

# Cancer Immunotherapy: Challenges and Advancements in CAR T Cell Technology

Pa-thai Yenchitsomanus, Ph.D.<sup>1,2</sup>

<sup>1</sup>Siriraj Center of Research Excellence for Cancer Immunotherapy (SiCORE-CIT), <sup>2</sup>Division of Molecular Medicine, Research Department, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.

## ABSTRACT

Cancer, characterized by uncontrolled cell proliferation, poses a major global health threat, as evident in the 2022 World Health Organization-International Agency for Research on Cancer report, recording 20 million new cases and 9.7 million deaths worldwide. Thailand alone reported 183,000 new cases and 118,000 fatalities, underscoring the need for tailored prevention, early detection, and treatment strategies. Conventional therapies like surgery, radiation, and chemotherapy, while effective in early stages, face limitations in advanced cases, prompting the development of targeted therapies and immunotherapy, notably chimeric antigen receptor (CAR) T cell therapy. CAR T cell therapy employs genetic engineering to create receptors recognizing cancer-specific antigens. Despite successes in hematological malignancies, challenges such as toxicities, relapse, and high costs persist. Ongoing research, led by the Siriraj Center of Research Excellence for Cancer Immunotherapy (SiCORE-CIT), focuses on advancing fourth- and fifth-generation CAR T cell technologies. SiCORE-CIT's fourth-generation CAR T cells exhibit potent anti-tumor activity against various cancers, surpassing second-generation counterparts. The innovative fifth-generation "Siriraj fifth-generation CAR T cells" secrete anti-PD-L1 scFv, showing potential for diverse cancer applications, highlighting the transformative impact of ongoing research. Successful applications of fifth-generation CAR T cells in B-cell leukemia, lymphoma, and multiple myeloma underscore their transformative potential. This emphasizes the critical role of continuous research in refining therapeutic approaches for both hematologic and solid malignancies. The ongoing exploration and development in this domain have the potential to revolutionize cancer treatment paradigms, significantly contributing to alleviating the global health burden associated with this complex disease.

**Keywords:** Cancer immunotherapy; Adoptive T-cell transfer; Chimeric antigen receptor (CAR) T cells; Fourth-generation and fifth-generation CAR T cells; Solid tumor application (Siriraj Med J 2024; 76: 313-324)

## INTRODUCTION

Cancer, characterized by uncontrolled cell growth and proliferation, poses a substantial global health challenge with intricate biological complexities, including genetic mutations, tumor microenvironment dynamics, and immune system evasion. An epidemiological perspective enables the identification of patterns, recognition of risk factors, and acknowledgment of disparities in cancer occurrence, revealing its extensive impact across diverse

populations. Beyond individual health, cancer brings socio-economic ramifications, including substantial financial burdens, productivity losses, and healthcare access disparities. The World Health Organization's (WHO) cancer agency, the International Agency for Research on Cancer (IARC), has released 2022 estimates, revealing a global cancer burden of 20 million new cases and 9.7 million deaths.<sup>1</sup> Underserved populations bear a disproportionate impact, underscoring the urgent need

Corresponding author: Pa-thai Yenchitsomanus

E-mail: [ptyench@gmail.com](mailto:ptyench@gmail.com)

Received 2 March 2024 Revised 24 March 2024 Accepted 25 March 2024

ORCID ID: <http://orcid.org/0000-0001-9779-5927>

<https://doi.org/10.33192/smj.v76i5.268031>



All material is licensed under terms of the Creative Commons Attribution 4.0 International (CC-BY-NC-ND 4.0) license unless otherwise stated.

to address global cancer inequities. Lung, breast, and colorectal cancers collectively account for two-thirds of global cases and deaths. Lung cancer tops the list with 2.5 million cases, followed by breast cancer with 2.3 million and colorectal cancers with 1.9 million. The resurgence of lung cancer as the leading cause of cancer death (1.8 million deaths) is linked to persistent tobacco use in Asia. In Thailand, where cancer remains a significant health concern, recent statistics reveal an escalating burden, with an increase in both new cases and cancer-related fatalities in 2022. The prevalence in Thailand was 154.4 age-standardized rate (ASR) per 100,000 persons, with 183,541 new cases diagnosed and 118,829 people succumbing to cancer.<sup>2</sup>

Cancer, as highlighted by WHO, induces substantial human suffering and places a significant economic burden. The global economic cost of cancer from 2020 to 2050 is estimated at \$25.2 trillion (in constant 2017 prices), equivalent to an annual tax of 0.55% on the global gross domestic product.<sup>3</sup> This cost encompasses direct medical expenses, productivity losses due to premature mortality and disability, and strain on healthcare systems. To address this multifaceted issue, a comprehensive approach is imperative, involving public health interventions and policy initiatives. Recognizing and addressing cancer's impact is vital for advancing prevention, early detection, and treatment, contributing to a healthier and more resilient global community, with specific attention to the challenges faced by countries like Thailand.

Current standard cancer treatments, involving surgery, chemotherapy, and radiation therapy, aim to mitigate tumor burden and prevent metastasis. Surgery physically removes tumors, chemotherapy targets rapidly dividing cells, and radiation induces cellular damage. Despite efficacy, these treatments have limitations and adverse effects. Surgery may be constrained by tumor inoperability or risk to vital structures. Chemotherapy leads to systemic toxicity, affecting healthy cells. Radiation faces challenges in delivering high doses while sparing healthy tissues. Resistance mechanisms contribute to treatment failures. The need for personalized, targeted therapies and immunotherapeutic approaches arises in response to current limitations, driving the ongoing quest for more effective and tolerable cancer treatments.

The advancement of cancer therapeutics has witnessed a paradigm shift towards innovative treatment modalities, prominently featuring targeted drug therapy and immunotherapy.<sup>4</sup> Targeted drug therapy, involving the use of small molecules or monoclonal antibodies, aims to selectively inhibit specific molecular pathways critical to cancer cell proliferation and survival, minimizing

collateral damage to normal cells. Immunotherapy, on the other hand, harnesses the patient's immune system to recognize and eradicate cancer cells by modulating immune responses. An emerging frontier in cancer treatment involves the integration of chimeric antigen receptor (CAR) T cells, a revolutionary form of immunotherapy.<sup>5</sup> CAR T cell therapy entails the genetic modification of a patient's own T cells to express receptors targeting specific antigens on cancer cells, enabling enhanced and targeted immune responses. This promising approach has demonstrated remarkable success in treating certain hematologic malignancies<sup>6</sup>, heralding a new era in personalized and precise cancer therapeutics. As research continues to unravel the complexities of cancer biology, the integration of these novel strategies holds immense potential for improving treatment efficacy and mitigating the challenges posed by conventional cancer therapies.

Our research team at the Siriraj Center of Research Excellence for Cancer Immunotherapy (SiCORE-CIT) is actively engaged in the exploration of research, development, and innovation in the field of cancer immunotherapy, with a particular focus on advancing CAR T cell technologies. This review article aims to showcase the progress made in our research endeavors, highlighting the innovations in fourth- and fifth-generation CAR T cell therapy. We emphasize the significant potential of these advancements in treating both hematologic and solid malignancies.

### **Chimeric antigen receptor (CAR) T cell therapy**

CAR T cell therapy represents a groundbreaking therapeutic approach within the domain of cancer treatment. Characterized by the genetic engineering of T cells to express artificial receptors, CAR T cells are designed to recognize specific antigens on the surface of cancer cells, thereby enhancing the immune system's targeted response against malignancies. CAR T cells are activated through a unique mechanism compared to normal T cell activation (Fig 1). In the process of normal T cell activation, the T cell receptor (TCR) mediates the recognition of antigens on the surface of infected or cancer cells by interacting with the peptide antigen presented on the major histocompatibility complex (MHC). This interaction triggers a series of signaling events leading to T cell activation. On the other hand, CAR T cells are engineered to express an artificial receptor on their surface, the chimeric antigen receptor (CAR). This receptor combines the antigen-binding domain of an antibody, namely single chain variable fragment (scFv), with signaling domains from TCR (CD3 $\zeta$ ) and co-stimulatory molecules (such as CD28 or 4-1BB) (Fig 1). When the CAR binds to a specific antigen on the

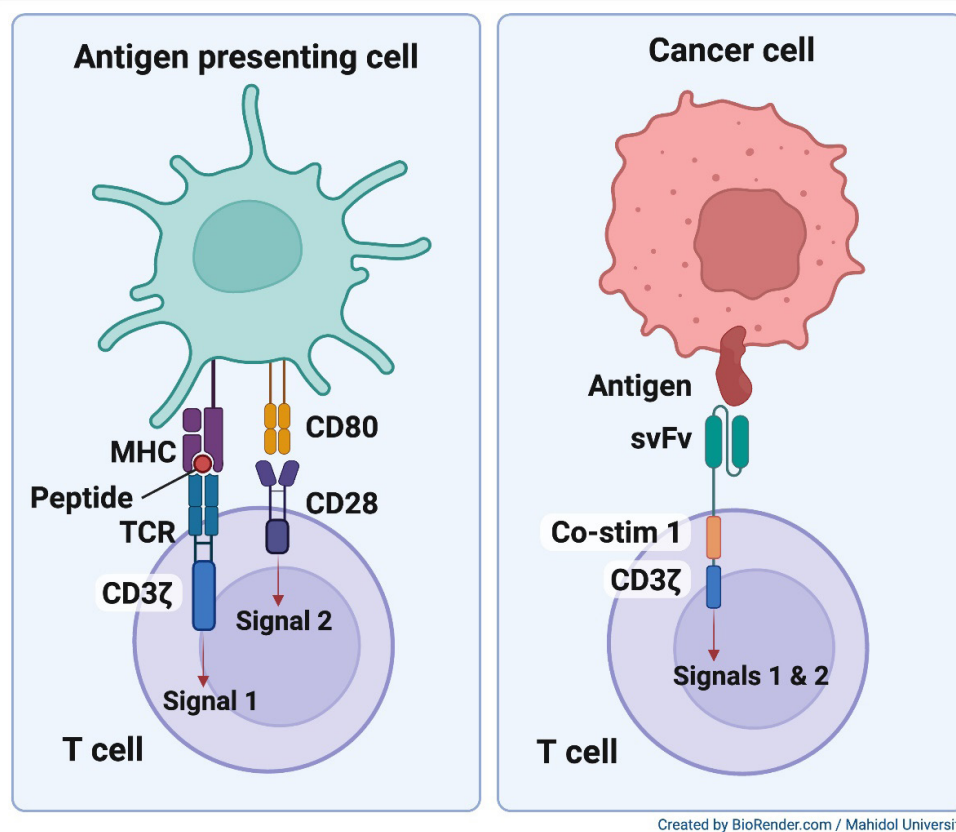


**Fig 1.** Schematic representation of T cell activation pathways comparing normal T cell and chimeric antigen receptor (CAR) T cell responses. In conventional T cell activation (left), the T cell engages with an antigen-presenting cell, initiating signal 1 by the interaction between the T cell receptor (TCR) and the antigen peptide presented on the major histocompatibility complex (MHC) molecule. Signal 2 is facilitated by the binding of a co-stimulatory ligand (e.g., CD80) to the co-stimulatory receptor (e.g., CD28). In CAR T cell activation (right), the engineered CAR molecule, comprising an extracellular single-chain variable fragment (scFv), directly binds to a specific antigen on the cancer cell surface, triggering the simultaneous activation of signals 1 and 2. Notably, CAR T cell activation is MHC independent. Upon activation, CAR T cells undergo proliferation, release cytokines, and exhibit cytotoxic functions, ultimately leading to the elimination of cancer cells.

target cell, it initiates intracellular signaling, bypassing the need for MHC presentation. This direct activation of CAR cells allows for targeted and enhanced immune response against cancer cells or other diseased cells. The salient characteristics of CAR T cells include their capacity for antigen-specific recognition, activation, and proliferation, allowing for a tailored and potent immune response. This innovative therapeutic strategy has demonstrated notable success in the treatment of hematological malignancies, particularly in cases where conventional therapies have shown limited efficacy.

The historical progression of CAR T cells marks a remarkable journey within the realm of immunotherapy. The concept of CAR T cells began to materialize in the late 1980s, culminating in the development of initial, first-generation CAR T cells (Fig 2). However, these early iterations faced limitations due to the absence of co-stimulatory signals, impacting their efficacy. A significant breakthrough occurred in the early 2000s when researchers, notably Carl June from the University of Pennsylvania, USA, successfully pioneered the second

generation of CAR T cells (Fig 2). Carl June played a pivotal role in elevating CAR T cell effectiveness by incorporating a co-stimulatory molecule such as CD28 or CD137/4-1BB into their design.<sup>7</sup> This innovative step substantially enhanced the activation, proliferation, and persistence of CAR T cells, resulting in more potent and enduring anti-cancer responses. Subsequent advancements led to the development of third-generation CAR T cells, integrating two co-stimulatory domains (e.g., CD28 and CD137/4-1BB) (Fig 2). Recently, our group and others have created fourth-generation CAR T cells, which include three co-stimulatory domains-CD28, CD137/4-1BB, and CD27 (Fig 2) - aimed at further enhancing CAR T cell efficiency and function for the targeted elimination of cancer cells.

### Designing CAR molecules and the production of CAR T cells for therapy

The strategic development of a CAR molecule involves a series of crucial steps to ensure its effectiveness and safety. Initially, the careful selection of a target antigen is

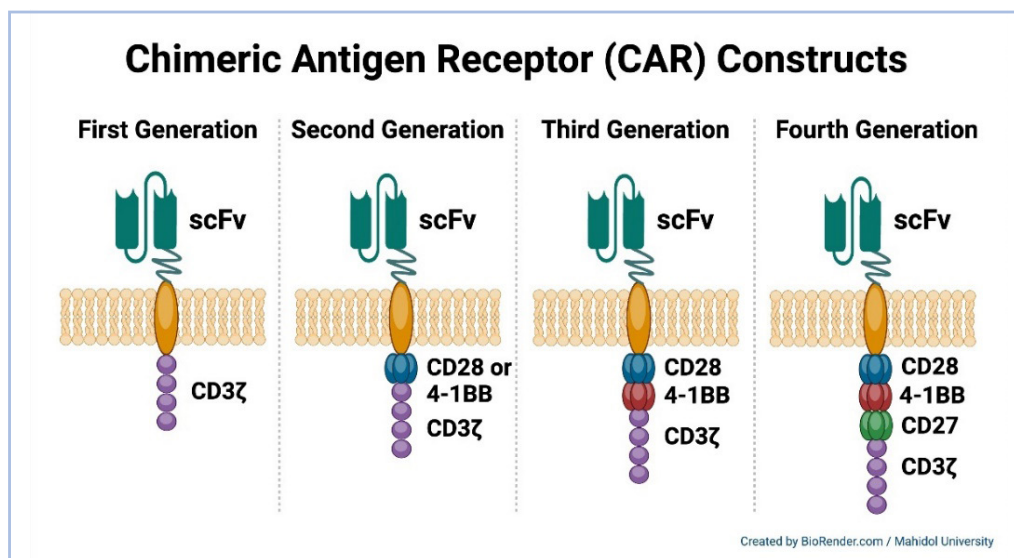


**Fig 2.** The evolution of CAR T cell generations. The first-generation CAR T cell features a single chain variable fragment (scFv) specific to a target antigen, a transmembrane domain (Tm), and CD3 $\zeta$ , lacking a co-stimulatory signal. The second-generation CAR T cell includes scFv, Tm, one co-stimulatory molecule (e.g., CD28 or CD137/4-1BB), and CD3 $\zeta$ . The third-generation CAR T cell is equipped with scFv, Tm, two co-stimulatory domains (e.g., CD28 and CD137/4-1BB), and CD3 $\zeta$ . In the fourth-generation CAR T cells, scFv, Tm, three co-stimulatory domains (e.g., CD28, CD137/4-1BB, and CD27) are integrated along with CD3 $\zeta$ .

paramount, focusing on the precise targeting of cancer cells while minimizing the risk of on-target but off-tumor effects. Following this, the meticulous choice of a specific antigen-binding scFv or peptide is essential to facilitate selective binding to the identified target antigen on cancer cells, thereby reducing immunogenicity. The inclusion of the intracellular signaling domain of CD3 $\zeta$ , essential for generating signal 1, along with co-stimulatory molecules such as CD28, CD137 (4-1BB), or CD27, which contribute to signal 2, constitutes a critical advancement in augmenting the functional capabilities of CAR T cells. Rigorous preclinical validation procedures are then conducted to confirm the expression, functionality, efficiency, and safety of the designed CAR molecule. The conclusive phase involves clinical validation through meticulously designed trials, focusing on the comprehensive assessment of the safety and efficacy of CAR T cell treatment in human subjects. These sequential processes highlight the systematic and rigorous approach necessary for the successful design and implementation of CAR molecules in cancer immunotherapy.

In CAR T cell production, a systematic approach involves identifying and confirming target antigen expression in cell lines and cancer tissues. Subsequently, there is the exploration and selection of a suitable scFv, followed by the design and cloning of the CAR construct into a lentiviral vector. Lentiviruses, engineered to carry the CAR construct that encompasses genes encoding the CD3 $\zeta$  intracellular signaling domain and co-stimulatory molecules, are

employed for transduction into mammalian cells to assess the expression of the CAR protein. Alternatively, other viral or non-viral vectors may be employed for delivering the CAR construct into the cells. The next step involves extracting lymphocytes from a blood sample and transducing them with lentiviruses carrying the CAR construct. Finally, cultivated CAR T cells undergo expansion, and their phenotypes and functions are assessed to ensure desired therapeutic characteristics. This sequential procedure is essential for the precise development of CAR T cells in cancer immunotherapy.

CAR T cell treatment involves several key steps (Fig 3). First, T cells are collected from the patient through leukapheresis. These T cells are then genetically modified in the laboratory to express a chimeric antigen receptor (CAR) on their surface, enabling them to target specific proteins on cancer cells. The modified CAR T cells are cultured and multiplied to generate a sufficient quantity. Before infusion back into the patient, a conditioning chemotherapy may be administered to enhance the CAR T cells' effectiveness. Once infused, the CAR T cells recognize and bind to the targeted cancer cells, leading to their activation and subsequent destruction. Patients are closely monitored for potential side effects, and follow-up assessments are conducted to evaluate the treatment's long-term efficacy. This innovative immunotherapy has shown promise, particularly in treating certain blood cancers like leukemia and lymphoma.

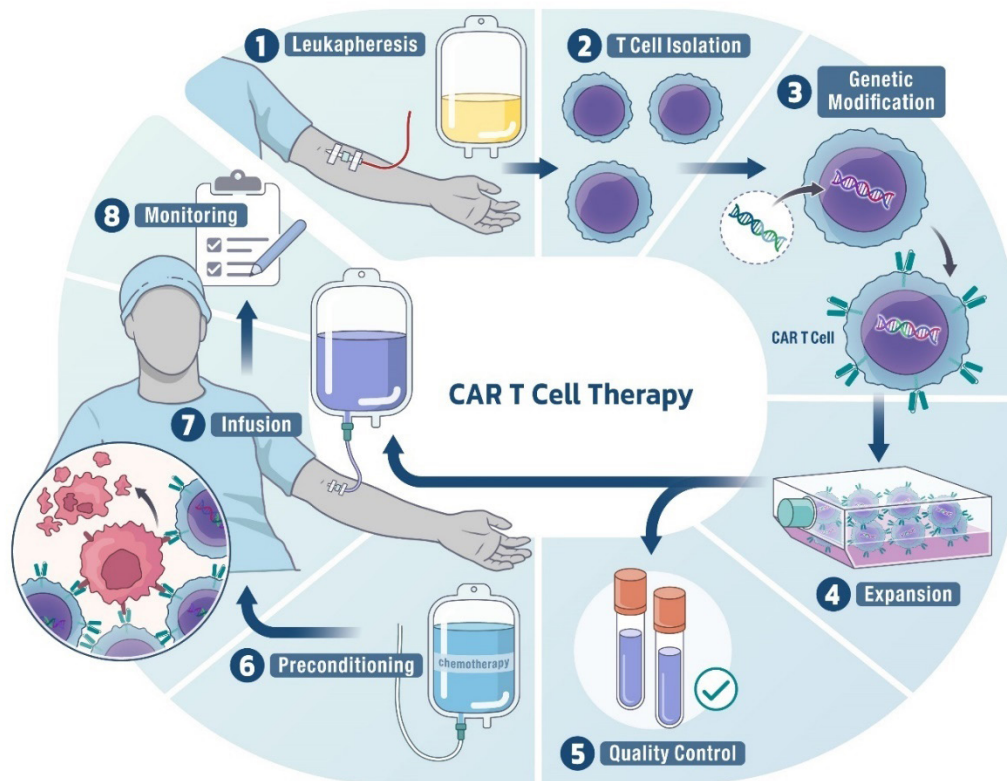


**Fig 3.** Overview of CAR T cell therapy process. (1) Leukapheresis: White blood cells are collected from the patient. (2) T Cell Isolation: T cells are extracted from the collected sample. (3) Genetic Modification: CAR gene is introduced into T cells, often using viral vectors. (4) Expansion: Modified T cells are multiplied in the laboratory. (5) Quality Control: Modified cells undergo assessment to ensure safety and potency. (6) Preconditioning: Lymphodepleting chemotherapy is administered to the patient. (7) Infusion: CAR T cells are reintroduced into the patient. (8) Monitoring: Regular assessment of the patient's response and management of potential side effects.

### FDA-approved CAR T cell therapies for B-cell malignancies in the United States

In 2012, a groundbreaking moment in cancer treatment unfolded with the administration of the first-ever CD19-directed CAR T cell therapy to a 6-year-old pediatric patient suffering from relapsed/refractory B-cell acute lymphoblastic leukemia (r/r B-ALL).<sup>8</sup> This event marked a significant turning point in cancer therapy. Subsequently, on August 30, 2017, the FDA granted approval for the world's first CAR-T therapy, Tisagenlecleucel, specifically for patients under the age of 25 with r/r B-ALL.<sup>9</sup> Impressively, the initial patient treated in 2012 has remained cancer-free for over 11 years.<sup>8</sup> Following this breakthrough, multiple studies showcased outstanding clinical outcomes with CAR-T therapies, resulting in FDA approvals for a total of six CAR T cell products<sup>10</sup>, all targeting hematological malignancies (Table 1). While CD19 and B-cell maturation antigen (BCMA) are the only antigens currently approved by the FDA, ongoing research is actively exploring new targets for CAR design. In the realm of acute lymphoblastic leukemia (ALL), CD19-directed CAR T cell treatments like Tisagenlecleucel have demonstrated remarkable response rates. Nevertheless, challenges such as relapse with CD19 antigen loss have

driven investigations into alternative targets like CD22.<sup>11</sup> T-cell malignancies present obstacles for CAR T cell therapy due to a lack of appropriate target antigens, but encouraging progress is being made with CAR T cells against CD7 and CD5.<sup>12</sup>

Chronic lymphoblastic leukemia (CLL) poses challenges, including lower success rates and issues with CD19 loss. Research efforts are focused on alternative targets such as CD20, CD23, receptor tyrosine kinase-like orphan receptor 1 (ROR1), and Fc receptor for immunoglobulin M (FcμR).<sup>13</sup> Acute myeloid leukemia (AML) is currently without an FDA-approved CAR-T therapy due to the absence of a specific antigen, but promising targets like CD33, CD123, CD117, and others are actively under investigation.<sup>14</sup> Non-Hodgkin's B-cell lymphoma (NHL) has witnessed remarkable responses to CD19 CAR-T therapies, resulting in FDA approval for multiple products.<sup>15</sup> However, challenges like CD19 antigen loss have prompted exploration of alternative targets like CD20, CD22, CD30, CD33 and CD123.<sup>16</sup> In multiple myeloma (MM), CAR-T therapies targeting BCMA have secured FDA approval<sup>17</sup>, while trials targeting non-BCMA antigens like CD38, GPRC5D, SLAMF7, and CD138 are currently underway.<sup>18</sup>

**TABLE 1.** FDA-Approved CAR T Cell Therapies.<sup>22</sup>

| Brand Name | Generic Name              | Target Disease                                                                | Target Antigen | Generation and Co-stimulatory Domain | Cost Per Single Dose |
|------------|---------------------------|-------------------------------------------------------------------------------|----------------|--------------------------------------|----------------------|
| Kymriah™   | Tisagenlecleucel          | Follicular Lymphoma, Diffuse Large B-cell Lymphoma, or Lymphoblastic Leukemia | CD19           | Gen 2/4-1BB                          | \$475,000            |
| Yescarta™  | Axicabtagene ciloeucel    | Follicular Lymphoma or Diffuse Large B-cell Lymphoma                          | CD19           | Gen 2/CD28                           | \$373,000            |
| Tecartus™  | Brexucabtagene autoleucel | Mantle Cell Lymphoma or Acute Lymphoblastic Leukemia                          | CD19           | Gen 2/CD28                           | \$373,000            |
| Breyanzi®  | Lisocabtagene maraleucel  | Large B-cell Lymphoma                                                         | CD19           | Gen 2/4-1BB                          | \$410,300            |
| Abecma®    | Idecabtagene vicleucel    | Relapsed or Refractory Multiple Myeloma                                       | BCMA           | Gen 2/4-1BB                          | \$419,500            |
| Carvykti™  | Ciltacabtagene autoleucel | Relapsed or Refractory Multiple Myeloma                                       | BCMA           | Gen 2/4-1BB                          | \$465,000            |

Despite the successes, CAR T cell therapy confronts various challenges, including common toxicities such as cytokine release syndrome (CRS) and neurotoxicity.<sup>19</sup> Tumor relapse, both antigen-positive and antigen-negative, remains a lingering concern.<sup>20</sup> Moreover, the exorbitant cost of the therapy, ranging from \$300,000 to \$500,000 per dose, restricts accessibility.<sup>21</sup> Ongoing research endeavors aim to tackle these challenges, enhance CAR T cell therapy, and broaden its applicability in hematological malignancies.

### Challenges of using CAR T cells in solid tumors

The application of CAR T cells in the context of solid tumors presents a complex set of challenges that significantly hinder their therapeutic efficacy. The physical barriers inherent to solid tumors, characterized by dense stromal tissue, extracellular matrix, and increased interstitial fluid pressure, impede the effective infiltration and distribution of CAR T cells within the tumor microenvironment.<sup>23</sup> Moreover, the immunosuppressive milieu within solid tumors, orchestrated by factors such as regulatory T cells (Treg), myeloid-derived suppressor cells (MDSC), cytokines, and the expression of immune checkpoint proteins like PD-L1 on cancer cells (Fig 4), poses a formidable obstacle to the sustained activity of CAR T cells. This inhibitory

landscape dampens the cytotoxic potential of both native T cells and CAR T cells, compromising their ability to mount an effective anti-tumor response. Overcoming these challenges necessitates innovative strategies, including the engineering of CAR T cells with enhanced tumor-penetrating capabilities, resistance to inhibitory signals, and incorporation of additional functionalities to counteract the suppressive microenvironment. Ongoing research endeavors are focused on unraveling the intricacies of solid tumor biology and tailoring CAR T cell designs to address the unique hurdles posed by these malignancies. As the development of CAR T cells for solid tumors progresses, a comprehensive understanding of these challenges becomes imperative to guide the refinement of therapeutic approaches, ultimately advancing the potential of CAR T cell therapy in the broader spectrum of cancer treatment.<sup>24</sup>

Challenges inherent in CAR T cell therapy necessitate thoughtful consideration and strategic approaches to address critical concerns. Firstly, there is a pressing need to enhance the efficacy of CAR T cells specifically in the treatment of solid tumors. This prompts exploration into novel methodologies and technologies geared towards augmenting their performance in the context of these challenging malignancies. Secondly, the mitigation of

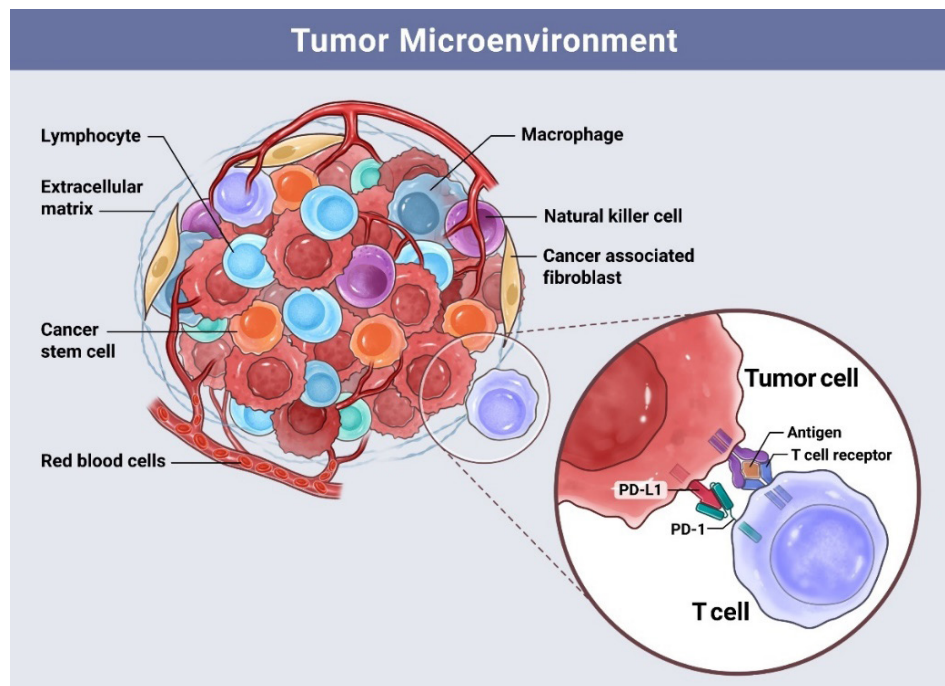


**Fig 4.** The formidable physical barriers within solid tumors are marked by dense stromal tissue, extracellular matrix, and a tumor microenvironment infiltrated by immunosuppressive cells. Additionally, cancer cells in these tumors express immune checkpoint proteins, such as PD-L1, which interacts with PD-1 to suppress T cell activity.

cytokine release syndrome (CRS), a potentially severe side effect associated with CAR T cell therapy, poses a significant challenge. The development and implementation of effective strategies to minimize CRS constitute a crucial avenue of research and innovation. Thirdly, the formidable hurdles presented by the immunosuppressive tumor microenvironment, marked by the expression of immune checkpoint blockade proteins (such as PD-L1), necessitate innovative approaches to overcome these barriers. Strategies aimed at modulating the microenvironment to favorably impact CAR T cell efficacy are of paramount importance. Lastly, the economic considerations surrounding CAR T cell therapy demand attention, prompting a search for avenues to reduce its cost. Exploring cost-effective technologies, streamlining production processes, and optimizing resource utilization are imperative in addressing this pertinent challenge. In summary, the identified challenges underscore the need for multidisciplinary research and strategic interventions to advance the field of CAR T cell therapy towards enhanced efficacy, safety, and accessibility.

#### **Innovative Fourth-generation CAR T cell therapy for hematologic and solid malignancies**

SiCORE-CIT is committed to optimizing CAR T cell therapy, aligning with the global trend of refining designs to overcome challenges in the tumor microenvironment and enhance overall efficacy. Our research group at SiCORE-CIT has pioneered the development of fourth-generation

CAR T cells, aiming to improve functionality, efficiency, and persistence. These advanced cells incorporate three co-stimulatory domains (CD28, CD137/4-1BB, and CD27) fused to CD3 $\zeta$  (Fig 5), leading to a significant enhancement of anti-tumor activities, proliferation, and survival. Each co-stimulatory domain offers distinct and shared advantages. CD28 supports T cell proliferation and cytokine production<sup>25</sup>, providing resistance against activation-induced cell death (AICD).<sup>26</sup> CD137/4-1BB not only stimulates T cell proliferation but also enhances T cell survival by inhibiting AICD and boosts cytokine production.<sup>27</sup> On the other hand, CD27 fosters T-cell proliferation and facilitates differentiation into effector and memory T cells, positioning it as a potential immune modulatory target for cancer treatment.<sup>28</sup> Moreover, we engineered fifth-generation CAR T cells, featuring co-stimulatory domains identical to fourth-generation counterparts and an additional capacity to secrete anti-PD-L1 scFv for inhibiting PD-L1 protein on cancer cells (Fig 5 and subsequent section). Our fourth- and fifth-generation CAR T cells target specific antigens overexpressed in various cancers, such as CD19 in B-cell leukemia and lymphoma; BCMA in multiple myeloma; CD133, MUC1 and integrin  $\alpha\beta 6$  in cholangiocarcinoma; and folate receptor  $\alpha$  and Trop2 in breast cancer. Table 2 provides a comprehensive compilation of cutting-edge fourth- and fifth-generation CAR T cells meticulously designed by SiCORE-CIT for the treatment of hematologic and solid malignancies.

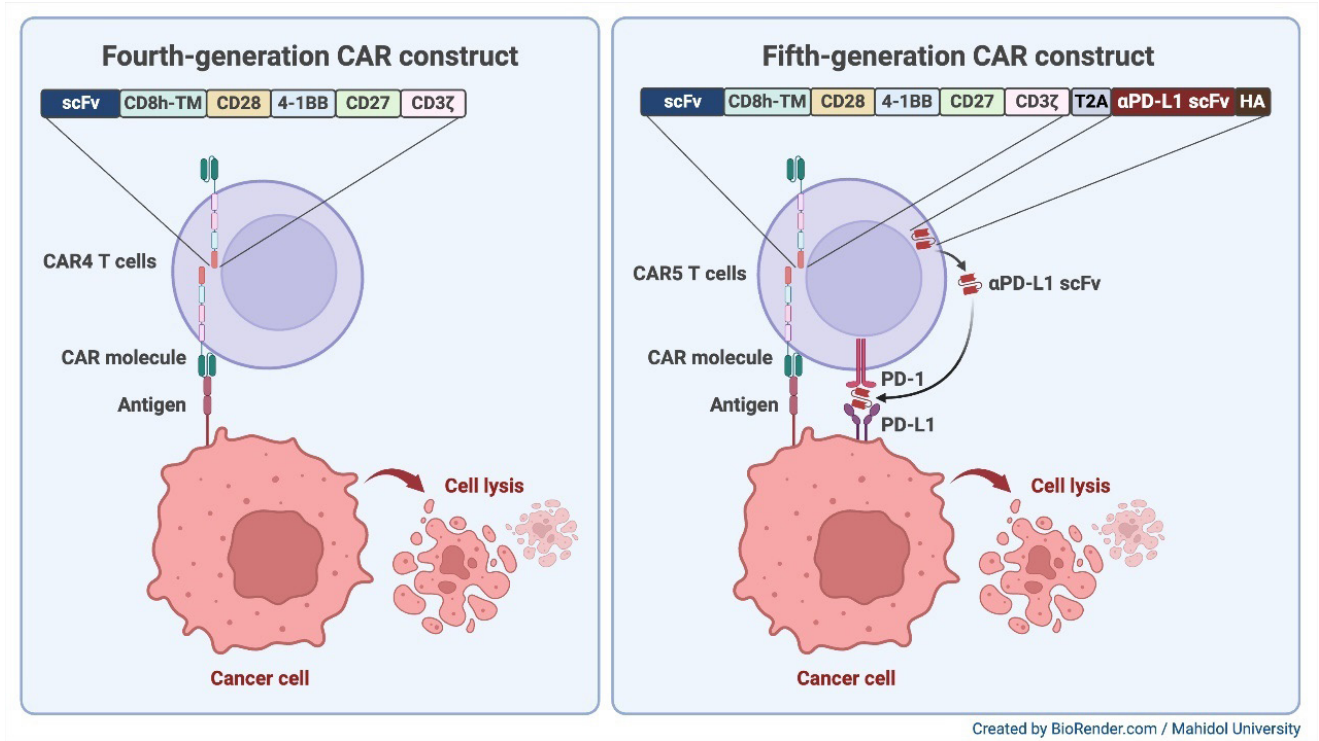


**Fig 5.** SiCORE-CIT has developed fourth- and fifth-generation CAR T cells. The fourth-generation CAR integrates three co-stimulatory domains (CD28, CD137 or 4-1BB, and CD27) fused to CD3ζ (left). The fifth-generation CAR T cells share similarities with the fourth-generation ones but are additionally engineered to secrete anti-PD-L1 scFv (right).

**TABLE 2.** Cutting-Edge Fourth- and Fifth-Generation CAR T Cell Therapies for Hematologic and Solid Malignancies.

| Fourth-Generation CAR T Cells   |                                                               |           |
|---------------------------------|---------------------------------------------------------------|-----------|
| Specific CAR T Cells            | Target Cancer                                                 | Reference |
| Anti-CD19 CAR4 T cells          | Acute lymphoblastic leukemia (ALL) and B cell lymphomas (BCL) | 29        |
| Anti-CD133 CAR4 T cells         | Cholangiocarcinoma (CCA)                                      | 30        |
| Anti-MUC1 CAR4 T cells          | Cholangiocarcinoma (CCA)                                      | 31        |
| Anti-Integrin αvβ6 CAR4 T cells | Cholangiocarcinoma (CCA)                                      | 32        |
| Anti-FRα CAR4 T cells           | Breast cancer (BC)                                            | 33        |
| Anti-Trop2 CAR4 T cells         | Breast cancer (BC)                                            | 34        |
| Fifth-Generation CAR T Cells    |                                                               |           |
| Specific CAR T Cells            | Target Cancer                                                 | Reference |
| Anti-CD19 CAR5 T cells          | Acute lymphoblastic leukemia (ALL) and B cell lymphomas (BCL) | 35        |
| Anti-BCMA CAR5 T cells          | Multiple myeloma (MM)                                         | 36        |

#### **Fourth-generation anti-CD19 CAR T cells for B-cell malignancies**

Autologous T cells expressing CD19-CAR therapy has shown promise for B-cell malignancies. Despite FDA-approved second-generation CD19-CAR T products' clinical efficacy, challenges like adverse effects and cell persistence exist. Fourth-generation CARs (CAR4) containing three co-stimulatory domains (CD28, CD137/4-1BB, and CD27) were developed to address these issues. We generated anti-CD19 CAR4 T cells with fully human scFv (Hu1E7-CAR4) and compared them to murine scFv-based counterparts (mFMC63-CAR4).<sup>29</sup> Comparative analyses revealed similar anti-tumor activities and proliferation, with Hu1E7-CAR4 T cells displaying lower cytokine secretion. These findings underscore Hu1E7-CAR4 T cells' clinical viability, warranting further studies and clinical trials.

#### **Fourth-generation anti-CD133 CAR T cells for cholangiocarcinoma**

The current treatment paradigm for cholangiocarcinoma (CCA), a lethal bile duct cancer prevalent in the northeast of Thailand, proves ineffective due to the disease's late and advanced stage diagnosis. Urgently required is a novel therapeutic modality, exemplified by the creation of fourth-generation chimeric antigen receptor (CAR4) T cells designed to target CD133, a well-known cancer stem cell marker associated with cancer progression.<sup>30</sup> Demonstrating high efficacy against CD133-expressing CCA cells, the anti-CD133-CAR4 T cells induced tumor cell lysis in a dose- and CD133 antigen-dependent manner. Concurrently, significant upregulation of IFN- $\gamma$  and TNF- $\alpha$  was observed upon tumor treatment. The effectiveness of these anti-CD133-CAR4 T cells extends beyond CD133-expressing CCA, proving beneficial for other CD133-expressing tumors. This study lays the groundwork for future *in vivo* investigations and clinical trials.

#### **Fourth-generation anti-MUC1 CAR T cells for cholangiocarcinoma**

Moreover, mucin 1 (MUC1) emerges as an attractive candidate antigen for CCA, given its high expression and association with poor prognosis. Anti-MUC1-CAR4 T cells, evaluated in CCA models<sup>31</sup>, exhibited increased production of TNF- $\alpha$ , IFN- $\gamma$ , and granzyme B compared to untransduced T cells when exposed to MUC1-expressing KKU-100 and KKU-213A CCA cells. These CAR4 T cells demonstrated specific killing activity against KKU-100 and KKU-213A cells, while showing negligible cytolytic activity against immortal cholangiocytes. Furthermore, anti-MUC1-CAR4 T cells effectively disrupted KKU-

213A spheroids, supporting their development as an adoptive T cell therapeutic strategy for CCA.

#### **Fourth-generation A20 CAR T cells for cholangiocarcinoma**

Additionally, integrin  $\alpha\beta6$ , upregulated in several solid tumors but minimally expressed in normal epithelial cells, emerges as a promising target antigen for CAR T cell immunotherapy in CCA. Investigating integrin  $\alpha\beta6$  expression in pathological tissue samples from liver fluke-associated CCA patients revealed overexpression in 23 of 30 (73.3%) cases, with a significant association between high integrin  $\alpha\beta6$  expression and shorter survival time.<sup>32</sup> Lentiviral constructs encoding CARs targeting integrin  $\alpha\beta6$  were engineered, resulting in highly expressed A20-CAR2 and A20-CAR4 in primary human T cells, both exhibiting significant cytotoxicity against integrin  $\alpha\beta6$ -positive CCA cells.<sup>32</sup> Notably, A20-CAR2 and A20-CAR4 T cells displayed anti-tumor function against integrin  $\alpha\beta6$ -positive CCA tumor spheroids. Upon specific antigen recognition, A20-CAR4 T cells produced a slightly lower level of IFN- $\gamma$  but exhibited higher proliferation than A20-CAR2 T cells, positioning them as a promising adoptive T cell therapy for integrin  $\alpha\beta6$ -positive CCA.

#### **Fourth-generation anti-FR $\alpha$ CAR T cells for breast cancer**

To address advanced breast cancer (BC), we developed fourth-generation CAR (CAR4) T cells targeting folate receptor alpha (FR $\alpha$ ), a BC-associated antigen.<sup>33</sup> These CAR T cells, with FR $\alpha$ -specific scFv and three costimulatory domains (CD28, CD137/4-1BB, and CD27) linked to CD3 $\zeta$ , demonstrated potent anti-BC activities. Cocultured with FR $\alpha$ -expressing MDA-MB-231 BC cells, anti-FR $\alpha$ -CAR4 T cells exhibited specific cytotoxicity, with enhanced activity against cells with higher surface FR $\alpha$  expression. This specific cytotoxicity was absent when cocultured with FR $\alpha$ -negative normal breast-like cells (MCF10A). In a 3D spheroid model, anti-FR $\alpha$ -CAR4 T cells effectively reduced spheroid size and induced breakage, highlighting their anti-tumor potential. This proof-of-concept study shows the feasibility and promise of anti-FR $\alpha$ -CAR4 T cells for adoptive T cell therapy in BC, offering a potential strategy for future clinical exploration.

#### **Fourth-generation anti-Trop2 CAR T cells for breast cancer**

The overexpressed trophoblast cell surface antigen 2 (Trop2) in BC is a promising immunotherapeutic target. A fourth-generation CAR (CAR4) was developed, featuring

an anti-Trop2 single-chain variable fragment (scFv) with three costimulatory domains CD28/4-1BB/CD27) and CD3 $\zeta$ , enhancing BC therapy.<sup>34</sup> Anti-Trop2 CAR4 T cells demonstrated heightened cytotoxicity and interferon-gamma (IFN- $\gamma$ ) production against Trop2-expressing MCF-7 cells compared to conventional second-generation CAR (CAR2; CD28). Notably, anti-Trop2 CAR4-T cells exhibited superior long-term cytotoxicity, proliferation, and specific targeting of Trop2-positive BC cells in both two-dimensional (2D) and three-dimensional (3D) cultures. Post-antigen-specific killing, these cells robustly secreted interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF- $\alpha$ ), IFN- $\gamma$ , and Granzyme B compared to non-transduced T cells. This study emphasizes the therapeutic potential of anti-Trop2 CAR4-T cells in adoptive T cell therapy for BC, holding significant promise for advancing BC treatment strategies.

### **Innovative Fifth-Generation CAR T cell Therapy for Hematologic and Solid Malignancies**

In our continuous effort to enhance the effectiveness of fourth-generation CAR T cells against cancers, our research group at SiCORE-CIT introduces a cutting-edge advancement: fifth-generation CAR T cells. These innovative CAR T cells, named '*Siriraj fifth-generation CAR T cells*', are engineered to secrete anti-PD-L1 scFv (Fig 5), distinguishing them from their predecessors. These fifth-generation CAR T cells possess the dual capacity to target and eliminate cancer cells expressing specific antigens while concurrently secreting anti-PD-L1 scFv, inhibiting PD-L1 on cancer cells and augmenting their killing potential. Recent reports highlight successful developments for B-cell leukemia and lymphoma (CD19)<sup>35</sup> and multiple myeloma (B-cell maturation antigen or BCMA).<sup>36</sup> Ongoing efforts aim to extend this innovation to fifth-generation CAR T cells designed for cholangiocarcinoma, breast cancer, retinoblastoma, and osteosarcoma. In this review, we delve into two fifth-generation CAR T cells: anti-CD19 CAR5 T cells for lymphoma and anti-BCMA CAR5 T cells for multiple myeloma (MM) (Table 2).

#### **Fifth-generation anti-CD19 CAR5 T cells for B cell lymphoma**

Lymphomas, predominantly B cell in origin, exhibit varying behaviors from slow growth to high aggression. Despite successful responses to chemotherapy in certain lymphomas, approximately 30–40% of aggressive B cell lymphomas (BCL) like Burkitt lymphoma (BL) and diffuse large B cell lymphoma (DLBCL) fail to respond or relapse after standard treatment. Immunotherapies, including

adoptive T cell therapy with chimeric antigen receptor (CAR) T cells targeting CD19, have shown promise but face challenges in aggressive BCL. We engineered fourth-generation CAR T cells (CAR4-T) with three costimulatory domains targeting CD19 (anti-CD19-CAR4-T). Seeking to enhance their efficacy against PD-L1-positive tumors, we further developed anti-CD19-CAR5-T cells secreting anti-PD-L1 scFv.<sup>35</sup> Our study demonstrated that anti-CD19-CAR5-T cells exerted more effective cytotoxicity and superior proliferation compared to anti-CD19-CAR4-T cells. Importantly, the secreted anti-PD-L1 scFv not only promoted self-proliferation of anti-CD19-CAR5-T cells but also restored the cytotoxic effect of anti-CD19-CAR4-T cells inhibited by PD-L1 expression on target cancer cells. Anti-CD19-CAR5-T cells exhibited lower proinflammatory cytokine release and demonstrated cytotoxicity against PD-L1-positive tumors even at lower cell numbers. This study provides substantial evidence for the enhanced antitumor efficiency of anti-CD19-CAR5-T cells, highlighting their potential for further investigation in *in vivo* models and clinical trials against aggressive B cell lymphomas.

#### **Fifth-generation anti-BCMA CAR5 T cells for multiple myeloma**

Multiple myeloma (MM), representing 1% of all cancers, necessitates novel therapeutic approaches due to frequent relapses despite advancements in cancer treatments. Chimeric antigen receptor-T (CAR-T) cell therapy targeting B-cell maturation antigen (BCMA) has gained FDA approval for MM, but limitations persist in achieving durable responses. Our research group developed third-generation anti-BCMA CAR T cells with CD28/4-1BB costimulatory domains and demonstrated superior antitumor efficiency. To further enhance CAR T cell persistence, we explored the CD27 costimulatory domain and engineered fourth-generation CAR T cells (CAR4-T) with CD28/4-1BB/CD27, showcasing potent antitumor efficiency across various tumor models. Recognizing programmed death-ligand 1 (PD-L1) as an immune inhibitory factor, we hypothesized that disrupting the PD-1/PD-L1 interaction could enhance CAR-T responses. Our previous work demonstrated that fifth-generation CAR T cells (CAR5-T) secreting anti-PD-L1 scFv mitigate PD-L1-mediated T cell inhibition in B-cell lymphoma.<sup>35</sup> In this study, we constructed anti-BCMA-CAR5-T cells capable of secreting anti-PD-L1 scFv.<sup>36</sup> Both anti-BCMA-CAR4-T and anti-BCMA-CAR5-T cells exhibited comparable antitumor activity against parental MM cells. However, only anti-BCMA-CAR5-T cells maintained cytolytic activity against PD-L1 high

MM cells, demonstrating their superiority. Anti-BCMA-CAR5-T cells also exhibited increased proliferation, release of cytolytic mediators, and specific cytotoxicity against BCMA-expressing target cells, presenting a potential advancement in MM CAR-T therapy. Further validation through animal models and clinical trials is warranted to assess efficacy and safety comprehensively and facilitate translation into clinical practice.

## CONCLUSION

Our research at SiCORE-CIT delves into preclinical studies to thoroughly understand the therapeutic potential, safety, and mechanisms of action inherent in fourth- and fifth-generation CAR T cells. CAR4 T cells, demonstrating heightened cytotoxicity compared to CAR2 T cells, have proven effective against both leukemic and solid cancer cells. Furthermore, CAR4 and CAR5 T cells showed reduced cytokine release, particularly IL-6, suggesting a potential decrease in cytokine release syndrome (CRS) during CAR T therapy. Notably, the secretion of anti-PD-L1 scFv from CAR5 T cells effectively inhibits PD-L1 expressed on cancer cells, enhancing the cytotoxic function and proliferation of CAR5 T cells. Additionally, CAR5-T cells demonstrated efficient cytotoxicity against PD-L1-expressing cancer cells, achieving notable results with fewer cell numbers. Our research trajectory highlights our unwavering commitment to advancing the frontiers of cancer immunotherapy, aiming to translate these innovations into meaningful clinical interventions for the betterment of cancer patients.

## ACKNOWLEDGEMENTS

I express my heartfelt appreciation to the committed members of the Siriraj Center of Research Excellence for Cancer Immunotherapy (SiCORE-CIT) and the Division of Molecular Medicine (DMM), Research Department, Faculty of Medicine Siriraj Hospital, Mahidol University. Special thanks to Assistant Professor Mutita Junking, Associate Professor Aussara Panya, Assistant Professor Dr. Chutamas Thepmalee, Ms. Nunghathai Sawasdee, Dr. Jatuporn Sujitjoon, Dr. Thaweesak Chieochansin, Dr. Chalermchai Somboonpatarakun, Dr. Piriya Luangwattananun, Dr. Suyanee Thongchot, Dr. Yupanun Wutti-In, Dr. Kamonlapat Supimon, Dr. Thanich Sangsuwannukul, Dr. Nattaporn Phanthaphol, Dr. Punchita Rujirachaivej, Dr. Kwanpirom Suwanchiwasiri, Ms. Pornpimon Yuti, Ms. Petlada Yongpitakwattana, Ms. Katesara Kongkla, Ms. Kornkan Choomee, and Mr. Krissada Natungnuy for their groundbreaking contributions to advancing CAR T cell technologies and enhancing the research findings presented in this article. I am grateful for the

collaborative spirit and support from Professor Chanitra Thuwajit, Associate Professor Peti Thuwajit, Associate Professor Naravat Pongvarin, Professor Sopit Wongkham, Professor Montarop Yamabhai, Professor Lung-Ji Chang, and Professor John Maher. I express my sincere gratitude for the ongoing research grant support received from the Siriraj Research Fund at the Faculty of Medicine, Siriraj Hospital, Mahidol University (Grant No. R016334002), the Basic Research Fund for Fiscal Year 2023 at Mahidol University (Grant No. FF-026/2566), and the National Research Council of Thailand (Grant No. N34A650524). I am a co-founder and active member of the Thailand Hub of Talents in Cancer Immunotherapy (TTCI). The academic endeavors of TTCI receive support from the National Research Council of Thailand (NRCT) under grant [number N35E660102]. Additionally, I extend my acknowledgment and thanks to Miss Arisa Jantaralap of the Medical Education Technology Center, Faculty of Medicine Siriraj Hospital, Mahidol University, for her invaluable contribution in creating the figures featured in this review.

## Conflict of interest

The author declares no conflict of interest.

## REFERENCES

1. The International Agency for Research on Cancer (IARC), World Health Organization (WHO). Press release no. 345: Global cancer burden growing, amidst mounting need for services. 1 February 2024.
2. The International Agency for Research on Cancer (IARC), World Health Organization (WHO), Global Cancer Observatory, Cancer Today, GLOBOCAN 2022, Population Factsheets: Thailand.
3. Chen S, Cao Z, Prettnner K, Kuhn M, Yang J, Jiao L, Wang Z, Li W, Geldsetzer P, Bärnighausen T, Bloom DE, Wang C. Estimates and projections of the global economic cost of 29 cancers in 204 countries and territories from 2020 to 2050. *JAMA Oncol*. 2023; 9(4):465-72.
4. Editorial. Advancing Cancer Therapy. *Nat Cancer*. 2021;2(3): 245-246.
5. June CH, Sadelain M. Chimeric antigen receptor therapy. *N Engl J Med* 2018;379(1):64-73.
6. Boyiadzis MM, Dhodapkar MV, Brentjens RJ, Kochenderfer JN, Neelapu SS, Maus MV, et al. Chimeric antigen receptor (CAR) T therapies for the treatment of hematologic malignancies: clinical perspective and significance. *J Immunother Cancer*. 2018;6:137.
7. June CH. Q&A: Carl June on CAR T-cell Therapy. *Blood Cancer Discov* 2020;1(1):8-9.
8. Newitt VN. The incredible story of Emily Whitehead & CAR T-cell therapy. *Oncology Times* 2022;44(6):1,19-20.
9. Prasad V. Immunotherapy: Tisagenlecleucel - the first approved CAR-T-cell therapy: implications for payers and policy makers. *Nat Rev Clin Oncol*. 2018;15(1):11-12.

10. Cappell KM Kochenderfer JN. Long-term outcomes following CAR T cell therapy: what we know so far. *Nat Rev Clin Oncol*. 2023;20(6):359-71.
11. Fry TJ, Shah NN, Orentas RJ, Stetler-Stevenson M, Yuan CM, Ramakrishna S, et al. CD22-targeted CAR T cells induce remission in B-ALL that is naïve or resistant to CD19-targeted CAR immunotherapy. *Nat Med*. 2018;24(1):20-28.
12. Dai Z, Mu W, Zhao Y, Cheng J, Lin H, Ouyang K et al. T cells expressing CD5/CD7 bispecific chimeric antigen receptors with fully human heavy-chain-only domains mitigate tumor antigen escape. *Signal Transduct Target Ther*. 2022;7(1):85.
13. Can I, Cox MJ, Siegler EL, Sakemura R, Kenderian SS. Challenges of chimeric antigen receptor T-cell therapy in chronic lymphocytic leukemia: lessons learned. *Exp Hematol*. 2022;108:1-7.
14. Turkalj S, Radtke FA, Vyas P. An overview of targeted therapies in acute myeloid leukemia. *Hemasphere* 2023;7(6):e914.
15. Susanibar-Adaniya S, Barta SK. 2021 Update on Diffuse large B cell lymphoma: A review of current data and potential applications on risk stratification and management. *J Hematol*. 2021;96(5):617-29.
16. Schultz L, Mackall C. Driving CAR T cell translation forward. *Sci Transl Med*. 2019;11(481):eaaw2127.
17. Hasanali ZS, Razzo B, Susanibar-Adaniya SP, Garfall AL, Stadtmauer EA, Cohen AD. Chimeric antigen receptor T cells in the treatment of multiple myeloma. *Hematol Oncol Clin North Am*. 2023 Dec 28:S0889-8588(23)00171-5.
18. Braunstein M, Wertz J, Davies F. A new decade: novel immunotherapies on the horizon for relapsed/refractory multiple myeloma. *Expert Rev Hematol*. 2021;14(4):377-89.
19. Chohan KL, Siegler EL, Kenderian SS. CAR-T cell therapy: the efficacy and toxicity balance. *Curr Hematol Malig Rep*. 2023; 18(2):9-18.
20. Dourthe M-E, Rabian F, Yakouben K, Chevillon F, Cabannes-Hamy A, Méchinaud F, et al. Determinants of CD19-positive vs CD19-negative relapse after tisagenlecleucel for B-cell acute lymphoblastic leukemia. *Leukemia*. 2021;35(12):3383-93.
21. Hay AE, Cheung MC. CAR T-cells: costs, comparisons, and commentary. *J Med Econ*. 2019;22(7):613-5.
22. National Cancer Institute. CAR T cells: engineering patients' immune cells to treat their cancers. <https://www.cancer.gov/about-cancer/treatment/research/car-t-cells>.
23. Simsek H, Klotzsch E. The solid tumor microenvironment-breaking the barrier for T cells: how the solid tumor microenvironment influences T cells. *Bioessays*. 2022;44(6):e2100285.
24. Albelda SM. CAR T cell therapy for patients with solid tumours: key lessons to learn and unlearn. *Nat Rev Clin Oncol*. 2024;21(1): 47-66.
25. Acuto O, Michel F. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat Rev Immunol*. 2003; 3(12):939-51.
26. Esensten JH, Helou YA, Chopra G, Weiss A, Bluestone JA. CD28 costimulation; from mechanism to therapy. *Immunity* 2016; 44(5):973-88.
27. Otano I, Azpilikueta A, Glez-Vaz J, Alvarez M, Medina-Echeverez J, Cortés-Domínguez I, et al. CD137 (4-1BB) costimulation of CD8+ T cells is more potent when provided in cis than in trans with respect to CD3-TCR stimulation. *Nat Commun*. 2021; 12(1):7296.
28. Starzer AM, Berghoff AS. New emerging targets in cancer immunotherapy: CD27 (TNFRSF7). *ESMO Open*. 2020;4 (Suppl 3):e000629.
29. Wutti-In Y, Sujitjorn J, Sawasdee N, Panya A, Kongkla K, Yuti P, et al. Development of a novel anti-CD19 CAR containing a fully human scFv and three costimulatory domains. *Front Oncol*. 2022;11:802876.
30. Sangsuwannukul T, Supimon K, Sujitjorn J, Phanthaphol N, Chieochansin T, Pongvarin N, et al. Anti-tumour effect of the fourth-generation chimeric antigen receptor T cells targeting CD133 against cholangiocarcinoma cells. *Int Immunopharmacol*. 2020;89(Pt B):107069.
31. Supimon K, Sangsuwannukul T, Sujitjorn J, Phanthaphol N, Chieochansin T, Pongvarin N, et al. Anti-mucin 1 chimeric antigen receptor T cells for adoptive T cell therapy of cholangiocarcinoma. *Sci Rep*. 2021;11(1):6276.
32. Phanthaphol N, Somboonpatarakun C, Suwanchiwasiri K, Chieochansin T, Sujitjorn J, Wongkham S, et al. Chimeric antigen receptor T cells targeting integrin  $\alpha\beta 6$  expressed on cholangiocarcinoma cells. *Front Oncol*. 2021;11:657868.
33. Luangwattananun P, Junking M, Sujitjorn J, Wutti-In Y, Pongvarin N, Thuwajit C, et al. Fourth-generation chimeric antigen receptor T cells targeting folate receptor alpha antigen expressed on breast cancer cells for adoptive T cell therapy. *Breast Cancer Res Treat*. 2021;186(1):25-36.
34. Somboonpatarakun C, Phanthaphol N, Suwanchiwasiri K, Ramwarungkura B, Yuti P, Pongvarin N, Thuwajit P, et al. Cytotoxicity of fourth-generation anti-Trop2 CAR-T cells against breast cancer. *Int Immunopharmacol*. 2024;129:111631.
35. Yuti P, Wutti-In Y, Sawasdee N, Kongkhla K, Phanthaphol N, Choomee K, et al. Anti-CD19 chimeric antigen receptor T cells secreting anti-PD-L1 single-chain variable fragment attenuate PD-L1 mediated T cell inhibition. *Int Immunopharmacol*. 2022;113(Pt B):109442.
36. Yuti P, Sawasdee N, Natungnuy K, Rujirachaivej P, Luangwattananun P, Sujitjorn J, et al. Enhanced antitumor efficacy, proliferative capacity, and alleviation of T cell exhaustion by fifth-generation chimeric antigen receptor T cells targeting B cell maturation antigen in multiple myeloma. *Biomed Pharmacother*. 2023; 168:115691.