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### The Biomedical Application of Chimeric Antigen Receptor T cell Therapy

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*Washington University in St. Louis*

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WASHINGTON UNIVERSITY IN ST. LOUIS

The Biomedical Application of Chimeric Antigen Receptor T cell Therapy

by

Jessica Merritt Devenport

A thesis presented to  
The Graduate School  
of Washington University in  
partial fulfillment of the  
requirements for the degree  
of Master of Biology

December 2019  
St. Louis, Missouri

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# **Chapter 1: Introduction**

Cancer is the second leading cause of death in the United States, with greater than 1,750,000 new cancer diagnoses and approximately 600,000 deaths projected for 2019<sup>1</sup>. Current treatments include surgery, chemotherapy, and radiation, and are invasive and associated with adverse toxicities. Because these therapies can be ineffective and often result in relapse, the push for new treatment options has continued. Chimeric antigen receptor (CAR) T cells, a type of adoptive cellular therapy, utilize transgenic receptors on T cells to recognize tumor-associated-antigens and induce target-specific killing. Targeted CAR-T therapies have shown potential for inducing remission and long-term relapse-free survival in some cancers, such as pediatric and adult B-ALL<sup>2-4</sup>. This review will look at the history of CAR-T cell research, including its application in clinical studies, adverse clinical side-effects, and more recent advances in the field.

## **1.1.1 The immune system and oncogenesis**

Paul Ehrlich first proposed that the immune system could suppress oncogenesis, and later advances by Burnet and Thomas built on that concept with the development of the cancer immunosurveillance hypothesis<sup>5,6</sup>. The cancer immunosurveillance hypothesis suggested that the adaptive immune system was responsible for preventing cancer development<sup>5</sup>. Other researchers debated the actuality of this hypothesis, suggesting that cancer development occurred due to a lack in tumor-cell signaling, resulting in a dampened immune response, or that the lack of an immune response against abnormal cancer cells resulted because the cancer cells were too similar to the surrounding tissues<sup>7,8</sup>.

The role that immunosurveillance plays in cancer development began to come into focus in the 1990's, when researchers discovered that mice lacking interferon  $\gamma$  (IFN- $\gamma$ ) responsiveness (either via the loss of IFN- $\gamma$  receptor or the loss of STAT1, a transcription factor required for IFN receptor signaling, as well as mice lacking a sufficient immune system, were more susceptible to the development of spontaneous and carcinogenic-induced tumors<sup>9,10</sup>. Because of these findings, researchers began investigating the specific role that the immune system plays in suppressing oncogenesis. The immune system was found to be important in preventing cancer via three different mechanisms: host protection against virally-induced tumors, reduction of inflammatory environments that lead to chronic wounds and tissue damage, and elimination of tumor cells via immune-recognition of specific antigens present on the tumor cell surface. Cancer immunotherapy stems from the fact that cancer cells express tumor-associated-antigens that allow them to be distinguished from their healthy-tissue counterparts. Tumor-associated antigens can be differentiation antigens, mutated antigens, viral antigens, or overexpressed antigens<sup>11</sup>.

## **1.1.2 Cancer immunosurveillance**

The cancer immunosurveillance hypothesis underwent a revision in 2001<sup>12</sup>. The newly defined cancer immune-editing hypothesis postulated that cancer immune-editing undergoes three processes: elimination, equilibrium, and escape<sup>12</sup>. The elimination phase occurs when both the adaptive and innate immune system work synergistically to eradicate abnormal cells before they become a problem<sup>12</sup>. The elimination phase requires immune cells, like T cells, to respond to tumor-associated antigens. An immune response against abnormal cells can reduce developing tumor cells and therefore prevent tumor formation. Despite the efforts of the immune system, some cancer cells can evade destruction during the elimination phase. These cells then enter into the

equilibrium phase, in which the immune system simultaneously dampens tumor growth while shaping the immunogenicity of the cells. The equilibrium phase is thought to be the longest phase of cancer immunosurveillance and can proceed throughout the duration of the host's life<sup>12</sup>. Tumor cells in the equilibrium phase are considered dormant, and these cells remain dormant until they overcome the equilibrium phase and grow into primary or metastatic tumors<sup>12</sup>. The equilibrium phase provides selective pressures that allow the genetically unstable tumor cells to develop mutations to evade immune detection<sup>12,13</sup>. Immuno-evasive mutations acquired during the equilibrium phase allow the oncogenic cells to grow aggressively and permit the development of an immunosuppressive microenvironment. Immuno-evasive mutations and an immunosuppressive tumor microenvironment prevent active moderation of the cancer cells by the immune system<sup>12</sup>.

The immune cells, such as T cells that infiltrate the tumor, are critical in determining the outcome of the immunoeediting process. T cells are a type of lymphocyte with many subtypes having a variety of functions. The relevant T cell types are discussed in section 1.2. Some T cells, known as T regulatory ( $T_{reg}$ ) cells, can suppress the local immune system, allowing tumors to grow uninhibited. However, accumulation of another subset of T cells, cytotoxic CD8 T cells, is directly associated with an increased immune response against the tumor cells<sup>14</sup>. When adequately primed and activated, the CD8 T cells can respond to the tumor-associated-antigens and elicit immune responses that result in tumor cell death, leading to a more favorable outcome among cancer patients<sup>15</sup>. Additional studies of the mechanisms driving T cell functions revealed that T cells could be redirected to more effectively target cancer, opening the door for new therapeutic immunotherapy advances.

## 1.2 T cells

Understanding T cell biology is critical in designing effective CAR-T cell therapy. As previously discussed, the adaptive immune response has a significant role in combatting tumor growth. Of the variety of cell types that are active in adaptive immune responses, T cells play the most crucial role in the context of CAR-T cell therapy. T cells are critical for the development of an adaptive immune response. T cell subsets have a variety of functions, but one of the most fundamental roles of T cells is to recognize and respond to pathogens and foreign antigens. Upon this recognition, T cells can induce immune-mediated cell death and secrete cytokines to drive immune responses. The way the T cell responds to a perceived threat directly impacts the type of immune response that the body produces<sup>16</sup>.

T cells can mature into a variety of subtypes. This section focuses on the relevant subtypes for CAR-T cell therapy. CD4+ helper T cells and CD8+ effector T cells represent two critical T cell populations that are necessary to drive immune responses against cancer. T cells can recognize epitopes presented via major histocompatibility complexes (MHC) on the surface of some cell types. There are two classes of MHC molecules: MHC class I and MHC class II. MHC molecules form a stable conformation when they bind with peptides, allowing the presentation of the complex on the surface of a variety of cell types, including macrophages, dendritic cells, and B cells<sup>16</sup>. MHC class I molecules bind short peptides, limited to 8-10 amino acids in length, while there is no limit for the length of a peptide that can bind to MHC class II molecules<sup>16</sup>.

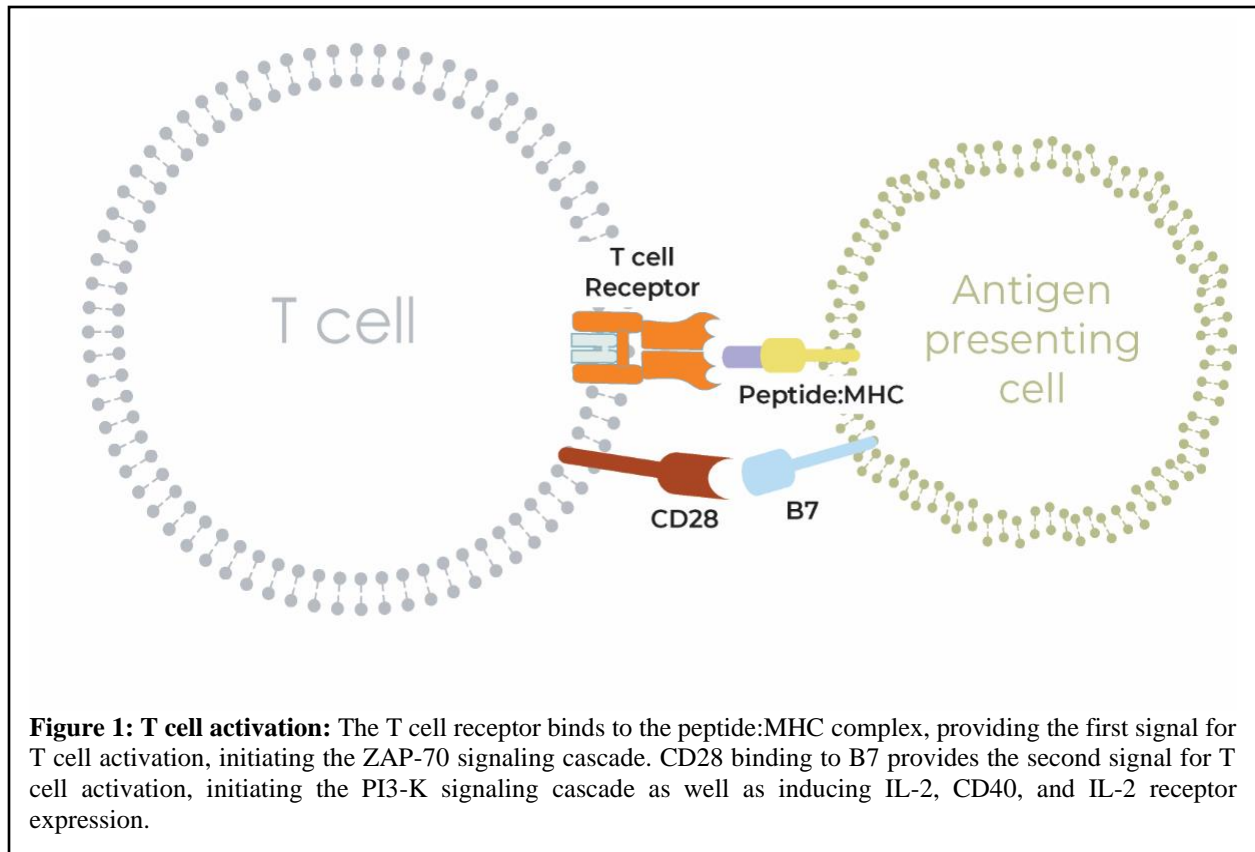
### 1.2.1 CD4+ T-Helper cells.

CD4 T cells can differentiate into a number of subsets, including T<sub>H1</sub>, T<sub>H2</sub>, T<sub>H17</sub>, T<sub>FH</sub>, and T<sub>reg</sub>. For the purposes of this review, only T<sub>H1</sub> cells will be discussed in-depth. CD4 T cells receive

three signals to become active and differentiate. The first signal in CD4 T cell activation occurs when the T cell receptor (TCR) binds to the peptide: MHC class II complex present on an antigen-presenting cell (APC). CD4, present on the surface of the T cell, also binds to MHC class II to stabilize the interaction. The second co-stimulation signal in activation occurs when B7 molecules on the APC bind to CD28 on the T cell (Figure 1). Importantly, B7 binding to CD28 induces expression of interleukin 2 (IL-2) and CD40 ligand (CD40L) as well as enhancing the affinity of the IL-2 receptor. Extracellular binding of IL-2 to its receptor promotes T cell growth and differentiation<sup>16</sup>. Additionally, B7 binding to CD28 activates the PI3-kinase intracellular signaling pathway. The PI3-kinase pathway is responsible for phosphorylating a protein kinase, AKT. Phosphorylation of AKT results in enhanced cell survival and upregulation of cellular metabolism<sup>16</sup>.

Binding of the TCR and the CD28 co-stimulatory receptor initiates a signaling cascade within the T cell. Once bound, Lck, a tyrosine kinase, phosphorylates residues present on the intracellular domain of the TCR complex. Phosphorylation of the intracellular domain of the TCR complex leads to phosphorylation of another protein kinase, ZAP70, resulting in differentiation, proliferation, and effector actions of the T cells.

Finally, cytokines secreted by the APC provide a third signal responsible for directing T cell differentiation. IL-12 and IFN- $\gamma$  secreted by the APC drive the differentiation of CD4<sup>+</sup> T cells to their T<sub>H</sub>1 subtype. The T<sub>H</sub>1 cells secrete cytokines such as IFN- $\gamma$  to activate macrophages, enabling the elimination of intracellular pathogens<sup>16</sup>. T<sub>H</sub>1 T cells are also critical for CD8 T cell activation, as discussed in section 1.2.2<sup>16</sup>.



### 1.2.2 CD8+ T-effector cells

Activation of CD8 T cells differs from the activation of CD4 T cells in some regards. In some cases, dendritic cell presentation of peptide: MHC class I can be sufficient for CD8 T cell activation without a co-stimulatory signal. However, in most cases, CD8 T cells require assistance from CD4 T cells to become fully activated. As previously discussed in section 1.2.1, activation of CD4 T cells results in the upregulation of IL-2 and CD40L. CD40L, now present on the CD4 T cell surface, binds to CD40 on the opposing cellular surface. The binding of CD40 to its ligand drives an increase in B7 and 4-1BBL, a co-stimulatory molecule, on the cell surface, providing additional stimulation to the CD8 T cell. Additionally, the IL-2 secreted by the CD4 T cell acts as a growth factor, inducing CD8 T cell differentiation<sup>17</sup>.

The primary role of activated CD8 T cells is to kill cells that present foreign peptides often derived from intracellular pathogens. Adhesion molecules present on the surface of the effector T cell, such as LFA-1, direct the T cell to sites of infection. LFA-1 transiently binds to ICAM, a cell surface glycoprotein present on varying tissues. Binding of the TCR to an antigen on the cell surface increases the affinity of LFA-1 and ICAM binding, allowing the CD8 T cells to elicit cytotoxic effects on the target cell<sup>16</sup>.

Cytotoxic effector molecules and cytokines are responsible for driving the effector function of the CD8 T cells. Four primary cytotoxic effector molecules produced by effector CD8 T cells are perforin, granzymes, granulysin, and Fas ligand. Perforin assists in the delivery of granules into the cytoplasm of the target cell. Granzymes are serine proteases that stimulate apoptotic pathways upon delivery to the cytoplasm of the host cell. Granulysin is an antimicrobial protein that also induces apoptosis. Fas ligand binds to Fas on the surface of the target cell, causing apoptotic cell death. CD8 T cells secrete cytokines, including IFN- $\gamma$ , LT- $\alpha$ , and TNF- $\alpha$ . IFN- $\gamma$  is the primary cytokine released by the effector cells. IFN- $\gamma$  has multiple roles, including but not limited to blocking viral replication, activating macrophages, and inducing MHC class II expression. LT- $\alpha$  primarily activates macrophages and B cells and can be directly toxic to target cells. Finally, TNF $\alpha$ , along with CXCL1, a chemokine that recruits and activates neutrophils, recruits neutrophils to the target cells to enhance the immune response<sup>16,18</sup>.

T cell populations begin to diminish upon clearance of the infection. However, some T cells persist, differentiating into effector and memory subsets that survey the body in case of a re-infection. Effector memory subsets can rapidly mature into effector T cells in the presence of large amounts of cytokines, such as IFN- $\gamma$ , IL-4, and IL-5, and can quickly enter an area of infection to begin eliminating the target cells. Central memory T cells primarily remain in the lymphoid tissue,

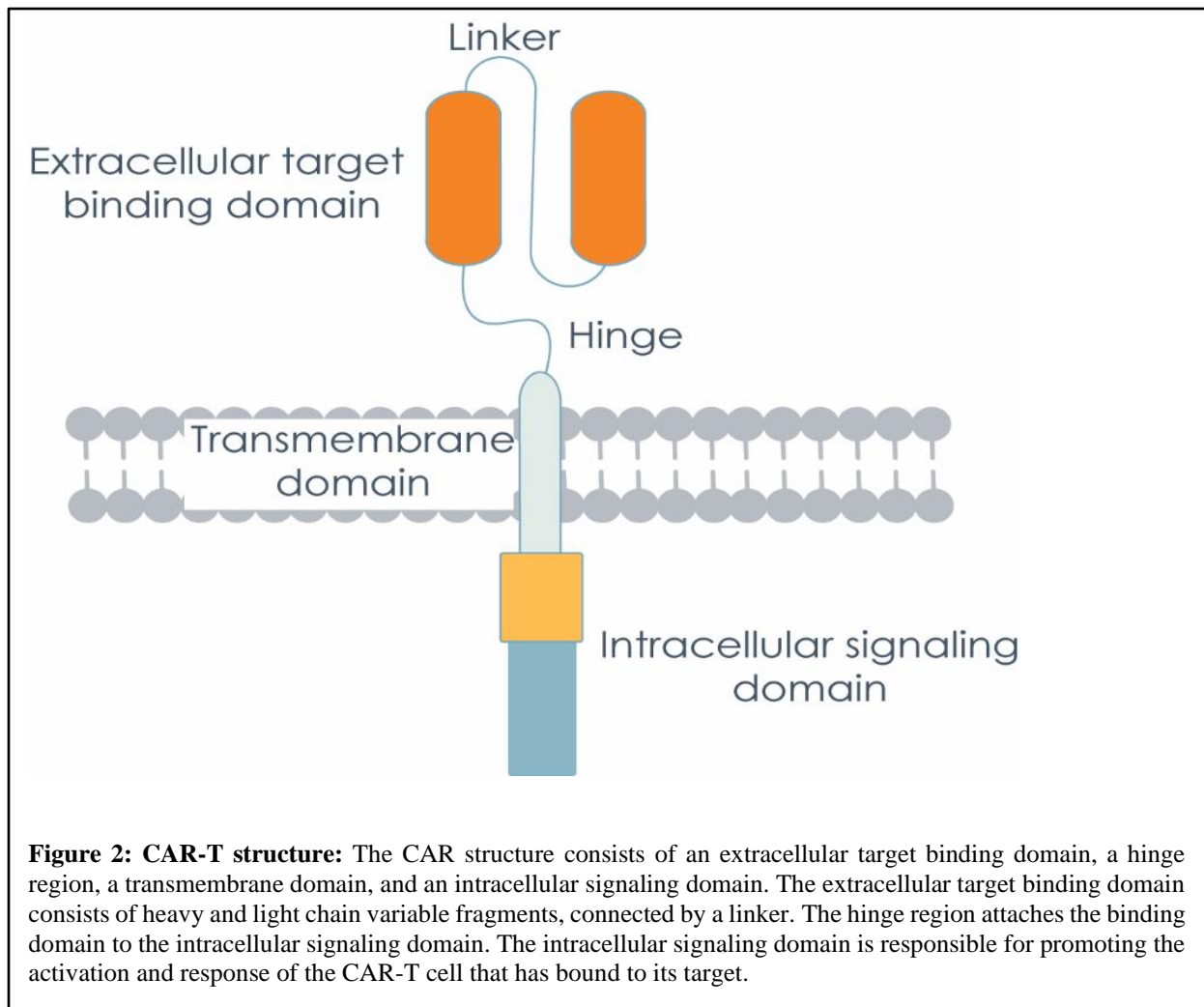
where, like effector memory cells, they differentiate into effector T cells<sup>16</sup>. The constant surveillance provided by memory T cells makes them very beneficial for CAR-T cell therapy, as memory CAR-T cells can provide prolonged anti-tumor effects<sup>19</sup>.

## **Chapter 2: CAR-T Cell Therapy**

### **2.1 CAR-T Structure**

In 1989, Eshhar and his team engineered the first T cell that was modified to recognize and respond to specific antigens<sup>20</sup>. His team generated chimeric TCR genes and functionally expressed them in T cells, allowing them to redirect the T cell response<sup>20</sup>. These recombinant cells were created to better understanding the signaling components necessary to induce T cell activation mediated by a signaling domain<sup>21-23</sup>. The recombinant T cells demonstrated that T cells could be engineered to engage specific peptides, utilizing receptor-ligand-mediated interactions<sup>23</sup>. The ability to redirect T cells to engage specific target antigens allowed for the engineering of T cells that could be used to target specific antigens present on cancer cells.





CAR-T cells are T cells engineered with recombinant receptors that combine the antigen-binding properties of monoclonal antibodies with the killing capacity and self-renewal of T cells<sup>24</sup>. The chimeric receptors on CAR-T cells are a fusion of four essential components: an extracellular target-binding domain, a hinge domain, a transmembrane domain, and an intracellular signaling domain<sup>25</sup> (Figure 2). CAR-T cells vary from other T cell receptor-modified cells in that they can recognize cell surface tumor antigens in an HLA-independent manner, meaning that the CAR-T cell can identify surface molecules that have not been processed and presented by MHC molecules<sup>26</sup>. Additionally, CAR-T cells can target non-protein antigens, such as tumor expressing

carbohydrates and glycolipids<sup>27-29</sup>. These factors are essential because tumors are often able to escape T cell-mediated killing by inhibiting antigen processing and presentation in an MHC dependent manner<sup>30</sup>.

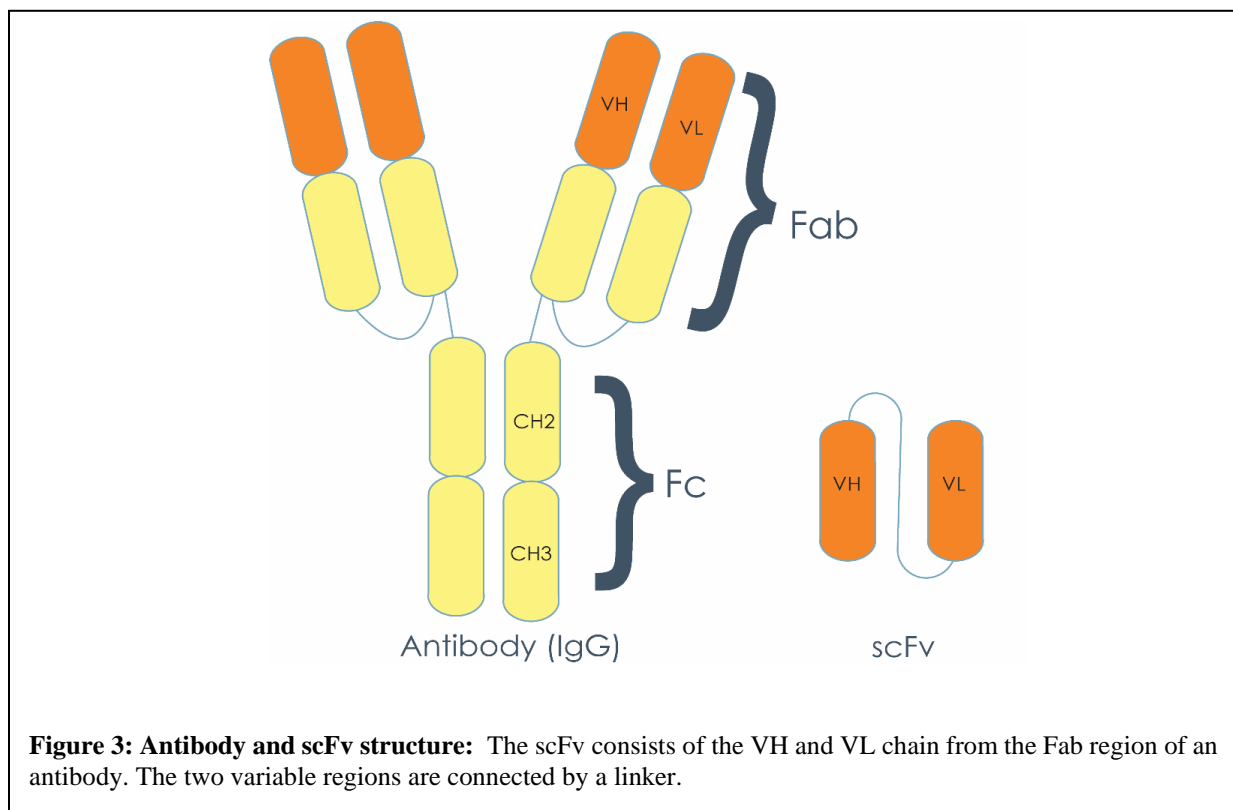
### **2.1.1 Extracellular target binding domain**

#### **scFv:**

The extracellular target binding domain is the portion of the CAR construct that binds to the target antigen. Some CAR-T cells contain extracellular target binding domains derived from nanobodies or natural binding partners of the target antigen. However, the most commonly used extracellular target binding domain is a single-chain fragment variable (scFv) derived from the antigen-binding fragment of an antibody (Fab)<sup>24,31</sup>. The Fab consists of a heavy-chain and a light-chain connected via a linker that allows the two peptide segments to fold over each other, mimicking their native conformation<sup>30,32</sup>. ScFv's retain the specificity and affinity, referring to the strength of the bond between the antibody and the antigen, for the target antigen as the original antibody while being expressed as an intact protein on the CAR-T surface<sup>33</sup> (Figure 3). The use of an scFv in CAR design, rather than the native TCR, enables the T cell to recognize antigens that are not presented by an MHC complex, allows for T cell activation in a single binding event, and permits recognition of low-density antigens on the surface of the tumor cell<sup>34</sup>. Additionally, scFv's derived from antibodies naturally have a higher affinity for their target than their TCR counterparts<sup>35</sup>. Higher affinity is vital because the affinity and avidity of the scFv for its target impact the release of cytokines from T cells, influencing the rate of tumor-killing and T cell persistence<sup>36-39</sup>.

ScFv binding avidity or the overall strength of the connection, relative to tumor antigen density, is vital to consider when designing scFv's for CAR construction. Notably, cloning the variable chain

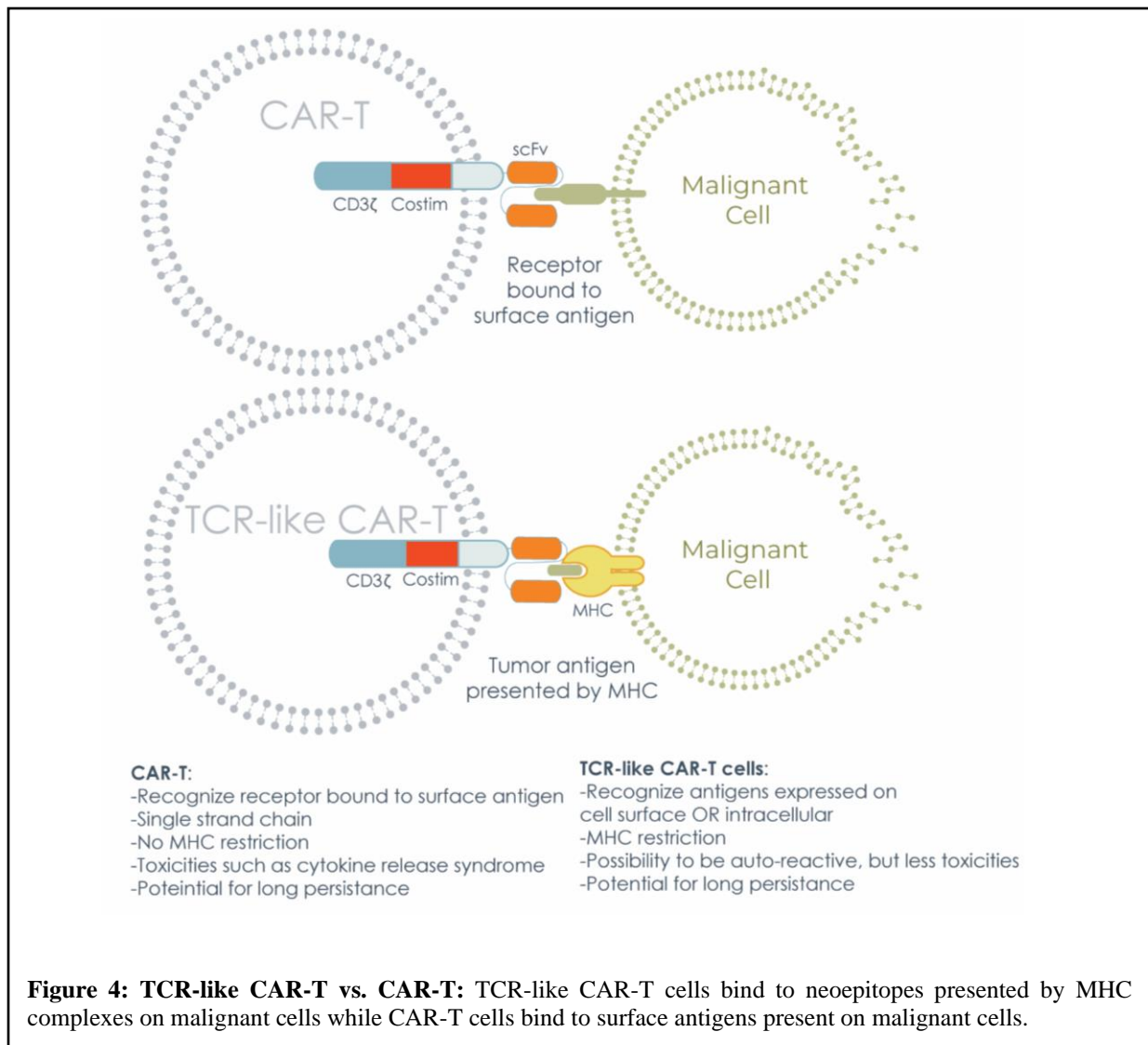
from a full antibody sequence can result in a reduction of binding avidity<sup>40</sup>. Activation of a CAR is dependent on the binding affinity and avidity of the scFv and the antigen density on the target cell. Therefore, assessing the binding affinity and avidity of the CAR to its target is essential in establishing a threshold level of activation. However, past the threshold level needed for activation, binding affinity and avidity do not directly correlate with the strength of the effector response<sup>33</sup>. Caruso *et al.* at MD Anderson Cancer Center designed CAR-T cells that varied in affinity for their target antigen. They found that CAR binding affinity alone does not define the effective rate of CAR-T cell activation<sup>37</sup>. The findings of Caruso *et al.* support the statement that CAR binding affinity does not direct the effector response, likely due to the inability of the CAR-T cell to activate further once it has bound to its antigen<sup>41</sup>. Furthermore, excessively high affinity and avidity interactions between the scFv and the target antigen can potentially result in T cell exhaustion and activation-induced cell death<sup>42</sup>



If the scFv is targets antigens that are present on tumors and healthy tissues, on-target off-tumor effects can occur, whereby normal tissues are damaged by the CAR-T cells<sup>36</sup>. Studies performed to test CAR-T's with lower binding affinities found that CAR-T's expressing low-affinity scFv were shown to have strong activity against tumors overexpressing the antigen of interest while reducing activity on tissues expressing the antigen at normal physiological levels<sup>36</sup>. However, in certain instances, CAR-T's with high binding affinities are necessary to induce complete T cell activation and tumor clearance<sup>33</sup>. Overall, it is important to engineer scFv's so that their binding affinity is sufficient to effectively bind to its target and activate T cell effector functions while causing minimal, on-target, off-tumor side effects.

### **TCR-like:**

The TCR-like class of TCR-engineered T cells expresses scFv's from antibodies that are specific for MHC class molecules bound to a loaded peptide (Figure 4). Unlike traditional CAR-T cells, in which the scFv's can recognize an antigen on the surface of a cell that is not bound to an MHC complex, TCR-engineered T cells can be designed to attach to neoepitopes, which are tumor-specific antigens present in the context of MHC. Neo-epitopes, generated when malignantly-transformed cells load mutated peptides onto the MHC, can be classified into three subtypes: tumor-specific antigens arising from mutated proteins, differentiation antigens expressed on specific cell lineages, and antigens derived from gene overexpression or amplification<sup>43</sup>. Neo-epitopes consist of intracellular and extracellular antigens, expanding the repertoire of targetable ligands. Targeting neo-epitopes allows for the range of targetable ligands to be broadened by targeting both intracellular and extracellular antigens. Comprehensive neo-antigen screening allows for the identification of recurrent neo-antigens<sup>44</sup>. These methods aim to increase the efficacy of cancer immunotherapy by utilizing recurrent neo-antigens among specific tumor types.



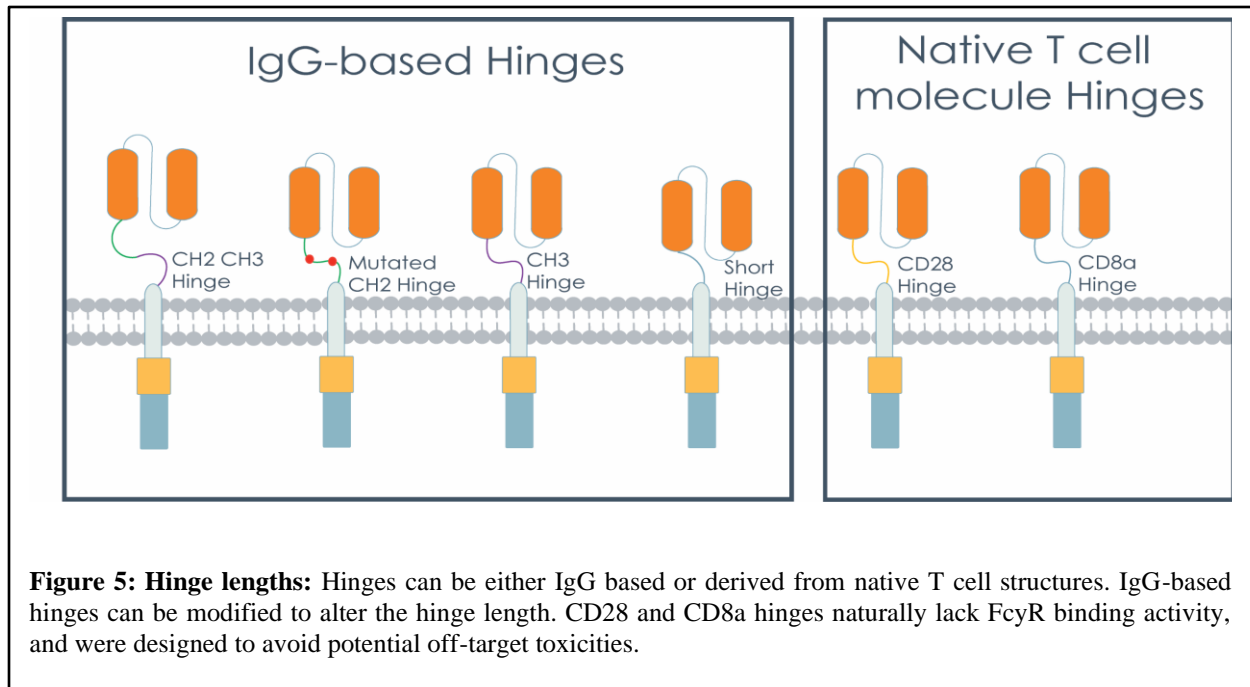
TCR-engineered T cells, like standard scFv CARs, have optimal affinities that can affect the outcome of the treatments. Both high-affinity and low-affinity TCR-engineered T cells have been generated and can induce T cell activation upon recognition of the MHC-peptide complex<sup>45</sup>. However, both high-affinity and low-affinity TCR-engineered T cells can have unexpected autoreactivity<sup>33</sup>. High-affinity TCR-engineered T cells can have lower viability than the low-affinity TCR-engineered T cells. Additionally, low-affinity TCR-engineered T cells can have reduced interactions with the targeted MHC-peptide complex than natural TCRs<sup>33</sup>.

### 2.1.2 Hinge/Spacer region

The hinge region is the non-antigen binding segment of the CAR's extracellular domain. This region commonly consists of an immunoglobulin Fc (CH2 or CD3), CD8 $\alpha$ , or a CD28 spacer region<sup>46</sup> (Figure 5). The hinge domains allow the CAR-T extracellular domain to be flexible, reducing special restrictions on the surface of the cell, thereby promoting the formation of synapses between the scFv and its target antigen<sup>47-50</sup>. Variations within the length of the hinge region can alter flexibility, dimerization, and stability, influencing T cell-to-target cell interactions and affecting activation signal strength<sup>33,38,47</sup>.

In the context of IgG-based hinges, portions of the Fc region can be deleted to generate variability within the length of the spacer, impacting CAR-T function in some, but not all, types of CAR-T cells<sup>50,51</sup>. Long spacers may be more advantageous when the antigen-binding site is close to the tumor cell membrane<sup>33,38,47</sup>. However, short spacers have been shown to lead to an increase in cytokine production and CAR-T cell proliferation in certain types of CAR-Ts, possibly due to the rise in the ability of the CAR to dimerize and exhibit tonic signaling<sup>46</sup>. Therefore, the distance of the target antigen from the tumor surface and properties of the varying hinge lengths both need to be considered when designing CAR-T cells for different targets.

Initial designs for IgG Fc hinge regions allowed the hinge to maintain interactions with the FC $\gamma$  receptors<sup>33,52,53</sup>. Because of this, the hinge regions were able to non-specifically activate the CAR-T in the presence of FC-receptor expressing cells<sup>33,54,55</sup>. Future hinge designs consisted of mutations within the hinge domain, altering the ability of the hinge to bind to the Fc $\gamma$  receptor. These alterations prevented off-target CAR-T activation and improved the overall persistence and antitumor effect of the CAR-T cell therapy<sup>54,55</sup>.



Other hinge regions, derived from native T cell molecules CD28 and CD8 $\alpha$  that naturally lack Fc $\gamma$ R binding activity were designed to avoid potential off-target toxicities. Current CAR-T cell designs utilize these native domains instead of IgG-based hinges. In a direct comparison, CAR-T cells containing the CD28 or CD8 $\alpha$  hinge were found to perform similarly to their IgG-based counterparts in degranulation, cytotoxicity, and proliferation *in vitro*<sup>56</sup>. However, CAR-T's containing the CD8 $\alpha$  hinge were found to produce fewer cytokines than the CAR-T comprising the CD28 hinge, which could be beneficial for counteracting some CAR-T toxicities<sup>57</sup>.

### 2.1.3 Intracellular signaling domain

#### First-generation CARs

First-generation CAR-T's consist of the CD3  $\zeta$ -chain, intracellular domains of CD4 or CD8, and an scFv ( $\zeta$ -CAR) (Figure 6). The CD3  $\zeta$ -chain was first cloned in the early 1990's<sup>58</sup> and is responsible for transmitting signals from the endogenous T-cell receptor<sup>20</sup>. Initially, the cloned

CD3  $\zeta$ -chain was fused with transmembrane domains of the TCR co-receptors CD8 or CD4 to study its function within leukemic T cells<sup>21-23,32,58-61</sup>. These chimeric receptors were able to induce early T cell activation, laying the foundations for the future of CAR-T generation.

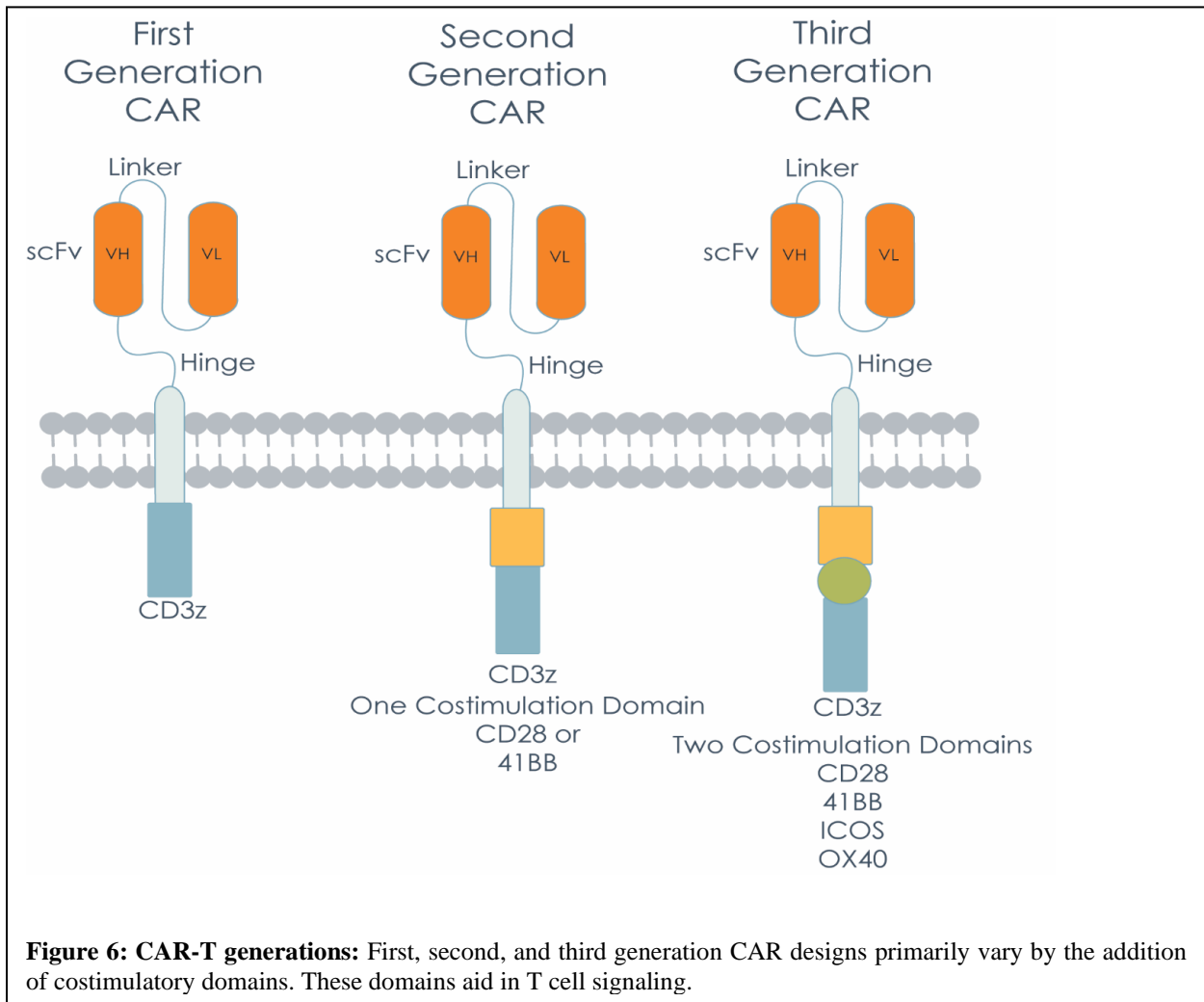
The chimeric receptor, consisting of the cloned CD3  $\zeta$ -chain fused with transmembrane domains of the TCR co-receptors CD8 or CD4, was later merged with an scFv to specifically redirect the T cell response<sup>32</sup>.  $\zeta$ -CAR-T cells were able to specifically redirect the T cell response and induce proliferation *in vitro*<sup>32,60</sup>. However,  $\zeta$ -CAR-T cells were only able to modestly delay the growth of tumors *in vivo*<sup>59</sup>. The failure of early CAR-T cells can be attributed to the lack of co-stimulatory domains, resulting in insufficient cytokine production (such as IL-2) and, therefore, an inadequate CAR-T cell response. As stated in section 1.2, T cells require multiple signals to proliferate and function properly. The CD3  $\zeta$ -chain is the sole signal transduction domain of  $\zeta$ -CAR-T's, and without the support of co-stimulatory domains,  $\zeta$ -CAR-T cells are not able to produce sufficient IL-2 to fully activate and induce proliferation, resulting in weak T cell expansion and anti-tumor activity *in vivo*<sup>61-64</sup>.

Under normal physiologic conditions, co-stimulatory receptors, such as CD28 and 4-1BB, play an essential role in the functional outcome of TCR signaling<sup>65</sup>. Co-stimulatory molecules co-localize with the TCR and work in cooperation with TCR signaling to determine T cell activation, differentiation, effector function, and survival<sup>66</sup>. Lack of co-stimulation can fail T cell progression beyond initial cell cycle stages. Additionally, the receptors on the  $\zeta$ -CAR-T cells are unable to induce secretion of optimal amounts of IFN- $\gamma$  causing the  $\zeta$ -CAR-T cells to rapidly anergize resulting in the inability of the CAR to delay tumor response<sup>67</sup>.



## Second-generation CARs

Second generation CAR-T's overcame limitations demonstrated by  $\zeta$ -CAR-T's by including a cytoplasmic domain derived from various co-stimulatory receptors (Figure 6). Co-stimulation in second-generation CAR-T's is most commonly provided by the signaling elements CD28 or 4-1BB, and less widely provided by ICOS, OX40, and CD27 among others<sup>59,68,69</sup>. The co-stimulatory domains utilized in second-generation CAR-T cells are analogous to the natural T cell activation domain promoting greater signaling strength and therefore enhancing CAR-T cell proliferation and *in vivo* persistence<sup>68,70-72</sup>.



### **Endogenous CD28:**

CD28 plays a vital role in T cell activation by amplifying the TCR signaling pathway. Because of the role of CD28 in normal T cell function, it was logical to utilize CD28 co-stimulation when designing second-generation CAR-T's. Binding of CD28 leads to phosphorylation and activation of various complexes, discussed in section 1.2 and later in this section, that boost TCR signaling. Co-stimulation provided by CD28 lowers the minimum level of TCR engagement required to activate the T cell, enabling the TCR to have increased sensitivity to antigenic stimulation<sup>73</sup>. The amplification of the TCR signaling pathway increases cytokine production, cell cycle progression and induces anti-apoptotic factors<sup>59,73-81</sup>.

Under normal physiologic conditions, the cytoplasmic tail of CD28 can associate with several intracellular signaling pathways. Binding of CD28 on T cells to co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) on APCs results in phosphorylation of tyrosine residues on the cytoplasmic tail of CD28, providing a docking site in which src homology (SH) domain-containing proteins can bind<sup>16</sup>. The binding of the SH domain-containing proteins promotes binding of the p85 subunit of PI3K, subsequently initiating the PI3K-AKT pathway (Figure 7)<sup>82</sup>. CD28 signaling allows for chromatin remodeling, enhancing transcription factor accessibility to the IL-2, IL-4, and IFN- $\gamma$  loci<sup>79,82-85</sup>. Increased accessibility of these loci provides for a more rapid secondary T cell response<sup>79,85</sup>. Activation of the PI3K-AKT pathway results in increased cytokine production, such as IL-2, IL-4, and IFN- $\gamma$ , and promotes T cell proliferation. As reviewed in section 1.2, B7 binding to CD28 also leads to phosphorylation of Lck, leading to downstream activation of the Ras pathway. Induction of the Ras pathway induces an IL-2 autocrine response and further promotes IL-2 gene expression<sup>76,82,86</sup>.

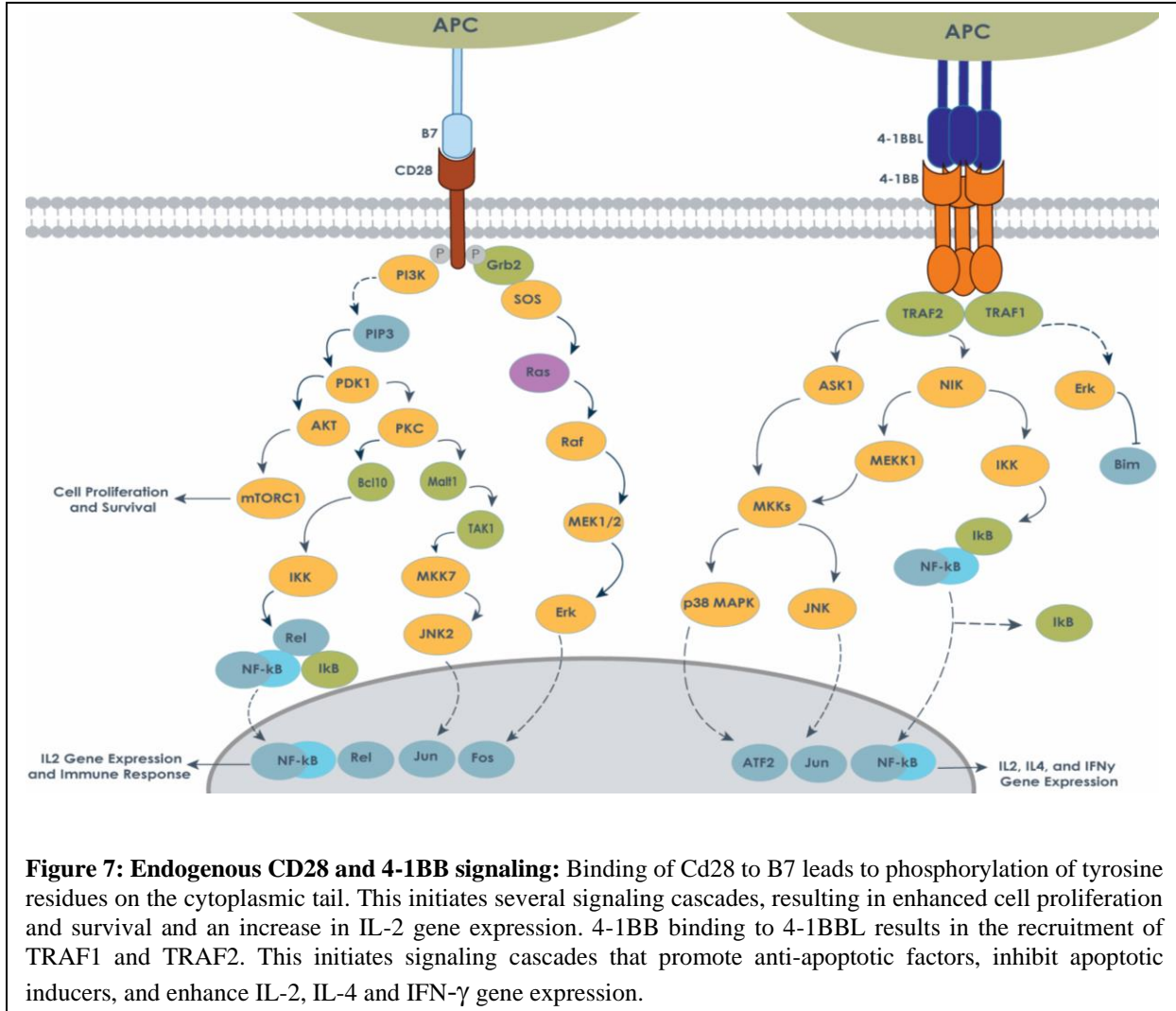
CD28 stimulates factors that are necessary for cell cycle progression and T cell survival. CD28 co-stimulation upregulates Cyclin-D, a cell cycle regulator, driving cell cycle progression to late G1 and S phases<sup>87-90</sup>. As previously stated, CD28 activates the PI3K-AKT pathway, enhancing the expression of transcription factors and transporters that are required for metabolism within the T cell (Figure 7)<sup>88-90</sup>. The PI3K-AKT pathway is also responsible for promoting survival of the T cell by inhibiting tumor suppressor p73 and apoptotic inducers, such as Bcl-2-like protein 11 (BIM), as well as upregulating anti-apoptotic proteins like Bcl-X<sub>L</sub><sup>91-94</sup>.

Regulatory factors can negatively affect the function of CD28 and, subsequently, T cell activation. Cytotoxic T lymphocyte-associated antigen 4 (CTLA4), which is induced upon T cell activation, can downregulate CD28 expression as well as inhibit its function by competing for receptor binding of CD80 and CD86, which are the ligands on which CD28 binds<sup>95-98</sup>. Programmed cell death protein (PD1), induced 24 hours after TCR stimulation, is also able to inhibit CD28 by blocking the CD28/PI3K pathway, leading to T cell exhaustion<sup>99</sup>. These factors could play an essential role in the function of CD28 in second-generation CAR-Ts.

#### **Endogenous 4-1BB:**

4-1BB is a costimulatory receptor that forms a trimeric complex at the surface of the T cell. 4-1BB becomes active upon binding to its ligand, 4-1BBL, which is present on activated dendritic cells, macrophages, and B cells. 4-1BB activation results in downstream signaling that helps to sustain T cell activation after the T cell has been primed<sup>59</sup>. 4-1BB is transiently induced by TCR and CD28 signaling in CD4 and CD8 T cells and by the presence of IL-15 in the absence of antigen stimulation in memory cells<sup>100,101</sup>. Induction of 4-1BB leads to enhanced TCR signaling through phosphorylation of adaptor signaling proteins, SLP-76, and signaling subunits CD3 $\epsilon$  and CD3 $\zeta$ <sup>102</sup>.

Additionally, 4-1BB recruits protein kinases that promote an increase in calcium levels within the cell, assisting with intracellular signaling<sup>59,102</sup>.



**Figure 7: Endogenous CD28 and 4-1BB signaling:** Binding of Cd28 to B7 leads to phosphorylation of tyrosine residues on the cytoplasmic tail. This initiates several signaling cascades, resulting in enhanced cell proliferation and survival and an increase in IL-2 gene expression. 4-1BB binding to 4-1BBL results in the recruitment of TRAF1 and TRAF2. This initiates signaling cascades that promote anti-apoptotic factors, inhibit apoptotic inducers, and enhance IL-2, IL-4 and IFN-γ gene expression.

TNF receptor-associated factors 1, 2, and 3 mediate 4-1BB signaling and are responsible for the activation of ERK and MAPK pathways and downregulate pro-apoptotic proteins via regulation of the NF-κB pathway (Figure 7)<sup>59,103–105</sup>. The ERK pathway is responsible for moderating a pro-apoptotic transcription factor, BIM, which is critical for T cell survival<sup>106</sup>. Activation of the MAPK pathway induces cytokine production and promotes Th1 T cell differentiation, while regulation of

NF- $\kappa$ B helps to moderate T cell activation by stimulating expression of the gene responsible for producing IL-2<sup>16,59,104,107,108</sup>.

4-1BB signaling can enhance T cell proliferation, cell cycle progression, cytokine secretion, and cytolytic potential<sup>102,109</sup>. Additionally, 4-1BB signaling has a positive effect on the differentiation of the memory CD8 T cell pool, which is responsible for driving T cell expansion upon exposure to a secondary challenge<sup>59,110,111</sup>. 4-1BB enhances cytokine secretion via the MAPK pathway, leading to increased production of IFN- $\gamma$ , IL-2, and IL-4. Finally, 4-1BB signaling can rescue T cells from anergy and exhaustion even after the downregulation of CD28<sup>59,112</sup>. The properties associated with 4-1BB co-stimulation have prompted researchers to commonly utilize 4-1BB as a co-stimulatory receptor in the second generation CAR-T.

#### **CD28 or 4-1BB use in CAR-T cells**

The biological properties of co-stimulatory domains CD28 and 4-1BB make them desirable candidates for incorporation into second-generation CAR-Ts. However, how these domains will function in the context of a CAR-T cell must be considered. The altered structure of the co-stimulatory domains within the CAR-T could affect the function of the domains. There are both temporal and spatial differences between the endogenous domains and the domains within the CAR-T. For example, endogenous 4-1BB is a monomer that trimerizes upon T cell activation. However, in the context of the CAR-T, 4-1BB is a forced dimer<sup>59</sup>. Another variation is the expression of the co-stimulatory domain, as co-stimulatory domains within the CAR-T are constitutively expressed. Additionally, the function of the domains could vary due to the covalent linkage of the co-stimulatory domain and the activating domain. Finally, the CAR-T function does not wholly rely on the cytoplasmic signaling domains and the nature of the immunological synapse

that second-generation CAR-T's form with the antigen may not be the same as the endogenous TCR synapse.

Signaling pathways that are activated by endogenous CD28 and 4-1BB are found to be induced in the CD28 and 4-1BB second-generation CAR-T's, respectively. CAR-T's containing either CD28 or 4-1BB co-stimulatory domains were able to induce signaling pathways such as NF- $\kappa$ B, AKT, and ERK<sup>113-116</sup>. Additionally, transcription factors that were induced in endogenous T cells by the co-stimulatory molecules were also found to be induced in second-generation CAR-Ts<sup>59,116</sup>. However, second-generation CD28 CAR-Ts (28- $\zeta$ -CAR) were found to activate the PI3K pathway, which is one of the pathways responsible for cell proliferation, more consistently than second-generation 4-1BB CARs (BB- $\zeta$ -CAR)<sup>59,113,115-117</sup>.

One critical factor in determining the efficacy of co-stimulatory domains within the second generation CAR-T is the secretion of cytokines. Both CD28 and BB- $\zeta$ -CAR-T's were able to secrete higher levels of cytokines than relative  $\zeta$ -CAR-T's<sup>59,118-120</sup>. Th1 cytokines, such as IL-2, IFN- $\gamma$ , TNF, and GM-CSF, and Th2 cytokines, such as IL-4 and IL-10, were all induced by these second-generation CAR-T's<sup>59,71,113,114,117,121-124</sup>. However, the addition of a CD28 co-stimulatory domain induced these cytokines faster than the addition of 4-1BB, while 4-1BB had a more delayed response.

Perhaps the most critical cytokine for T cell function and adoptive cellular therapy is IL-2. As previously stated, IL-2 promotes CAR-T cell proliferation and sustains effector function. Additionally, IL-2 affects neighboring cells, such as NK cells and T<sub>regs</sub><sup>59,125-128</sup>. T<sub>regs</sub>, an immunosuppressive population of T cells, are undesirable in immunotherapy due to their propensity to attenuate the effector T cell response<sup>126</sup>. 28- $\zeta$ -CAR-T's are less sensitive than  $\zeta$ -CAR-

T's to T<sub>reg</sub> inhibition due to IL-10 and TGFB secretion<sup>126</sup>. The presence of IL-2 through constitutive CAR-T cell activity is also able to partially restore the cytolytic function of 28- $\zeta$ -CAR-T's in the presence of Tregs without affecting their ability to proliferate and secrete IFN- $\gamma$ <sup>59,127</sup>.

The antigen specificity of second-generation CAR-T's is critical in determining the safety and efficacy of the CAR-T. Some second-generation CAR-T's were found to induce tonic signaling or constitutive activity<sup>59,71,129</sup>. Tonic signaling could be possible in CD28 CARs if the scFv fragments were to oligomerize and induce CAR clustering, therefore increasing downstream signaling<sup>59,116</sup>. BB- $\zeta$ -CAR-T's were found to increase proliferation *in vitro* and showed enhanced survival *in vivo* in the absence of an antigen. Proliferation and survival of these CAR-T cells, even with a lack of stimulation could be due to the forced dimeric structure of the co-stimulatory domain, as native 4-1BB requires a conformational change within the 4-1BB domain to initiate the signaling cascade<sup>59,130</sup>. However, properly designing the scFV and hinge regions can circumvent these adverse effects. Well-designed second-generation CARs should be able to avoid exhaustion or anergy due to structural problems while retaining their antigen-dependent function.

### **Third-generation CARs**

Third generation CAR-T's have the same basic design as second-generation CAR-T's but with the addition of a second co-stimulatory domain (Figure 6)<sup>113</sup>. 28- $\zeta$ -CAR-T's are attributed to rapid T cell expansion and lead to a robust T cell response shortly after treatment, while 4-1BB promotes T cell persistence, potentially contributing to protection from relapse<sup>129</sup>. Third generation CAR-T's incorporate both CD28 and 4-1BB in an attempt to combine the positive effects attributed to these co-stimulatory domains.

The combined co-stimulatory domains in the third generation CAR must be able to retain their original function to be superior to second-generation CARs. As previously stated, the addition of a CD28 co-stimulatory domain enhances T cell proliferation and persistence, enhances the CAR's ability to secrete IFN- $\gamma$ , and initiates a signaling cascade promoting the differentiation and survival of effector T cells<sup>130-133</sup>. Incorporation of a 4-1BB co-stimulatory domain reduces exhaustion, allowing the CAR-T to persist longer than 28- $\zeta$ -CAR-T's, and promotes survival and differentiation of T memory cells<sup>131,134,135</sup>. In a comparison of the second generation and third generation CAR-T's, the addition of a second co-stimulatory domain did not negatively affect the properties of either co-stimulatory domain<sup>132</sup>. Importantly, third-generation CAR's exhibited enhanced expansion when compared to their second-generation counterparts, while maintaining their ability to support long-term persistence<sup>59,132</sup>.

Third-generation CARs designed to include both CD28 and 4-1BB were found to enhance both pathways stimulated by CD28 and 4-1BB, leading to enhanced proliferation, expansion, and persistence<sup>132,136</sup>. Third generation CAR-T's were found to have higher levels of phosphorylation status upon binding to their target antigen than their second-generation counterparts, indicating that the intracellular signaling is increased in third-generation CAR-T's<sup>132,136</sup>. The enhanced phosphorylation and increase in intracellular signaling allow the third generation CAR-T's to have greater expansion and supports differentiation into memory subsets<sup>132,137</sup>.

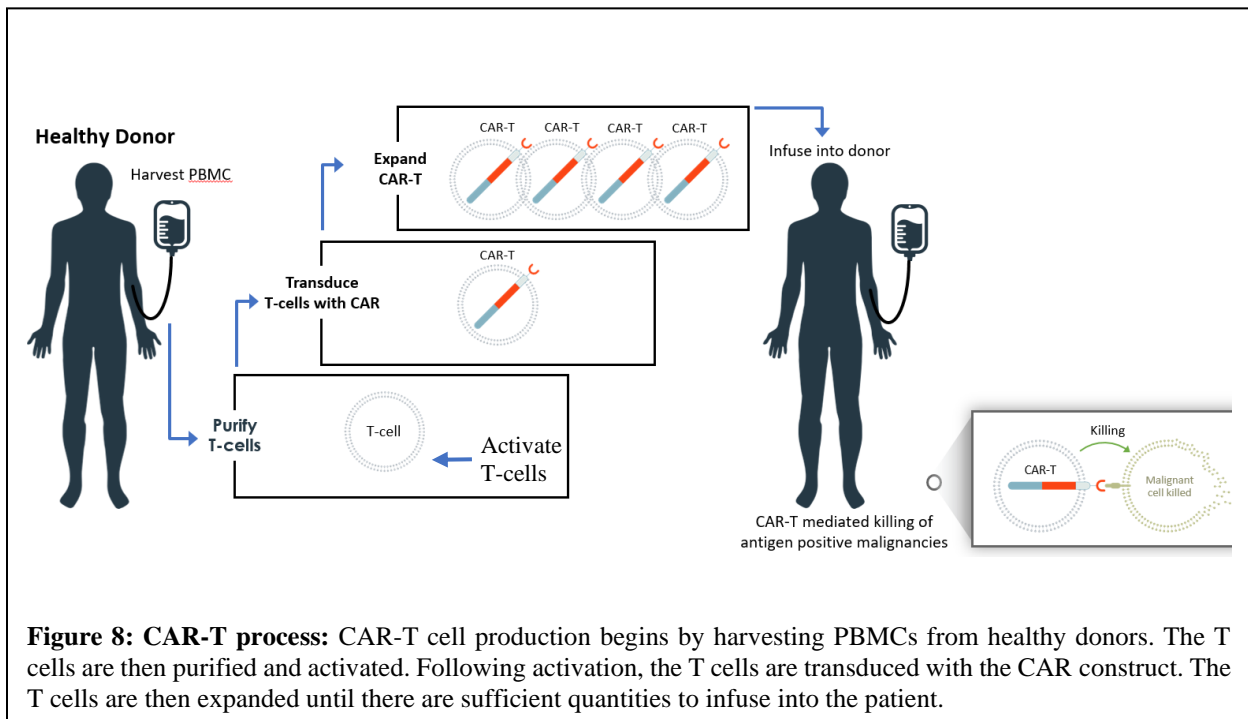
Overall, the incorporation of two co-stimulatory domains into the CAR-T design enhances the efficacy of the CAR-T. The design of third generation CAR-Ts alleviates the inadequacies associated with having a single co-stimulatory domain by mitigating obstacles such as ligand-independent tonic signaling and T cell exhaustion. However, in the context of CAR-T cell therapy for B cell malignancies, third-generation CAR-T cells may lead to increased B cell aplasia,



resulting in an increased risk for infection in the patients<sup>138</sup>. Despite this, the favorable properties of third-generation CAR-T's allow them to be a promising alternative to previous CAR generations.

## 2.2 Targets and clinical studies

### 2.2.1 CAR-T manufacturing



**Figure 8: CAR-T process:** CAR-T cell production begins by harvesting PBMCs from healthy donors. The T cells are then purified and activated. Following activation, the T cells are transduced with the CAR construct. The T cells are then expanded until there are sufficient quantities to infuse into the patient.

CAR-T cell manufacturing has five primary phases. First, peripheral blood mononuclear cells (PBMCs) are collected from the patients. The T lymphocytes are then isolated from the PBMCs, and activation beads are added to provide the stimulation necessary for expansion. Genetic modification methods are used to express the CAR construct within the T cell. The manufactured CAR-T cells are then expanded until there are sufficient CAR-T cells for treatment (Figure 8).

## **T cell preparation**

To date, the majority of CAR-T cell trials have used the patient's T cells for the generation of CAR-T (allogenic products will be discussed in section 2.2.4). Leukapheresis is used to collect the patient's peripheral blood mononuclear cells in various ways depending on the protocols of the centers (Figure 8)<sup>139–153</sup>. Isolation of the lymphocytes occurs following the removal of the red blood cells and monocytes. Counterflow centrifugal elutriation and magnetic bead isolation systems allow for the enrichment of T lymphocytes from the leukapheresis product. Clinical-grade machines such as the Clinimacs plus and Prodigy use magnetic bead isolation to allow for the enrichment of specific T cell subsets<sup>154</sup>.

T cells have to be stimulated *ex vivo* through sustained and adequate activation to generate sufficient CAR-T numbers for infusion into the patient<sup>154</sup>. Activation is commonly performed using beads coated with anti-CD3/antiCD28 monoclonal antibodies. These beads act as artificial antigen-presenting cells and activate the cells by inducing signaling through the TCR and CD28 costimulatory pathways (as described in section 1.2)<sup>151,153,155–158</sup>. Two types of beads that are commonly used to stimulate T cells are antibody-coated magnetic beads and antibody-coated nanobeads. The magnetic beads must be removed at the end of the manufacturing process via magnetic separation, while the nanobeads are biodegradable and do not have to be removed<sup>151,157</sup>.

## **Genetic modification**

CAR-T therapies rely on the ability to stably express the CAR on the T cell surface. Primarily three types of stable gene expression vectors are used in manufacturing CAR-T cells: retroviral vectors, lentiviral vectors, and transposon/transposase systems<sup>151,158</sup>.

### **Gamma-retroviral vectors**

Gamma-retroviral vectors were the first type of stable gene expression vectors used to create a CAR-T that stably expressed a CD19 CAR<sup>151,159</sup>. These vectors can transduce dividing cells, demonstrating another reason T cell activation is important prior to transduction<sup>160</sup>. Retroviral vectors allow for high gene expression and are available in multiple stable packaging cell lines, allowing the generation of a cell line that produces CAR virus<sup>151,161,162</sup>. Long term follow-up studies of these vectors show a high safety profile<sup>163</sup>. By using a large-scale bioreactor, retroviral vectors also can be generated in cGMP grade vector stocks in high enough quantities to support phase three clinical studies<sup>151,164</sup>.

### **Lentiviral vectors**

Lentiviral stable gene expression vectors can transduce dividing and non-dividing cells; however the integration of lentiviral vectors into dividing cells is much more efficient than integration into non-dividing cells<sup>165</sup>. These types of expression vectors have high gene transfer efficiency, drive stable levels of CAR expression, and have a safer genomic integration profile<sup>160</sup>. The third-generation lentiviral vectors split the viral genome into three separate plasmids<sup>166</sup>. Separating the viral genome into three different plasmids results in a safer vector that has a low possibility of viral recombination. Viral recombination could result in the production of viral particles, which would lead to the patient producing virus.

One of the primary obstacles presented by lentiviral vectors is their ability to be made in large quantities. Lentiviral vectors require the packaging cell line to be transiently transfected with multiple plasmids<sup>166</sup>. Transient transfection with multiple plasmids is different than gamma-retroviral transfection, which uses a cell line with a stable transfection of the core packaging plasmids<sup>164,166</sup>. The multi-plasmid transient transfection protocol introduces variations that can be

problematic in scaling up lentiviral production for clinical use. Recent research has utilized a Cre recombinase-mediated insertion of the viral plasmids into a constitutively expressed locus on the packaging cells<sup>166,167</sup>. Cre recombinase-mediated insertion could allow for the production of stable packaging cells that can produce lentivirus. Despite these obstacles, lentiviral vectors are one of the most commonly used vehicles for CAR delivery<sup>167</sup>.

### **Transposon/Transposase**

Although there is frequent use of viral gene expression vectors in the clinic, there are still risks associated. Non-viral vector systems, such as transposon/transposase systems, have been designed to overcome the problems of viral vectors. The transposon/transposase system introduces the CAR as a naked DNA plasmid into the T cells via electroporation<sup>151</sup>. These systems have more straightforward manufacturing methods, cost less to produce, and have direct release testing. However, currently, these systems often result in low gene transfer, can be toxic to cells, and can require long culture times<sup>151</sup>. Because of these factors, viral vectors remain more widely used.

A transposon/transposase system, Sleeping Beauty (SB), has been developed to try to overcome some of the obstacles presented by traditional transposon/transposase systems<sup>168</sup>. The SB system can be used in combination with mini-circles, which are supercoiled DNA vectors, offering an alternative source of SB transposons and transposases<sup>169</sup>. Minicircle systems were designed to be more effective and less toxic when compared to conventional naked DNA plasmids. Because the SB transposons are mRNA, they degrade, eliminating the risk of unintentional integration of transposases into the host genome<sup>169</sup>. A head-to-head study of optimized CAR-T's, modified with both viral and non-viral vectors, found that the CAR-T's had the same anti-tumor function and potency both *in vivo* and *in vitro*<sup>170</sup>. Because of these findings, in addition to the fact that viral

vectors are the single most substantial cost of making CAR-T cells, non-viral methods may be used to clear regulatory hurdles and accelerate clinical translation of CAR-T cell trials.

### **CAR-T expansion**

Following T cell transduction, the CAR-T cells must undergo expansion to reach levels sufficient for therapeutic doses (Figure 8). Bioreactors are used to expand large quantities of CAR-T cells in a sterile cell manufacturing facility. GMP clean facilities house bioreactors and other equipment necessary to create the CAR-T cells.

GE bioreactors use a cell bag on a rocking base. The equipment maintains the inflation of the bag while rocking the cells, allowing for rapid gas transfer and mixing. The design of this bioreactor enables automatic cell-feeding and waste removal<sup>151,156,165,171</sup>.

GRex bioreactors use a culture flask that has a gas-permeable membrane, allowing for the cells to grow to a high density without compromising gas exchange. This system provides for a one-time feeding regimen and reduces the volume at the time of harvest. However, expansion kinetics can become unbalanced if the cells are disturbed while in culture. Consequently, the cells cannot undergo testing until the culture is complete<sup>171,172</sup>.

Miltenyi Prodigy bioreactors are an all-in-one, closed CAR-T production system. This system combines a cell washer, magnetic separation column, and cell cultivation device. The Prodigy also supports the lentiviral transduction of T cells. Because of the Prodigy's multi-functionality, the complex multistep CAR-T processing and manufacturing procedures can be automated and decentralized, resulting in the generation of fresh product and eliminating the need to send cells away for lengthy manufacturing procedures<sup>157,173–176</sup>.

## **CAR-T infusion**

The CAR-T is prepared for infusion once it has expanded to sufficient levels (Figure 8)<sup>177-182</sup>. Testing of the safety profile of CAR-T therapy occurs before infusion. Testing includes sterility and mycoplasma checks, analysis of endotoxin levels, and analysis of copies of transgene insertion<sup>155,156,177</sup>. Examination of the percent CD3 positive T cells and the percent CAR positive T cells allows for the assessment of the CAR-T cell purity. Following the safety and purity analysis, the potency of the CAR-T cells can be examined. Potent CAR-T cells are cytotoxic and have high levels of IFN- $\gamma$  secretion<sup>151,183,184</sup>.

### **2.2.1 Clinical Applications**

#### **B cell malignancies**

Preliminary proof-of-concept clinical trials for the treatment of hematologic malignancies were conducted using CAR-T cells targeting B cell-specific antigens. CD20 and CD19 are B cell-specific antigens expressed on the surface of healthy B cells and are overexpressed on malignant B cells<sup>93</sup>. The first CAR-T cell therapies were developed to treat patients with chronic lymphoid leukemia (CLL), B cell acute lymphoblastic leukemia (B-ALL) and B cell lymphoma.

The Press group at the Fred Hutchinson Cancer Research Center in Seattle, Washington, was the first group to use CAR-T cell therapy in patients with hematologic malignancies. This group developed a CD20- $\zeta$ -CAR-T's and delivered it to nine patients who either had follicular lymphoma or mantle cell lymphoma<sup>186</sup>. The use of a  $\zeta$ -CAR-T construct required IL-2 infusions to boost CAR-T cell proliferation. This trial demonstrated the safety of CAR-T cell therapy, but overall, the treatment was ineffective due to a lack of CAR-T cell proliferation and persistence<sup>186</sup>.

The Brenner group at Baylor performed a trial to directly compare the efficacy of first and second-generation CAR constructs. Six patients with relapsed or refractory non-Hodgkin lymphoma received simultaneous infusions of CD19- $\zeta$ -CAR-T cells and second-generation CD19 CAR-T cells containing a CD28 co-stimulation domain (CD19-28- $\zeta$ -CAR). Results of this trial demonstrated that CD28 co-stimulation in the second generation constructs greatly improved the *in vivo* expansion and persistence, leading to better overall clinical efficacy<sup>187</sup>. The researchers also suggested that IL-2 infusions could further promote CAR-T cell persistence<sup>187</sup>. The benefits attributed to co-stimulation provided by the second generation CAR design prompted the use of second-generation CAR-T cells in future clinical trials.

The results reported by Rosenberg group at the NCI confirmed the Baylor group's findings. A single patient who had progressive lymphoma involving all major lymph nodes received 19-28- $\zeta$ -CAR-T cell therapy<sup>159</sup>. Flow cytometry analysis of a cervical lymph node biopsy showed that the patient's follicular lymphoma consistently expressed CD19<sup>188</sup>. 19-28- $\zeta$ -CAR-T cells were generated using the patient's peripheral blood mononuclear cells, and the patient received an infusion of the 19-28- $\zeta$ -CAR-T cells following a lymphodepletion regimen<sup>188</sup>. Following the CAR-T infusions, the patient also received IL-2 every eight hours for a total of eight doses to support CAR-T expansion and persistence<sup>188</sup>. This first patient went into partial remission that lasted 32 weeks<sup>188</sup>. Additionally, the analysis of the patient's bone marrow revealed prolonged B cell depletion<sup>188</sup>. The results of this study were encouraging and indicated the potential use of CD19 CAR-T cells as antigen-specific therapy.

In 2011, the June group at the University of Pennsylvania tested the efficacy of CD19-BB- $\zeta$ -CAR-T cells in three patients with chemotherapy-resistant CLL<sup>3</sup>. Two of the three patients in this trial had complete responses, and the third had a partial response that lasted greater than eight months<sup>3</sup>.

The researchers examined levels of cytokines, chemokines, and other soluble factors for more than 100 days post-treatment to allow for an in-depth analysis of potential toxicities<sup>3</sup>. Cytokine levels associated with the induction of specific immune responses increased in two of the patients, and peaked around day 20, demonstrating that the CAR-T cells were eliciting a cytotoxic response<sup>3</sup>.

The design of the CAR-T in this trial differed from the previous trial due to the incorporation of the 4-1BB co-stimulatory domain versus CD28. The authors hoped that this domain would lead to sustained clinical efficacy and eliminate the need to deliver exogenous cytokines. Cytokine analysis of the patient's serum revealed that the patients did not have elevated levels of IL-2 and TNF- $\alpha$ , which is important because elevated levels of IL-2 have been associated with T<sub>reg</sub> cell suppression of CAR-T's<sup>189</sup>, while TNF- $\alpha$  is associated with cytotoxic storm-related effects<sup>3</sup>. Non-elevated levels of IL-2 and TNF- $\alpha$  could suggest that the 4-1BB CAR-T demonstrate increased efficacy and decreased cytotoxicity relative to CD28 CAR-T's. The June group also examined the phenotypes of the CAR-T cells at various time points following treatment. The researchers found that the persisting CAR-T population consisted of central and effector memory cells. These phenotypes could be associated with prolonged survival of the CAR-T cells as well as prolonged immunosurveillance<sup>3</sup>. The persisting CAR-T cells also retained their ability to kill target cells *in vitro*<sup>3</sup>.

The Sadelain group at Memorial Sloan-Kettering reported on a phase I clinical trial utilizing CAR-T's containing a CD28 co-stimulatory domain for patients with CLL later in 2011<sup>190</sup>. This trial included two separate arms to assess the impact of conditioning on CAR-T cell efficacy. The first arm involved direct CAR-T cell infusions, and the second arm included a lymphocyte depleting regimen followed by CAR-T cell infusions<sup>190</sup>. All of the patients who did not receive a lymphocyte



depleting regimen died of progressive disease<sup>190</sup>. The results varied among the four patients who received a lymphocyte depleting regimen and CAR-T infusions; one patient had a reduction in disease followed by stable disease for six months, while two patients had a stable disease that lasted two to four months. The fourth patient did not respond to treatment and died of progressive disease<sup>190</sup>. The CAR-T's used in this trial were cleared from circulation more rapidly than the 4-1BB CAR-T's used in earlier trials but did not induce complete responses. This trial demonstrated that lymphodepletion regimens could potentially increase the efficacy of CAR-T cell therapies in patients with hematologic malignancies<sup>190</sup>.

The Sadelain group was the first group to report their findings of using CD19 CAR-T's in patients with B-ALL<sup>190</sup>. The researchers found that the CAR-T cells exhibited higher expansion rates from the patient who had B-ALL than the patients with CLL<sup>190</sup>. B cell aplasia was evident in the patient with B-ALL only 48 hours after treatment, meaning that the CAR-T was clearing CD19+ B cells<sup>190</sup>.

Updated results for this study were reported in 2013 after the inclusion of five more relapsed B-ALL patients. These patients had undergone chemotherapy but not a stem cell transplant. Four of the five patients had persistent chemotherapy-refractory disease at the time of the CAR-T infusion. As with previous trials, the patients underwent a lymphodepletion regimen followed by CD19 CAR-T cell infusion. All five of the patients rapidly went into complete remission, independent of the tumor burden at the start of the trial, following CAR-T infusions, demonstrating remarkable clinical efficacy of CAR-T cell therapy against B-ALL<sup>191</sup>.

Despite these promising results, cytokine related toxicities affected the outcome for some of the patients. Patients with higher disease burden at the time of treatment experienced increased

cytokine toxicities<sup>191</sup>. Four of the five patients were able to undergo stem cell transplants following treatment, while the fifth patient underwent lymphotoxic steroid therapy shortly after the CAR-T infusion to lessen the effects of the cytokine toxicities<sup>191</sup>. The four patients that were able to follow up their treatment with a stem cell transplant had not relapsed at the time of the studies publication, while the fifth patient did relapse three months after therapy ended, presumably to the shortened duration of the CAR-T therapy<sup>191</sup>.

This trial was pivotal in the progression of CAR-T therapy. Aggressive, relapsed B-ALL that previously had a statistically dismal outcome was able to be effectively treated by CD-19 CAR-T therapy. The CAR-T treatment provided complete remission to patients who previously would not have been eligible for potentially life-saving stem cell transplants.

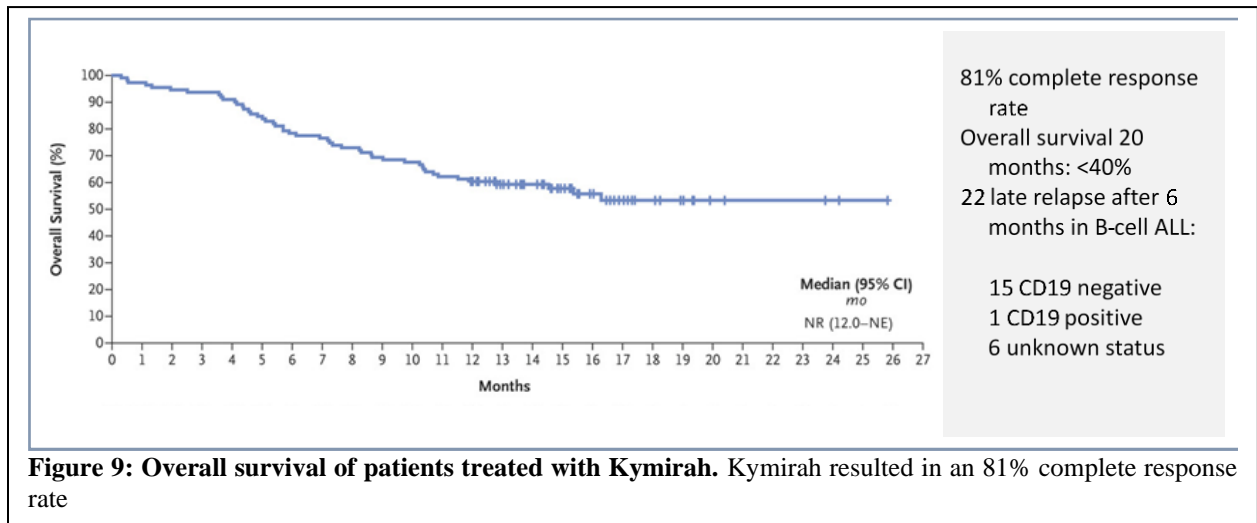
After the success of this trial, Memorial Sloan Kettering Cancer Center (MSKCC) initiated a more extensive phase one clinical trial with 45 patients using their CD19-28- $\zeta$ -CAR-T<sup>192</sup>. Of the 45 patients enrolled, 37 achieved or maintained clinical remission. This more extensive study did not find a significant difference between patients who followed up with a stem cell transplant and those who did not. 80% of the patients were still minimal residual disease negative and maintaining a complete response six months after treatment<sup>192</sup>.

Another larger trial at Fred Hutchinson Cancer Research Center treated 29 adult patients with CD19-BB- $\zeta$ -CAR-T cell therapy. However, the lymphodepletion regimens in this trial differed, leading to variability in the response rates<sup>192</sup>.

Relapsed pediatric B-ALL trials began to follow suit after the success of CAR-T therapy in adults with relapsed B-ALL. UPenn reported on their Juliet trial in which 53 children treated with CD19-BB- $\zeta$ -CAR-T cell therapy (CTL019). 50 of 53 patients achieved or maintained minimal residual

disease negative complete responses. However, 20 patients did later relapse post-CAR-T therapy, with 13 patients having CD19 negative disease<sup>192</sup>.

CTL019 renamed Kymirah, became the first CAR-T approved by the FDA in August of 2017 for the treatment of B-ALL in patients up to 25 years of age<sup>193</sup>. Clinical trials leading up to FDA approval of Kymirah included four phase two trials resulting in 90% of patients having greater than one-year survival, 43% showing a complete response, 33% showing a partial response, and 22% maintaining stable disease (Figure 9)<sup>2,193</sup>. A tally of clinical trials using CD19 CAR-T cells to treat B-ALL can be found in Table 1<sup>2,194,195,195-198</sup>.



The first CAR-T cell study for relapsed refractory diffuse large B cell lymphoma (DLBCL) was conducted by the NCI, using second-generation CD28 CD19 CAR-T cells<sup>199</sup>. Nine patients underwent a lymphodepletion regimen followed by CAR-T infusion. Five patients had complete responses, and two had partial responses. Duration of the responses spanned 38 to 56 months, with some responses ongoing at the time of the studies publication<sup>199</sup>.

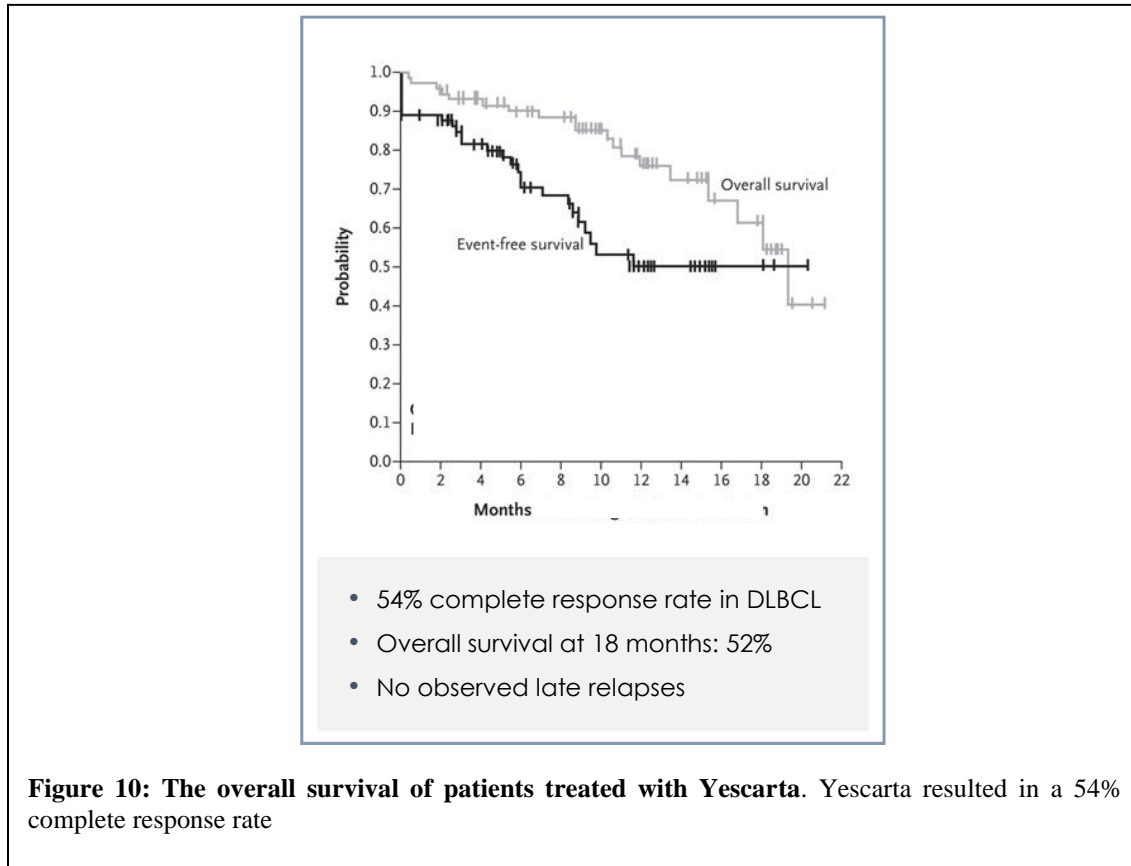
Treating institute	Patient populations	Patient number	Co-Stimulatory Domain	Antigen-recognition moiety	Signalin g domain	Vector	Infused cell dose cells/ kg	Responses
MSKCC	Ped	32	CD28	FMSJ25C1-28z	CD3z	yRV	1-3x10 <sup>6</sup>	Ped: CR: 83% OS: 12.9 mo
UPenn	Pediatric and young adult	59	CD28	FMC63-CD8a	CD3z	LV	10 <sup>7</sup> -10 <sup>8</sup>	Ped and adults: CR: 90%, 6 mo OS:78%, Ped: CR: 81%, 12 mo OS: 76%
NCI	Young adult	38	CD28	FMC63-28z	CD3z	yRV	1-3x10 <sup>6</sup>	Ped: CR: 70%
FHCRC	Adult	29	4-1BB	FMC63-IgG4	CD3z	LV	2x10 <sup>5</sup> , 2x10 <sup>6</sup> , 2x10 <sup>7</sup>	Adults:CR:93%, Ped: CR: 93% 12 mo OS: 69.5%
MDACC	Adult		CD28	FMC63-IgG4	CD3z	SBT	10 <sup>6</sup> -10 <sup>8</sup>	Adults: CR: 69% OS: 63%

**Table 1: CD19 CAR-T cell trials for relapsed B-ALL** <sup>195</sup> Abbreviations: FHCRC, Fred Hutchinson Cancer Research Center; LV, Lentivirus; MDACC, University of Texas MD Anderson Cancer Center; MSKCC, Memorial Sloan Kettering Cancer Center; NCI, National Cancer Institute; OS, overall survival; UPenn, University of Pennsylvania Health System; Ped, pediatric and young adults;  $\gamma$ RV, gamma-retrovirus; SBT, sleeping beauty transposon; CR, complete remission.

Subsequently, several other institutions began using CART19 for the treatment of DLBCL, including the Zuma trial at the NCI, the Transcend trial at Fred Hutchinson Cancer Research Center (34 patients), and the Juliet trial at UPenn (38 patients)<sup>199</sup>. The NCI used a CD19-28- $\zeta$ -CAR-T (KTE-C19) (Zuma Trial), FHCRC used a CD19-BB- $\zeta$ -CAR-T (JCAR017), and UPenn used their 4-1BB construct, Kymirah (Figure 9) (Juliet Trial). The Zuma trial had an overall response rate of 54%, the Transcend trial had an overall response rate of 59%, and the Juliet trial had an overall response rate of 40%<sup>200-202</sup>

KTE-C19 was the first CAR-T used in a multi-center trial to evaluate CAR-T cell therapy for DLBCL. One hundred one patients received KTE-C19 during the phase two portion of this trial. The overall response rate of this trial was 83%, with a complete response rate of 54%. Based on

the positive results of this extensive trial, KTE-C19 was approved by the FDA under the name Yescarta in October of 2017 (Figure 9)<sup>193,199,200</sup>.



Similar results were also found in the trial utilizing JCAR017. This study had the best overall response rate at 75%, with a complete response rate of 55%<sup>199</sup>. However, the six months follow up of this study was lower than the other reviews, with overall response rates of 33% and complete response rates of 29%. Despite the positive results of these trials, there are several challenges associated with CAR-T treatment for B cell malignancies, such as target negative relapse, T cell exhaustion, safety issues, and restricted approval. These challenges will be discussed in sections 2.2.2 and 2.2.3. A full list of clinical trials and results for CD19 CAR-T treatment of B cell lymphomas can be found in Table 2.

Treating institute	Lymphoma subtypes	Patient number	Co-Stimulatory Domain	Antigen-recognition moiety	Signalin g domain	Vector	Infused cell dose cells/ kg	Responses
NCI (KTE-C19)	DLBCL, TFL, PMBCL	101	CD28	FMC63	3z	yRV	2x10 <sup>6</sup>	ORR: 82%, CR: 54%, 12 mo OS: 59%
UPenn (Kymirah)	DLBCL, TFL	111	4-1BB	FMC63	3z	LV	3.1x10 <sup>8</sup>	ORR: 52%, CR: 40%, 12 mo, OS:49%
FHCRC (JCAR017)	DLBCL, TFL	114	4-1BB	FMC63	3z	LV	1x10 <sup>8</sup> or 5x10 <sup>7</sup> x2	ORR: 80%, CR: 59%, 12 mo OS: 63%

**Table 2: CD19 CAR-T cell trials for relapsed B cell Lymphoma** Abbreviations: CR, complete remission; DLBCL, Diffuse Large Cell B Cell Lymphoma; FHCRC, Fred Hutchinson Cancer Research Center; LV, Lentivirus; NCI, National Cancer Institute; ORR, overall response rate; OS, overall survival; UPenn, University of Pennsylvania Health System; Ped, pediatric and young adults; PMBCL, primary mediastinal large B cell lymphoma;  $\gamma$ RV, gamma-retrovirus; SBT, sleeping beauty transposon; TFL, transformed follicular lymphoma.

### Multiple Myeloma (MM):

MM is the second most common hematologic malignancy after non-Hodgkin lymphoma<sup>203,204</sup>.

MM is the malignancy of plasma cells that leads to the accumulation of plasma cells in the bone marrow<sup>205</sup>. Despite the multiple treatment options for MM, the disease remains incurable<sup>205–208</sup>.

Patients with MM commonly develop the drug-resistant disease, leading to untreatable relapsed and/or refractory MM<sup>203</sup>. CAR-T therapy aims to provide MM patients with a new treatment option.

The first step in developing a CAR-T for MM was to identify targetable antigens. As previously discussed, these antigens need to be expressed on the tumor cell but ideally not expressed on healthy tissues. Several surface antigens have been considered for targets in MM, including but not limited to: CD138, k light chain, CD19, and BCMA (Table 3)<sup>204,209–212</sup>.

### CD138:

CD138 is an adhesion molecule that is expressed on normal and malignant plasma cells<sup>204,211</sup>.

Patients with CD138 positive MM are associated with a negative prognosis<sup>204,213</sup>. However,

CD138 is also expressed on epithelial cells found in the liver, skin, and glands<sup>204,211,213–215</sup>. The first clinical trial to treat MM was performed at PLA General Hospital in Beijing<sup>214</sup>. This trial treated five patients with a CD138-BB- $\zeta$ -CAR-T. Four out of five patients achieved stable disease for seven months, with one patient showing a reduction in circulating plasma cells to less than 30% from baseline for 12 weeks<sup>214</sup>. However, the patients were not cured, and the CAR-T cells did not home well to the myeloma lesions<sup>214</sup>.

Surface antigen	Expression in MM cases (%)	Expression in normal hematopoietic cells	Known expression in other tissues
CD138	High expression	Plasma cells	Liver, skin, glandular epithelial cells
k light chain	Expression on k-restricted disease propagating cells	Mature B cells	
CD19	Low expression, possibly on disease propagating cells	B cells	
BCMA	60-100%	Mature B cells, plasma cells	

**Table 3:** Target antigens in clinical trials for MM

### **K light chain:**

The k light chain is associated with a specific subset of MM and was found to be expressed on some disease-propagating myeloma cells<sup>204</sup>. Because of this, researchers Baylor developed an anti-k light chain CAR-T<sup>204,216,217</sup>.

The Baylor group performed a clinical trial that was restricted to patients with k light chain positive disease<sup>216,217</sup>. They treated seven patients with a k light chain-28- $\zeta$ -CAR-T. Four out of seven patients maintained stable disease for 24 months<sup>217</sup>. The CAR-T treatment was well tolerated with minimal toxicities among patients. However, the k light chain is generally secreted rather than retained on the cell surface<sup>217</sup>. The loss of antigen expression on the surface of the MM cell makes targeting the k light chain less efficient than targeting a constitutively expressed antigen.

### **CD19:**

CD19, targeted extensively in B cell malignancies, was considered as a potential target for MM. While CD19 is absent on the majority of plasma cells, research has shown that CD19 can potentially be expressed on disease propagating MM clones<sup>204,210,218</sup>. The University of Pennsylvania conducted a clinical trial using a CD19-BB- $\zeta$ - CAR-T in ten patients. The CAR-T was detectable for up to six weeks in nine out of ten patients. Despite the presence of the CAR-T cells, patients ultimately went on to relapse<sup>218</sup>. However, the researchers did find that in one patient, the CART19 treatment did manage the MM better than any of the patient's other previous therapies<sup>218</sup>.

### **BCMA:**

Potentially one of the best options as a targetable antigen for MM is B cell maturation antigen (BCMA). BCMA belongs to a tumor necrosis factor receptor superfamily. It is expressed on the surface of all mature B cells and plasma cells, including MM cells. Importantly, and likely the reason that it is the current lead antigen for CAR-T therapy, BCMA is absent from other normal tissues<sup>209,219</sup>.

The first clinical trial to target BCMA was done by Ali *et al.* at the National Cancer Institute. They gave 12 patients with chemotherapy-resistant MM BCMA-28- $\zeta$ -CAR-T cells in varying doses<sup>219</sup>. All of the patients responded in varying degrees to treatment. Eight of the patients were able to maintain stable disease for up to 16 weeks. Three patients had a partial response, with one response lasting longer than 26 weeks, while one patient had a complete response lasting 17 weeks<sup>219</sup>.



Treating institute	Antigen	Patient number	Co-Stimulatory Domain	Antigen-recognition moiety	Signaling domain	Vector	Infused cell dose cells/ kg	Responses
PGHB	CD138	5	4-1BB	NK-92	3z	LV	0.44x10 <sup>6</sup> -3.78x10 <sup>6</sup>	SD: 4
Baylor	k light chain	7	CD28	CRL-1758	3z	yRV	2x10 <sup>7</sup> , 1x10 <sup>8</sup> , 2x10 <sup>8</sup>	SD: 4
UPenn	CD19	10	4-1BB	FMC63	3z	LV	1-5x10 <sup>7</sup>	Longer PFS than 1st SCT in 2
NCI	BCMA	26	CD28	11D5-3	3z	yRV	0.3-9x10 <sup>6</sup>	CR: 10 PR:8 SD:1 PD: 1
BBM	BCMA	33	4-1BB	NR, murine	3z	LV	5, 15, 45 and 80x10 <sup>7</sup>	CR: 10 PR: 8 SD: 1 PD: 2
UPenn	BCMA	24	4-1BB	NR, human	3z	LV	1-5x10 <sup>7</sup> or 1-5x10 <sup>8</sup>	CR: 2 PR: 9 SD:5 PD: 3
NLB	BCMA	35	NR	NR	NR	LV	1.5-7x10 <sup>6</sup>	CR:15 PR:20
MSKCC	BCMA	6	4-1BB	NR, human	3z	yRV	1x10 <sup>6</sup> , 15, 45, 80x10 <sup>7</sup>	PR:3 SD:1
FAH	BCMA/CD19	10	CD28/OX40	NR	3z	LV	5-50x10 <sup>6</sup>	CR:2 PR:7

**Table 4: MM clinical trials** Abbreviations: Baylor, Baylor College of Medicine; CR, complete response; BBM, Bluebird Bio multicenter; LV, lentivirus; MSKCC, Memorial Sloan Kettering Cancer Center; NCI, National Cancer Institute; NLB, Nanjing Legend Biotech; PD, progressive disease; PFS, progression-free survival; PGHB, PLA General Hospital, Beijing; PR, partial response; SD, stable disease; UPenn, University of Pennsylvania; yRV, gamma retrovirus

While not as dramatic as the B-ALL patient responses to the CART19, researchers were encouraged by these results and decided to pursue it further. The same group published a more recent study in 2018 with a larger cohort of 16 patients. The patients had an overall response of 81%, with 63% very good partial responses<sup>220</sup>. The event-free survival for this trial was 31 weeks. Patients in this trial showed the elimination of extensive bone marrow myeloma as well as soft-tissue plasmacytomas<sup>220</sup>.

Other studies using second-generation BCMA CAR-T cells are ongoing. Bluebird Biotech reported on treating 21 patients with varying doses of BB-ζ-CAR-T cells<sup>204,221</sup>. Some of the patients receiving smaller doses of CAR-T cells did not respond. However, 10 out of 18 patients

who received a higher dose of CAR-T cells responded to treatment, having a complete response that lasted 40+ weeks<sup>221</sup>. A full list of completed BCMA CAR-T trials and their results can be found in Table 4<sup>204,209,215,216,219–223</sup>. Overall, multiple myeloma patients treated with CAR-T cell therapy respond in varying degrees, from partial responses to complete responses. Since most patients ultimately relapse after CAR-T therapy, efforts are underway to identify and test new targets. One of these is CS1/(SLAMF7)<sup>224</sup>. While no clinical trial data is available, clinical trials are enrolling to test the efficacy of targeting CS1 in patients with multiple myeloma.

### **T cell malignancies**

T cell malignancies include a variety of subgroups of cancers arising from T cells<sup>225</sup>. These cancers can derive from T cell precursors or mature T cells and give rise to T cell lymphoma or T cell leukemia. Current treatment for T cell lymphomas and leukemia include intensive chemotherapy regimens that are not only extremely toxic but are ineffective at inducing and sustaining remission<sup>226–231</sup>. Due to the lack of effective treatments, researchers hoped to translate CAR-T therapy into the T cell malignancy setting.

The development of CAR-T cell therapies for T cell malignancies is associated with many challenges. One of the main obstacles is those targetable antigens present on malignant T cells are also present on CAR-T cells. The presence of the target antigen on the CAR-T cell results in fratricide, or self-killing of the CAR-T cells, preventing the manufacture of sufficient CAR-T cell quantities for infusion into the patient<sup>232–234</sup>. There are two approaches to target T cell cancers; the first method avoids fratricide by creating CAR-T against antigens not expressed on healthy T cells, while the second approach is to suppress the expression of the target antigen on CAR-T such that the CAR-T cell no longer recognizes itself as a target<sup>232,235</sup>.

## **CD5:**

The first published CAR-T cell therapy to target T cell malignancies was performed by Mamonkin et al. at Baylor College of Medicine in 2015<sup>236</sup>. The researchers designed a CAR-T that would target CD5, which is present on about 80% of T cell leukemias and lymphomas<sup>226,236</sup>. CD5 is typically expressed on thymocytes, peripheral T cells, and some B lymphocytes and is rapidly internalized upon binding to an antibody. The internalization of CD5 upon antibody binding has previously been used to deliver a drug to CD5 positive malignancies in the form of antibody-drug conjugates<sup>236</sup>. Mamonkin *et al.* hoped that they could expose the CD5 CAR-T cells to an antibody to cause loss of CD5 expression on the CAR-T cell, leading to a reduction in fratricide. However, even with extremely high doses of CAR-T cells ( $2 \times 10^7$  per mouse), this CAR-T cell therapy was only able to temporarily induce remission in mouse models. Despite this, there is currently a clinical trial enrolling patients with refractory or relapsed T-ALL and T cell lymphoma to test the efficacy of the CD5 CAR-T cell therapy<sup>237</sup>.

## **CD7:**

Another widespread target antigen for T cell malignancies CD7, which is found on more than 95% of lymphoblastic leukemias and lymphomas, as well as some peripheral T cell lymphomas<sup>226</sup>. CD7 is a transmembrane glycoprotein that is expressed on most peripheral T cells, NK cells, and their precursors<sup>226</sup>. CD7 expression on the CAR-T cell must be disrupted to overcome fratricide. Three groups published back-to-back on different methods of creating CART7 cell therapy. The first group used CRISPR/cas9 technology to gene edit CD7 from the CAR-T cell<sup>238</sup>. Fratricide was effectively prevented by the deletion of CD7 from the surface of the CAR-T<sup>238</sup>. As with the CD5 CAR-T, preliminary results suggested that the CD7 CAR-T could slow the progression of T-ALL

in xenograft mouse models<sup>238</sup>. A clinical trial is preparing to test the efficacy of this CD7 CAR-T in patients with high-risk T cell malignancies<sup>239</sup>.

The second group designed a fratricide-resistant CAR-T by developing a protein expression blocker (PEBL)<sup>240</sup>. The PEBL is essentially an anti-CD7 scFv bound to an intracellular retention domain<sup>240</sup>. The addition of the PEBL effectively prevented fratricide by preventing the expression of CD7 on the surface of the CAR-T cell. The PEBL-transduced CART7 cells were able to avert T-ALL progression in preliminary xenograft mouse models effectively.

However, a critical aspect of the CAR-T cell design was not taken into consideration in the first two experiments. Harvesting and creating CAR-T cells from a patient with T cell malignancies could result in contamination from malignant cells, as it is functionally impossible to isolate the healthy T cell population from the malignant T cell population during CAR-T manufacturing<sup>241–246</sup>. Because of this, CAR-T cells must be derived from allogeneic donor T cells rather than the patients. Deriving CAR-T cells from a source other than the patient could result in graft-versus-host disease (GvHD), a life-threatening condition that occurs when the graft (in this case, the CAR-T cells) attacks the hosts' tissues<sup>232</sup>.

The third group to develop a CD7 CAR-T considered the effects of GvHD. Cooper *et al.* found that CRISPR/cas9 technology could be used to delete not only CD7 but also the T cell receptor (TCR) from the CAR-T cell<sup>232</sup>. The deletion of the TCR prevented the development of GvHD in patient-derived xenograft T-ALL models<sup>232</sup>. By deleting the TCR, our group was able to develop a “universal” allogenic CAR-T (UCART7) that prevented both fratricide and GvHD while enabling targeting of CD7+ T cell malignancies. UCART7 was effective in preventing the progression of T-ALL and prolonged survival in preliminary xenograft mouse experiments<sup>232</sup>.

While these studies have been the most successful published CAR-T cell developments for T cell malignancies, other groups have tried to develop CAR-T cells that target more restricted antigens.

A complete table of target antigens can be found in Table 5<sup>226</sup>.

Antigen	Frequency in T-ALL	Frequency in TCL	Normal tissue expression	Clinical trial status
CD5	90%	85% (PTCL), 95% (AITL), 26-32% (ALCL), 36% (NK-T), 85% (ATLL), 91% (CTCL)	T cells, thymocytes, B-1 cells	Recruiting
CD7	>95%	50% (PTCL), 57% (AITL), 32-54% (ALCL), 79% (NK-T), 25% (ATLL), 18% (CTCL)	T cells, thymocytes, NK cells	Not yet recruiting
CD3	33%	60-66% (PTCL), 71% (AITL), 32-40% (ALCL), 36% (NK-T), 80% (ATLL), 91% (CTCL)	Mature T cells	
CD30	17%	16% (PTCL), 32-50% (AITL), 93% (ALCL), 64% (NK-T), 39% (ATLL), 18% (CTCL)	Activated T and B cells	Recruiting
TCR (TRBC1)	7-11%	27% (PTCL), 34% (AITL), 25% (ALCL)	~35% of T cells	Recruiting
CCR4	0%	34% (PTCL), 88% (ATLL), 31-100% (CTCL)	Tregs, Th2 and Th17 cells, platelets, kidney	
CD4	12%	60% (PTCL), 86% (ALCL), 29% (NK-T), 94% (ATLL), 92% (CTCL)	CD4+ T cells, some monocytes and dendritic cells	
CD37	0%	82%	Mature B cells, low levels in plasma and dendritic cells	

**Table 5:** Target antigens for T cell malignancies

## 2.2.2 CAR-T cell therapy toxicities

CAR-T cell therapies have demonstrated potent anti-tumor effects in some clinical models. However, as CAR-T use in the clinic become more common, toxicities associated with CAR-T cell therapies have emerged. These toxicities include cytokine release syndrome (CRS), CAR-T cell-related encephalopathy syndrome (CRES), haemophagocytic lymphohistiocytosis (macrophage-activation syndrome) (HLH), and on-target off-tumor effects (Figure 9).

## Cytokine release syndrome

CRS is a condition that is associated with CAR-T cell therapy. The frequency of CRS became apparent after larger cohorts participated in clinical trials (2.2.1)<sup>190,247–251</sup>. The incidence rate of CRS varies with the disease type treated with CAR-T cell therapy: CLL-38.8%, B-ALL 29.3%, NHL, 19.8%<sup>252</sup>. CRS is a massive, rapid release of cytokines from immune cells into the bloodstream. Upon activation, the CAR-T cells, as well as monocytes, macrophages, and dendritic cells, excessively release cytokines and chemokines causing systemic reactions. Some of these secreted factors include IL-2, IL-6, IL-6ra, and GM-CSF<sup>253–256</sup>.

CRS typically manifests within five days of CAR-T cell infusion. Symptoms include high fever, hypotension, hypoxia, and multi-organ failure. CRS can affect the respiratory, gastrointestinal, hepatic, and renal systems<sup>253,253–258</sup>. CRS is manageable in most patients but requires hospitalization, strict monitoring, and treatments in intensive care facilities<sup>259</sup>. Fever could also indicate infections that could be deadly due to elevated systemic inflammation<sup>260</sup>. Patients at an increased risk of developing CRS tend to have a higher disease burden. However, this correlation is not always predicative<sup>257</sup>. Predictive biomarkers can vary for the type of CAR-T product used, but elevated levels of IL-6, soluble gp130, IFN- $\gamma$ , IL-15, IL-8, and IL-10, could suggest potential CRS development<sup>259</sup>. Additionally, different CAR-T products induce CRS to varying degrees<sup>259</sup>. Because of this, there are unknown risks associated with the clinical development of CAR-T cell therapies against new targets.

IL-6, IL-6ra, and gp130 are associated with an increased risk of CRS development<sup>150,260–262</sup>. When IL-6 binds to IL-6ra, the complex can bind to membrane-bound gp130, elevating IL-6 levels through a process known as trans-activation<sup>263</sup>. The formation of this complex leads to activation of the JAK-STAT signaling pathway, which mediates pro-inflammatory effects<sup>264</sup>.

There are three treatment options available to mediate CRS. Tocilizumab, a humanized monoclonal antibody against IL-6R that is currently used to treat rheumatoid arthritis, is FDA approved for the treatment of CRS following CAR-T therapy<sup>253,254,261,262,265,266</sup>. Siltuximab, a monoclonal antibody against IL-6, can also be used to mitigate the effects of CRS<sup>267</sup>. Both of these monoclonal antibodies work by blocking the effects of IL-6. These treatments are used in low-grade CRS cases and do not impact the anti-tumor effects of CAR-T cell therapy. Corticosteroids can be used to treat high-grade CRS, but have the potential to reduce the anti-tumor effects of CAR-T cell therapy<sup>253–255,268–270</sup>. Rapid reversal of CRS symptoms can occur if the patients are treated appropriately<sup>253–255,257,262,271–274</sup>.

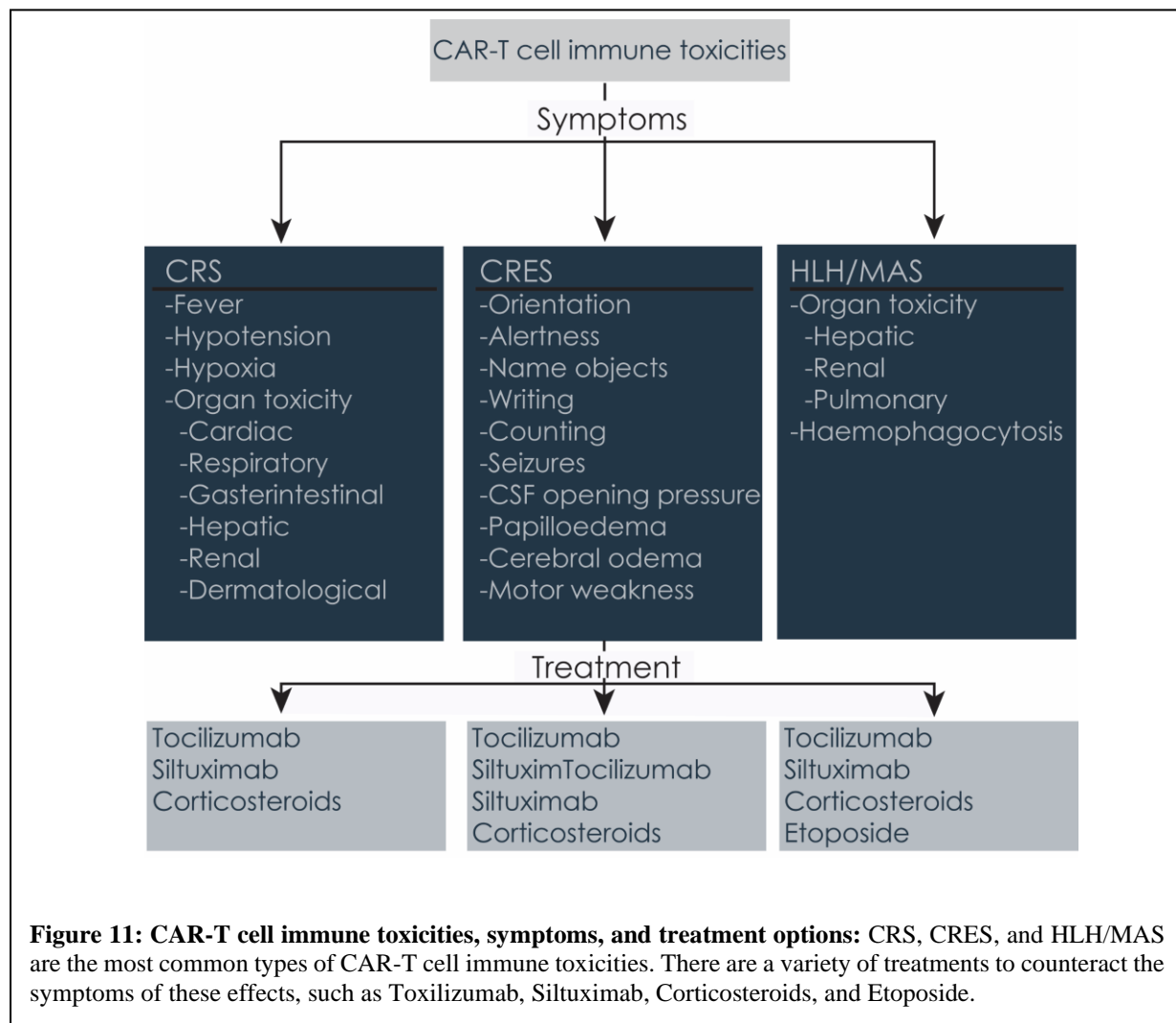
### **CAR-T cell-related encephalopathy syndrome**

CAR-T cell-related toxic encephalopathy syndrome (CRES) is associated with severe immune activation, lymphohistiocytic tissue infiltration, and immune-mediated multi-organ failure. Symptoms of CRES commonly include confusion, delirium, seizures, and cerebral oedema<sup>150,250,251,275–280</sup>. Patients with CRES often have a loss of attention, changes in language, and impaired motor skills.

The manifestation of CRES occurs in two phases. The first phase is associated with high fever and CRS-like symptoms within five days of CAR-T cell infusion, while the second phase of CRES usually occurs once the first phase has passed, or beyond five days. The second phase can present as delayed neurotoxicity up to three or four weeks following treatment<sup>259</sup>. CRES generally only lasts two to four days but can vary from several hours to weeks<sup>259</sup>.

There are three main mechanisms behind CRES development. The first occurs when IL-6 and IL-15 passively diffuse through the blood-brain barrier (BBB). High levels of these cytokines in serum

are correlated with increased severity of neurotoxicities<sup>272,273</sup>. The second mechanism behind CRES development occurs when T cell traffic into the central nervous system<sup>256,261,272,281</sup>. In some patients with CRES, CAR-T cells are detected in the cerebral spinal fluid in the absence of malignancies within the central nervous system. Additionally, these patients also have higher levels of protein in their cerebral spinal fluid, indicating that there is a disruption within the BBB<sup>278,279</sup>. The final proposed mechanism is that organ dysfunction, such as hepatic and renal system changes as well as hypoxia and infection, can contribute to the development of CRES (Figure 10)<sup>259</sup>.





The anti-IL-6 treatments used to treat CRS can be used to remedy the effects of phase one CRES, while corticosteroids are used to treat phase two CRES<sup>256</sup>. The ability of anti-IL-6 to reverse the effects of CRES suggests that the BBB is more permeable when the patient is experiencing CRS<sup>259</sup>. The increased permeability of the BBB during phase 1 of CRES enables the increased diffusion of anti-IL-6 drugs. Short durations of corticosteroid administration allow for the CRES to be resolved without inhibiting the anti-tumor effects of the CAR-T cell therapy (Figure 10)<sup>259</sup>.

Fatalities associated with CRES are rare. However, five CRES-related deaths resulting 24 hours after CAR-T infusion were associated with a multi-center trial using JCAR015, a CART19 used to target B cell malignancies. Patients who have severe CRES show endothelial activation, intravascular coagulation, capillary leak, and increased BBB permeability<sup>282</sup>. The increased BBB permeability can result in systemic cytokine infiltration into the brain<sup>282</sup>. One of these cytokines, IFN- $\gamma$ , induces brain vascular pericyte stress resulting in fatal toxicities<sup>282</sup>.

### **Hemophagocytic lymphohistiocytosis/macrophage activation syndrome**

Haemophagocytic lymphohistiocytosis (HLH), also known as macrophage activation syndrome (MAS), occurs when there is severe immune activation, lymphohistiocytic tissue infiltration, and immune-mediated multi-organ failure<sup>283,284</sup>. Manifestations of HLH are similar to that of CRS, which could suggest that these syndromes belong on the same spectrum of systemic hyper-inflammatory disorders (Figure 10)<sup>255,257,258,283,283–285</sup>. HLH is rare, occurring in about 1% of patients, but has a high mortality rate if it is not treated quickly<sup>259</sup>. Because symptoms of HLH are similar to that of low-grade CRS and advanced stages of hematologic malignancies in the absence of CAR-T therapy, HLH can be hard to diagnose<sup>286,287</sup>.

The primary goal in treating HLH is to suppress over-active CD8 T cells and macrophages<sup>288</sup>. Despite the differences between HLH and CRS, suspected HLH can be treated as though it were CRS. If this treatment does not resolve HLH, patients can be treated with etoposide, which is the preferred treatment for HLH, and rapid initiation of therapy is critical due to the high risk for death<sup>286-288</sup>.

### **On-target off-tumor effects**

On-target off-tumor effects are essential to consider when developing any CAR-T cell, even if the targeted antigen is lineage-specific, like the CART19. In the context of CART19 therapy, the CAR-T cells target CD19, which is present on both normal and malignant B cells. The shared expression of CD19 across B cell populations results in B cell aplasia<sup>188,281</sup> (Figure 10). Patients with B cell aplasia require intermittent infusions of immunoglobulins to prevent infections and infection-associated complications<sup>188,248</sup>. As previously stated, CART19 treatment can also target pericytes in the brain, leading to severe neurotoxicities<sup>282</sup>.

In the context of renal cell carcinoma, carboxyanhydrase-IX-specific CAR-T cells reacted to shared antigens present on the duct epithelium<sup>289,290</sup>. Damage of these tissues resulted in the immediate release of liver enzymes into the blood<sup>289</sup>. To circumvent this, the researchers proposed delivering monoclonal antibodies to block carboxyanhydrase-IX antigen sites within the liver<sup>289</sup>. While monoclonal antibodies could prevent the antigen sites present on healthy tissues from being targeted by CAR-T cells, there is also the potential for this to reduce the anti-tumor effects of CAR-T cell therapy.

Metastatic colorectal patients who received carcinoembryonic antigen (CEA)-specific CAR-T cell therapy experienced severe on-target off-tumor effects<sup>291</sup>. Patients experienced severe, transient

inflammatory colitis due to CEA present on healthy colonic tissue<sup>291</sup>. The researchers proposed that the transient nature of the colitis was due to the CAR-T cells in that area became quiescent over time<sup>291</sup>. The recommended treatment mechanisms for the inflammatory colitis was to reduce the load of commensal flora or to apply local steroids to reduce the T cell activity within the colon<sup>291</sup>.

While some on-target off-tumor effects can be managed, others have fatal results. In a trial using a Her2-neu-specific CAR-T, a patient experienced rapid respiratory failure and multi-organ dysfunction resulting in death<sup>292</sup>. This patient began experiencing respiratory distress only 15 minutes after the CAR-T cell infusion<sup>292</sup>. In this case, the CAR-T cells localized to the lungs, where there were low levels of the target antigen present on normal tissue<sup>292</sup>. The CAR-T cells then reacted to the lung tissues and produced a CRS-like response. Despite the clinical intervention, the patient died within five days of treatment<sup>292</sup>.

### **2.2.3 Mechanisms of resistance**

Due to the increasing number of patients who receive CAR-T cell therapy and extensive follow-up studies, more data is becoming available for therapy resistance. In the context of CD19 CAR-T treatments, approximately 10-20% of patients will fail to go into remission, while 30-50% of patients who achieve remission will generally relapse within one year of treatment<sup>2,293</sup>. Mechanisms of resistance can include incomplete response to the CAR-T cell therapy, often due to clonal expansion of a target-negative tumor cell. The relapsed disease can be either antigen-positive or antigen-negative.

## Antigen-positive relapse

Antigen-positive relapse is thought to be associated with insufficient CAR-T cell persistence or B cell aplasia resulting in loss of active CAR-T mediated leukemia surveillance<sup>294</sup>. CAR-T cell persistence is essential in continuing surveillance, and increased endurance is vital for durable remission<sup>293</sup>. Several factors can contribute to CAR-T cell persistence, including initial T cell quality, phenotype, and proportions of CD4 to CD8 positive T cells<sup>19,139,295,296</sup>. Initial T cell quality can vary from patient to patient, often dependent on the patient's prior treatments and the type of tumor that the patient has<sup>295</sup>. In addition to the patient-dependent T cell qualities, researchers have demonstrated that shifting the phenotypic ratio from effector to central memory or stem cell-like memory can enhance therapeutic responses and prolong CAR-T cell persistence<sup>19</sup>.

The CAR-T cell design is thought to play a role in persistence, and some researchers have suggested that varying the construct design can improve the durability of remission. However, while CAR-T cells containing a 4-1BB co-stimulatory domain persist longer than CAR-T cells with a CD28 co-stimulatory domain, the overall durability of remission is very similar in the context of B cell malignancies. This suggests that the relevance of CAR-T persistence could depend on the cancer type<sup>59,139,250,272,294,296</sup>.

Antigen-positive relapse offers the opportunity to re-treat the patient with CAR-T cell therapy. Several trials have attempted to re-infuse CAR-T cells into relapsed patients, but these showed modest to no clinical benefit<sup>150,272,294,297-299</sup>. One study found that only one out of eight patients had a response to their re-infusion<sup>297</sup>. Of those ten patients, eight lacked CAR-T cell persistence, and the other two lacked significant CAR-T cell re-expansion<sup>297</sup>.

Another strategy to re-treat CAR-T cells consisted of repeat re-infusions to try to combat the early lack of CAR-T persistence. In initial experiments, the CAR-T cells were unable to re-expand due to immune-mediated rejection of the CAR-T cells upon repeat dosing<sup>251</sup>. Optimized re-infusion protocols later consisted of intensified lymphodepletion regimens, which improved the expansion rates and persistence of the CAR-T cells. Future multiple-infusion strategies could include re-treating antigen-positive relapse with varying CAR constructs to prevent rejection<sup>299,300</sup>.

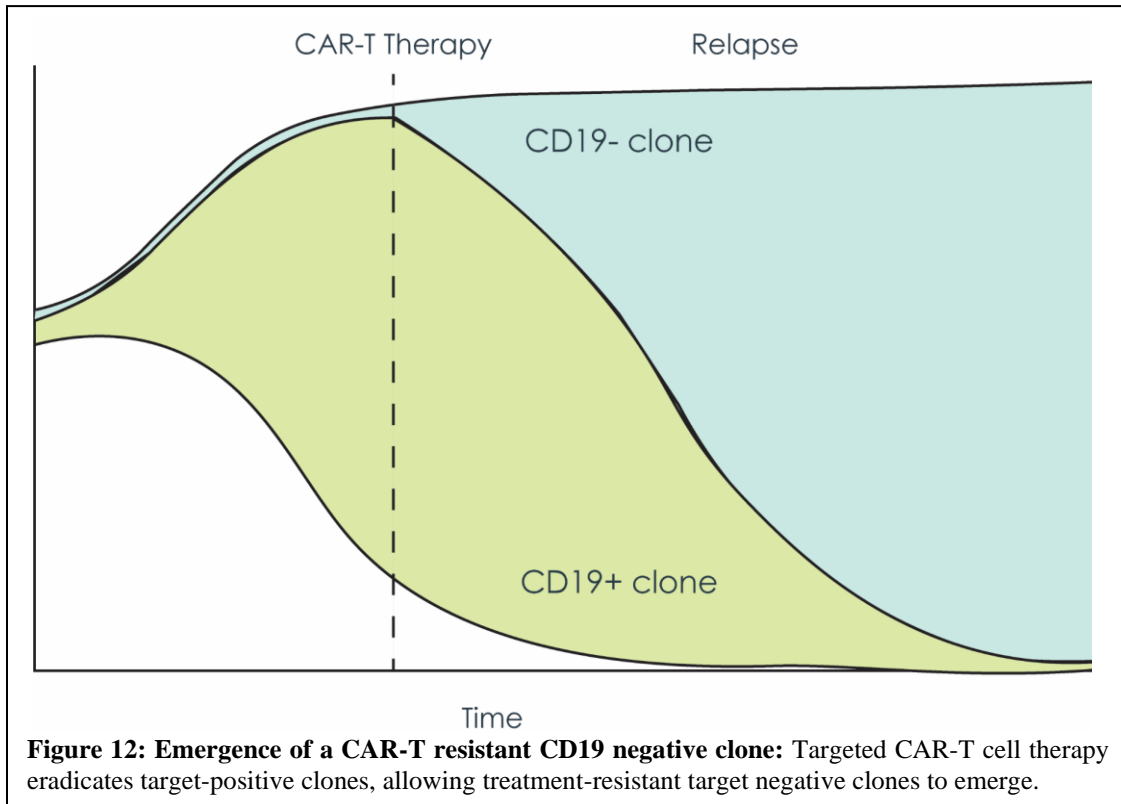
### **Antigen-negative relapse**

Antigen negative relapse can occur when an antigen-negative clone expands under selective pressure (Figure 10), or when the tumor mutates to evade treatment (Figure 11). Clonal expansion of an antigen-negative clone, or a clone that lacks the binding epitope, can occur due to tumor heterogeneity<sup>301-306</sup>. Identifying antigen-negative clones before treatment begins allows for CAR-T resistant clone to be monitored throughout therapy, and could act as a prescreening strategy to determine who is a good candidate for CART19 therapy<sup>297</sup>. Fisher *et al.* identified that malignant clones express variants of CD19 that lack the binding epitope, preventing recognition by the CAR-T cells<sup>301,307</sup>. These variant CD19 binding epitopes may be present at the start of treatment and can result in CAR-T treatment failure<sup>301</sup>.

Target modulation is one of the most well-known mechanisms of antigen-negative relapse following CAR-T induced remission. This type of resistance can be found among varying types of cancer, including ALL. Mechanisms of modulation include genetic receptor modifications, lineage switching, and epitope masking (Figure 11)<sup>301,303,307-314</sup>.

Genetic receptor modifications can include acquired mutations and alternative splicing<sup>307</sup>. Both of these mechanisms result in altered cell surface expression of CD19<sup>308</sup>. Modified cell surface

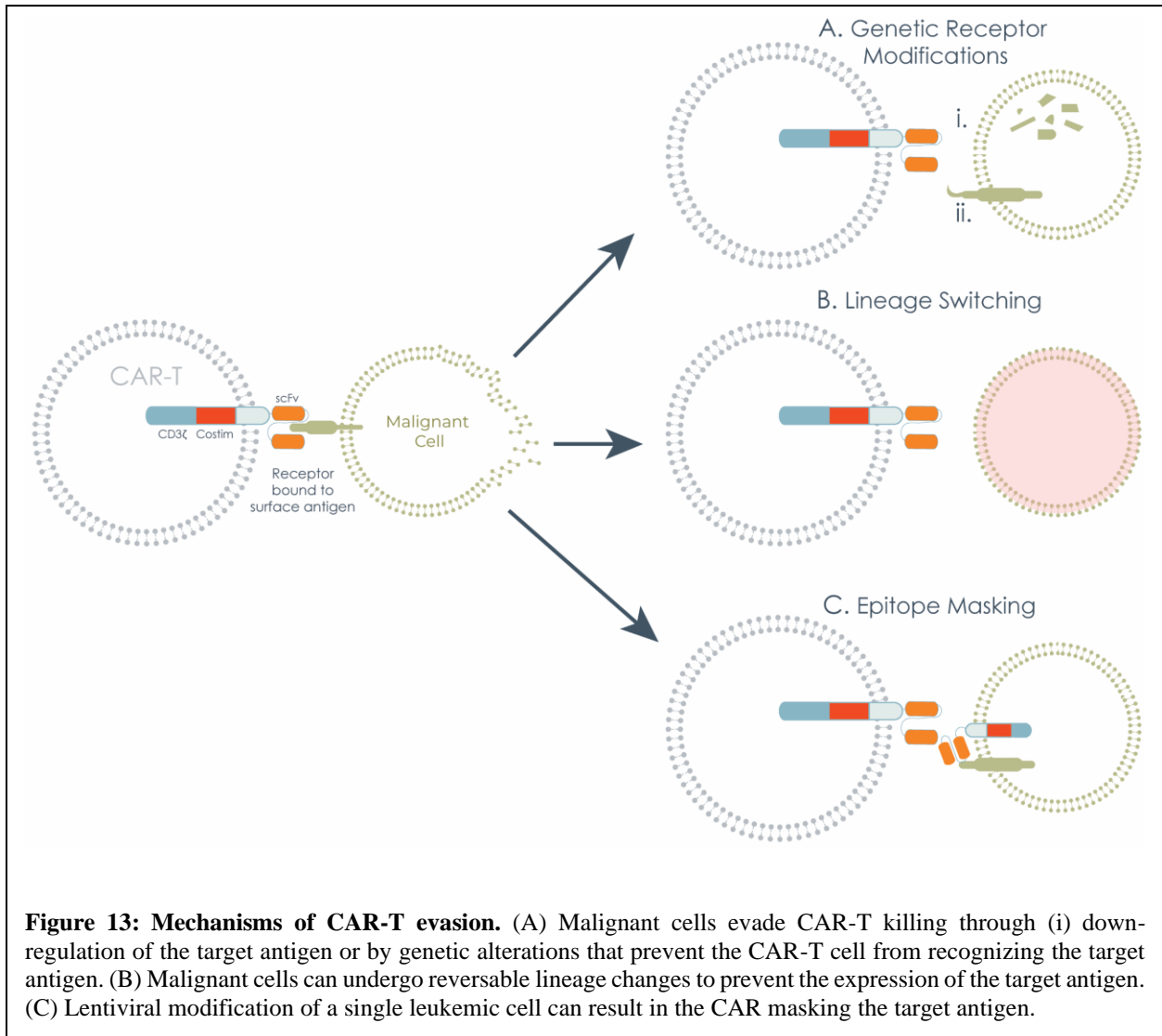
expression of CD19, such as a reduced expression or complete down-regulation of CD19, can prevent the CAR-T cells from recognizing the tumor cell<sup>301</sup>. While total antigen loss is not required for tumor cells to evade CAR-T cell therapy, one study found misfolded CD19 proteins in the endoplasmic reticulum of the tumor cells, suggesting a mechanism for complete antigen loss<sup>308,315</sup>.



Tumor cell lineage switching is reported in leukemic malignancies<sup>303,310–314</sup>. In one particular instance of cell lineage, switching was seen in a pre-clinical model using CAR-T cells to target FLT3 in ALL<sup>311</sup>. In this case, CAR-T cell treatment induced a reversible B cell to T cell lineage switch in malignant B cells<sup>311</sup>. By doing this, the tumor was able to evade CAR-T cell therapy, which later could result in antigen-negative relapse.

Finally, the transformation of a single leukemic cell occurring during CAR-T production can lead to treatment-resistant clones<sup>244</sup>. Ruella *et al.* at UPENN reported on resistance induced by

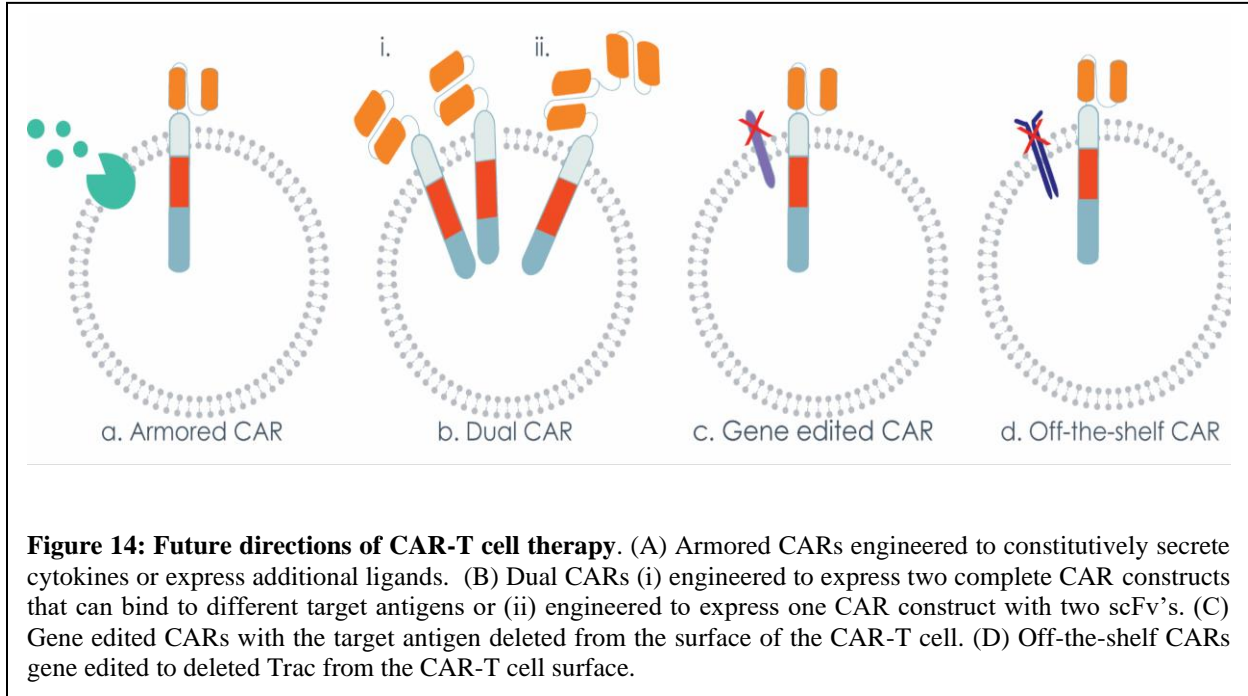
transforming a single leukemic B cell present in the leukaphoresis<sup>244</sup>. Transforming a leukemic cell with a CAR construct resulted in epitope masking, which occurs when the CAR expressed within the leukemic cell binds to the CD19 antigen on the cell surface. By binding the CD19 epitope, the leukemic cell is shielded from CAR-T cell killing<sup>244</sup>.



## 2.2.4 Future directions of CAR-T cell therapy

The success and limitations of current CAR-T cell therapy have prompted researchers to explore mechanisms to increase CAR-T cell efficacy. While this list is by no means exhaustive, some of

the more common avenues of CAR-T exploration include armored CARs, gene-edited CARs, off-the-shelf CARs, and combination therapies.



### Armored CARs

Armored CAR-T cells modify traditional CAR-T cell designs so that the CAR can inducibly or constitutively secrete cytokines, or express ligands that enhance CAR-T cell efficacy<sup>316</sup>. Cytokines, such as IL-7 or IL-15, have been used to increase CAR-T cell survival and cytotoxicity. Tamada *et al.* found that CAR-T cells modified to express IL-7 and CCL19 enhanced T cell proliferation and survival while acting as a chemoattractant for other T cells and dendritic cells<sup>317</sup>. This study found that they were able to improve survival in pre-clinical models with their IL-7-CCL19 armored CAR-T. Another study that designed a CAR-T that constitutively secreted IL-12, a pro-inflammatory cytokine that enhances the cytotoxic potential of CD8 T cells, found that their



armored CAR had improved proliferation and increased the survival when compared to a standard CAR-T in a pre-clinical murine model<sup>318</sup>.

### **Dual CARs**

A current obstacle in CAR-T cell therapy is the lack of specific target antigens present on the tumor cells. Tumors are often heterogeneous, resulting in an incomplete expression of a single antigen. The heterogeneity of tumors can result in relapse and the emergence of treatment-resistant clones. Many researchers are looking into improving the efficacy of their CAR-T cells by incorporating another scFv<sup>194,319–323</sup>. Some design approaches to dual-target CAR-T cells include incorporating two separate CAR constructs into the T cell or connecting the scFv via an additional linker, and common approaches include targeting CD19 and CD22<sup>319,320,324</sup>. Amrolia *et al.* reported complete remission in seven out of ten patients treated with CD22-OX40  $\zeta$  – CD19-BB  $\zeta$  CAR<sup>319</sup>. Schultz *et al.* reported that three out of four pediatric B-ALL patients were MRD negative following CD19-CD22-BB  $\zeta$  CAR-T treatment<sup>320</sup>. Huang *et al.* reported that 18 out of 36 NHL patients had a complete response following treatment with a CD19 CD22 third-generation cocktail<sup>325</sup>. Several dual CAR-T cell therapies are in clinical trials to reduce the rate of relapse<sup>319–321,323–325</sup>

### **Gene-edited CARs**

Gene-edited CAR-T cells enable researchers to target a wider variety of antigens<sup>232,326–332</sup>. Currently, certain types of cancers, such as T cell malignancies, are unable to take advantage of CAR-T cell therapies because of the shared antigen expression between the tumor and CAR-T cells. By utilizing gene-editing technology, such as TALEN, zinc finger nucleases, and CRISPR/cas9, researchers have developed a way to enable CAR-T cell use in a previously inaccessible setting. One study found that CRISPR/cas9 could be used to delete the target antigen

from the surface of the CAR-T cell<sup>232</sup>. The deletion of the target antigen from the surface of the CAR-T cell is critical when designing a CAR that targets malignant T cells. If the target antigen is present on the CAR-T cell, the CAR-T cells will kill recognizes themselves, resulting in fratricide or self-killing. Deletion of the target antigen allowed for the development of a CAR-T that could be used to effectively target T cell malignancies<sup>232</sup>. Furthermore, gene-editing technologies have enabled the generation of off-the-shelf CARs.

### **Off-the-shelf CARs**

Off-the-shelf CAR-T cells, also known as universal CAR-T cells, are CAR-T cells that are manufactured from donor T cells that can then be delivered to any patient<sup>232</sup>. A variety of genetic modification methods have been employed in the effort to develop universal CAR-T cells<sup>327,329,329–331,333–357</sup>. Developing CAR-T cells from healthy donors provides several advantages over CAR-T cells derived from the patient. The first is that patients often lack in T cells. Patients that are lacking in T cells are not able to donate sufficient quantities of cells for CAR-T development, and furthermore the patient's disease may impact the quality of the T cells they can donate, which could result in inhibited CAR-T expansion during the manufacturing process. Because of this, patients who have low T cell counts are frequently excluded from CAR-T cell trials<sup>232,295</sup>. Additionally, patients with T cell malignancies are unable to provide any T cells for the development of their treatment. As previously stated, this is because it is challenging and impractical to separate the malignant T cells from the healthy ones. Finally, CAR-T manufacturing is an extensive process, and many patients are unable to survive the three to six weeks that are required for CAR-T development<sup>358</sup>.

Furthermore, the accidental transformation of even a single malignant cell can result in epitope masking and the development of treatment-resistant malignant clones<sup>244</sup>. Universal CAR-T cells provide the advantage of using healthy donors as a T cell source. However, universal CAR-T cells could potentially be rejected by the host's immune system, resulting in reduced efficacy of the CAR-T cell therapy.

Despite the need, previous lack of progress resulted in the inability to use donor T cells in the CAR-T manufacturing process. Infusing donor T cells into immunosuppressed non-identical recipients results in severe, life-threatening GvHD<sup>232,236,238,240</sup>. GvHD occurs when the infused donor T cells recognize the recipients' tissues as foreign and elicit an immune response against them<sup>359,360</sup>. Qasim *et al.* utilized TALEN gene editing to disrupt the CD52 gene and the TCR $\alpha$  chain in CD19 CAR-T cells<sup>357</sup>. Patients receiving these universal CAR-T cells received a lymphodepletion regimen combined with anti-CD52 antibody therapy. The combination of these therapies allowed for the clearance of the patient's T cells while leaving the CAR-T cell therapy unaffected. Anti-CD52 antibody therapy, combined with TCR and CD52 deleted CAR-T cells, was administered to two patients. The first patient went into remission but unfortunately developed GvHD. However, the persisting CAR-T cells in this patient were CD3+, indicating contamination and expansion of a CAR-T cell population that was not TCR deleted<sup>357</sup>. The persisting CAR-T cells in subject two, however, were CD3-. Subject two responded well to the treatment, only exhibiting mild symptoms of GvHD that reversed after the administration of topical steroids<sup>357</sup>. To corroborate these findings, DiPersio *et al.* found that the deletion of a T cell-specific receptor, Trac, resulted in a GvHD resistant CAR-T cell product<sup>232</sup>. By deleting Trac from the CAR-T cell surface, the researchers were able to develop an off-the-shelf CAR-T cell that could be used to treat hematologic malignancies, such as B-ALL and T-ALL.

# **Chapter 3: Conclusion**

## **3.1 Immunotherapy**

The idea that the immune system can regulate tumor growth has come a long way since Paul Eldrich first suggested that the immune system could modulate tumor growth in the early 1900s<sup>12</sup>. Discoveries made in future generations allowed researchers to genetically engineer T cells that could be redirected to target specific antigens present on the surface of cancers<sup>20</sup>. Through a collective effort, multiple generations of CAR-T cells have been designed to optimize CAR-T cell therapy. Initial  $\zeta$ -CAR-T cell therapies established a solid foundation for future designs and provided necessary proof-of-concept studies that demonstrated the efficacy of CAR-T cell therapy. A solid understanding of the co-stimulatory receptors required for optimal T cell activation lead researchers to design two more generations of CAR-T cells, each with their benefits and limits. Through these studies, clinical trials were able to evaluate the safety and efficacy of CAR-T cell therapy in treating a variety of malignancies in patients who previously had no alternative treatment options.

## **3.2 CAR-T cell use in the clinic**

Preliminary, proof-of-concept, clinical trials demonstrated that CAR-T cells could be used to target CD19 and CD20 positive malignancies in some patients<sup>3,4,159,160,190</sup>. Many trials began to follow suit, resulting in the FDA approval of two CAR-T cells for the treatment of CD19 positive B cell malignancies<sup>193,361</sup>. CAR-T cell therapy is being investigated in a multitude of cancer types, ranging from diverse hematological malignancies to solid-organ malignancies. Each distinct CAR-

T cell therapy is associated with unique obstacles that researchers are diligently working to overcome.

The widespread use of CAR-T cell therapy has revealed several severe toxicities, examined in depth in section 2.2.2. Toxic side effects associated with CAR-T cell therapy demonstrate the need for a better understanding of the mechanisms responsible for adverse effects. Treatments are becoming available that can ameliorate nearly all of the symptoms of CAR-T cell-mediated toxicities; however, researchers are still working to develop safer CAR-T alternatives<sup>259,271,289,291</sup>.

### **3.3 CAR-T cell therapy resistance**

Current treatment options for cancer patients frequently result in relapse and treatment-resistant disease. CAR-T cell therapy is not an exception to that rule, and common mechanisms of relapse and resistance include antigen-positive and antigen-negative relapse. Because antigen-positive relapse is thought to be associated with a lack of CAR-T cell persistence, researchers are working to improve the overall quality of the CAR-T cell infusion as well as modifying CAR-T cell designs to establish a more robust CAR-T cell response<sup>362</sup>.

Antigen negative relapse can occur due to a variety of factors. A target-negative clone, present at the time of treatment, can expand, resulting in treatment-resistant disease<sup>308,362</sup>. Additionally, pressures presented by CAR-T cell therapy can cause the tumor to modify the antigen so that it no longer can be recognized by the CAR-T cell or undergo a transient lineage switch so that the malignant cell no longer expresses the target antigen<sup>301,308,311,315</sup>. Finally, errors in CAR-T manufacturing can result in the incorporation of a CAR construct into a single cancerous cell, resulting in epitope masking that effectively hides the malignant cell from the CAR-T cells<sup>244</sup>.

### 3.4 Future directions of CAR-T cell therapy

CAR-T cell therapy continues to progress as researchers improve upon previous CAR designs. Efforts to enhance the efficacy of CAR-T cell therapy has led to the development of armored CARs, dual CARs, gene-edited CARs, and off-the-shelf CARs. Armored CAR-T hopes to enhance CAR-T cell expansion, persistence, and trafficking<sup>317,318</sup>. Dual CARs hope to combat antigen-negative resistance and relapse by targeting multiple antigens on the surface of malignant cells<sup>194,363</sup>. The possibilities for gene-edited CARs are endless, allowing researchers to edit their CAR-T cells in any way they see fit. Gene-edited CARs enable the treatment of previously inaccessible diseases such as all T cell malignancies<sup>232</sup>. Finally, off-the-shelf CAR-T cells hope to provide a universal CAR-T cell therapy to patients who are unable to donate their T cells for CAR-T production<sup>232,244,339</sup>. Off-the-shelf CAR-T cells also aim to reduce the costs and time restraints for CAR-T production, improving the accessibility of CAR-T cell therapy.

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