplemented culture media exhibited better proliferate rate and increased IL-4 secretion upon antigen exposure, when comparing with those expanded with standard FBS, xeno-free HS or xeno-free HPL .



It has been known that memory phenotype of CAR T cell has an impact on the treatment outcome after CAR T cell administration [52]. Previous preclinical studies have provided evidence that naïve and stem cells like memory T cell are crucial for *in vivo* expansion, survival, and long-term persistence of CAR T cell [53] which corresponding to the finding from clinical study reported that a higher percentage of naïve T cells in the infused product resulting in higher post-infusion expansion and better response [54]. In this study, we observed more naïve phenotype in PB CD19 CAR T cell cultivated with

SR supplements culture media comparing with standard FBS HS and HPL supplements culture media. Similar finding has been observed in activated T cell and virus specific T cell expansion using SR supplemented culture condition which show less differentiated T cell memory immunophenotype comparing with FBS and SR supplemented culture condition [51, 55].

To further study the effects of serum supplement on T cell exhaustion, we set up a tumor rechallenge assay, that allows CAR T cell to encounter target antigen for multiple rounds. However, our PB CD19 CAR T cells, regardless of cultivation with different serum supplements, could sustain killing activity without significantly upregulation of exhaustion makers. Therefore, the differential effect of serum supplement on T cell exhaustion was not detected in our study. A previous study by Nakamura et al. demonstrated that PiggyBac anti-HER2 CAR T cells exhibited potent and sustained killing activity against target cells even after multiple rounds of tumor rechallenge without rendering T cell exhaustion. The relatively lower T cell exhaustion in PB based CAR T cell can be partly due to the transfection process that does not require T cell activation [47].



Interestingly, SR cultured CD19 PiggyBac CAR T cell exhibited better proliferation after multiple rounds of tumor rechallenged, which could be related to the higher level of naïve phenotype. In addition, we also observed a significant increase in IL-4 secretion in SR cultured CAR T cell which could contribute to better T cell

proliferation. IL-4 is a known growth factor for activated T cells and enhanced T cell proliferation after initial activation [56, 57]. Besides, IL-4 is shown to be produced by naïve T cells and regulate CD8 T cell development, proliferation, and function [58, 59]. Recent study also showed that both viral based and non-viral transduced CD19 CAR T cell exhibit significantly higher IL-4 level after antigen stimulation comparing with activated T cell which could lead to CAR T cell expansion [46].

On the other hand, we found a higher level of IL-2, IL-10 and IL-6 secretion in HPL cultured CAR T cell. IL-2 is known as a T cell growth factor that supports the proliferation and survival of T cells along with the generation of effector and memory T cells. Unfortunately, IL-2 also stimulates proliferation and enhances the function of other T-cells such as natural killer (NK) cells and B-cells [60].

Interleukin-10 (IL-10) has a role as a major immune suppressive factor that was significant for induction of tolerance through inhibition of TH1 immune response and T-cell cytotoxic activity [61]. IL-10 can impair the proliferation, cytokine production, and migratory capacities of effector T cells by acting directly on CD4 T cells to induce non-responsiveness or T cell anergy [62, 63]. Also, IL-10 can promote tumor immune escape by declining the anti-tumor response in the tumor microenvironment [64]. Previous publications reveal that IL-10 can be produced by the tumor cells and IL-10 can prevent the antigen-specific proliferation of human T cells by inhibiting the antigen-presenting capacity of monocytes through the downregulation of class 2 MHC, co-stimulation, and

adhesion molecules [63, 64]. Therefore, overproduction of IL-10 induces the failure to control tumour immunogenicity. In patients, IL-10 has been described in the tumor-microenvironment of different type of cancers and clearly linked to poor prognosis [62].

IL-6 is a pleiotropic cytokine that has a crucial role in the regulation of innate immunity, inflammation, and hematopoiesis [65]. IL-6 also affects T cells by inducing the specific differentiation of naive T cells into effector T-cells [66]. The overproduction of IL-6 and uncontrolled its signaling are important to the pathogenesis of cytokine storms and can lead to severe acute inflammatory disease. Increasing IL-6 levels were observed in patients with pediatric and adult B cell acute lymphoblastic leukemia who received CAR T cell therapy with cytokine storm, implying IL-6 has a major role in the progression and severity of cytokine storms. Furthermore, recent evidence in a patient who was administered CD19-targeted CAR T cells identified endothelial cells as major IL-6 producing cells that were responsible for the cytokine storm development and associated with vascular dysfunction due to endothelial activation [67].



Besides, Cytokine release syndrome (CRS) is one of the most clinically significant and feasibly life-threatening toxicities [68]. CRS is a systemic immune storm that involves the mass cytokines released after CAR-T cell therapy[69]. This phenomenon causes multisystem trauma, potentially life-threatening, and sometimes mortality. In the setting of CRS, a significant increase in interleukin-6 is noticed, in association with elevations of other cytokines such as interleukin-10 and interleukin-2

[69]. It is concluded that HPL cultured CAR T cells may not be suitable to use as an alternative serum supplement for piggyBac CD19 production in clinical setting.

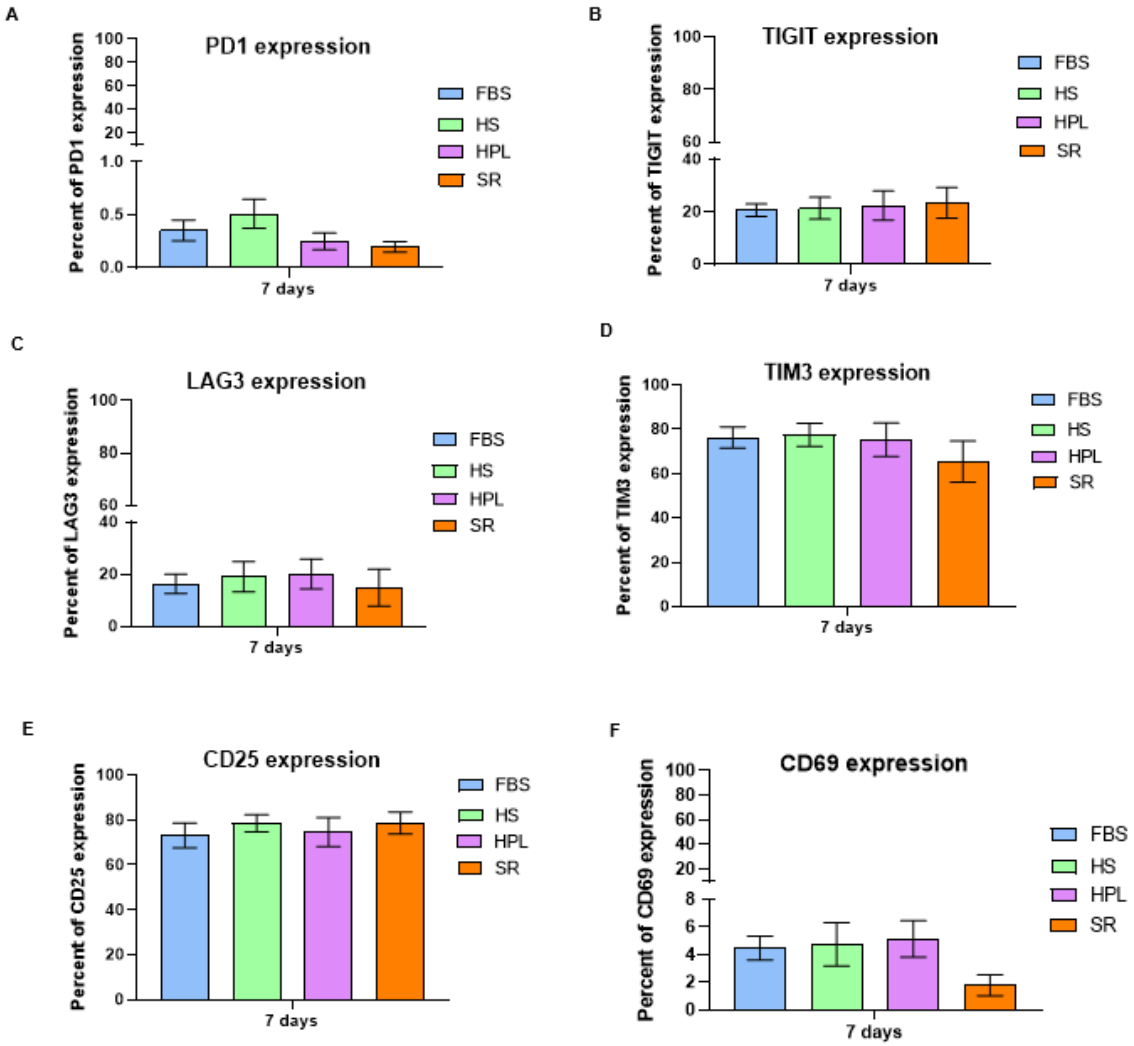
This study also showed that SR cultivated PB CD-19 CAR T cell exhibit lower expression of CD69 during sequential experiments. CD69 is an early activation marker of T cells. Generally, the expression of CD69 is transient and peaks after stimulation then it will diminish 18–24 h afterward [70]. The lower expression of CD69 in SR could possibly related to the enrichment of the naïve phenotype since previous evidence suggest that naïve T cells in peripheral blood had lower expression of CD69 (<0.1% expression) compared with central memory T cells (2% expression) and effector memory T cells (3% expression) [70, 71].

The limitations in this study including the unidentified components of each serum supplements that gave the different cytokine secretion pattern and different of memory phenotype. Secondly, the memory phenotype results didn't interpretation from CAR positive molecule because we select the CD3 positive molecule to analyze that mean these results may be interfered by untransduced T cells. Also, we didn't confirm the memory phenotype after co-culturing to ensure the memory phenotype of remaining cells.

In conclusion, our findings demonstrate the feasibility of using SR, a chemically defined supplement as an alternative serum source for CD19 PiggyBac CAR T cells production to support the expansion, function, and maintained less differentiated T cells.

Using xeno-free and serum-free media can offer advantages such as a defined media composition, and reduced risk of infectious contamination in GMP compliance manufacturing of CAR T cells.

# APPENDIX A




*Supplement Figure 1 The detailed exhaustion and activation markers of PiggyBac CD19 CAR T cells that co-cultured with leukemia cancer cell line (NALM6) at Effector: Target (E:T) ratio 1:5 without cytokine for 7 days.*

(A) The percentage of PD-1 (B) TIGIT (C) LAG3 (D) TIM3 (E) CD25 and (F) CD69 expressing on different serum supplements (FBS,HS, and SR) at ratio 1:5 for 7 days.

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