CAR-T Cell Therapy and its Engineering Marvels in Cancer Immunotherapy: A Comprehensive Review

By

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A thesis submitted to the School of Pharmacy in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (B. Pharm.)

School of Pharmacy

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Declaration

It is hereby declared that

1. The thesis submitted is my original work while completing a degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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Approval

The thesis titled "CAR-T Cell Therapy and its Engineering Marvels in Cancer Immunotherapy: A Comprehensive Review" submitted by Samarah Arjumand (18146068), of Spring, 2018 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy.

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Ethics Statement

This project does not involve any kind of animal and human trial.

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Abstract

Immunotherapy has emerged as a promising approach in the treatment of various malignancies, with adoptive cell therapy (ACT) showcasing significant efficacy. Among the diverse spectrum of ACT, Chimeric Antigen Receptor T-cell (CAR-T) therapy stands out as a groundbreaking modality, revolutionizing cancer treatment paradigms. This thesis delves into the intricate domains of CAR design, elucidating the structural components crucial for target specificity and efficacy. Insights into the administration process ensure a comprehensive understanding of the therapeutic protocol. The Mechanism of Action (MOA) of CAR-T therapy is expounded upon, unraveling the intricate signaling cascades that culminate in tumor cell destruction. Classification based on generation highlights the evolution and refinement of CAR-T technologies, while sophisticated approaches in CAR-T therapies explore strategies to enhance efficacy and mitigate adverse effects. Accessible-approved CAR-T therapies are scrutinized, emphasizing their clinical impact and therapeutic potential. Clinical trials and their implications shed light on ongoing research endeavors. Along with the discussed advantages, a section on the disadvantages of CAR-T therapy provide a balanced perspective on its clinical utility, while prominent targetable antigens delineate the antigenic landscape of this therapy for further future development. This thesis endeavors to provide a holistic understanding of CAR-T therapy, its challenges, and its transformative potential in the realm of cancer immunotherapy.

Keywords: Immunotherapy, CAR-T therapy, adoptive cell therapy, cancer treatment, targeted antigen recognition.

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Chapter 1

Introduction

Cancer is a group of diseased cells exhibiting a common phenomenon of abnormal uncontrolled cellular proliferation, with the tendency to trespass into adjacent, as well as distant, tissues, known as metastasis, that, if stays unmanaged, can lead to a slow and painful demise of patients (National Cancer Institute, NCI Dictionary of Cancer terms). Cancer is the second leading cause of worldwide death, placed just after heart disease related deaths (Center for Disease Control and Prevention, FASTSTATS - leading causes of death 2024). In the year 2020 alone, approximately 19.3 million cancer cases and 10.0 million cancer related deaths were recorded (Sung et al., 2021). The number became 20.0 million and 9.7 million for new case diagnosis and yearly death toll by the year 2022, respectively. The diagnosis rate is speculated to increase to 35 million by the year 2050 (American Cancer Society, Global cancer facts & figures). Regardless, finding a care-plan, if not a complete therapeutic cure, has become an unavoidable issue to deal with this epidemic.

Till now, the cancer treatment options can be broadly divided into 5 sections- surgery, radiotherapy, traditional chemotherapy, targeted precision therapy and lastly, immunotherapy (Falzone et al., 2018).

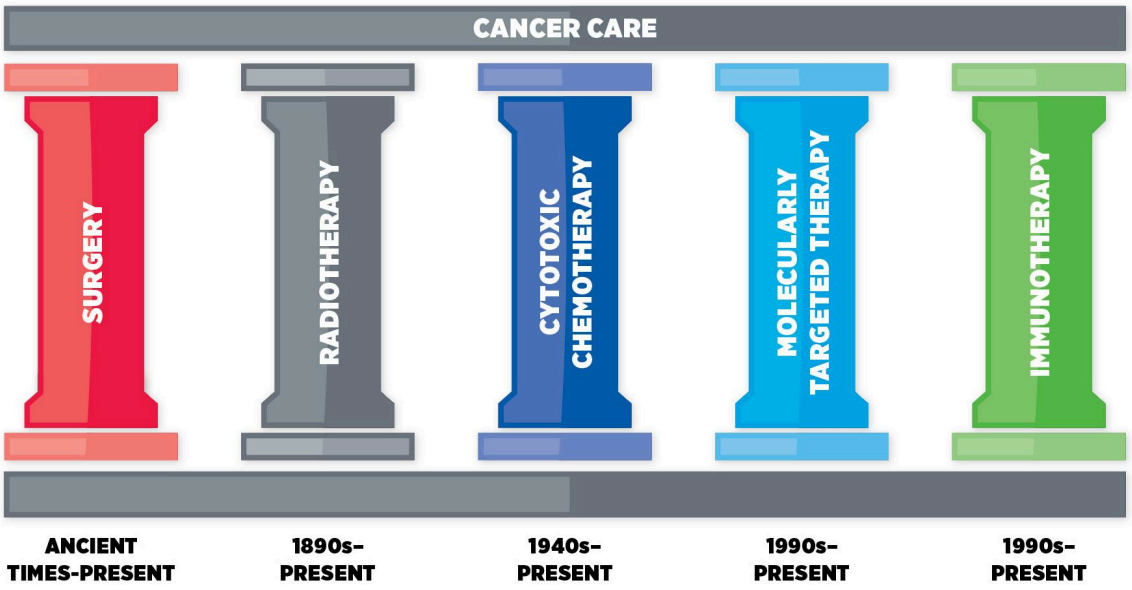

Figure 1. Five Pillars of Cancer Treatment (American Association of Cancer Research, 2021).

Among these, immunotherapy is the most recent and advanced approach where the patient's own innate and consequential adaptive immune system is leveraged to amplify or dampen in order to achieve a customizable therapeutic outcome regarding immune-system regulated conditions (Naran et al., 2018). In the context of cancer immunotherapy to modify the immune system to detect and cause anti-tumor activity, numerous techniques are being studied, namely, immune checkpoint inhibitors (ICB), Monoclonal Antibody (MAB) therapy, Cytokine therapy, Cancer vaccine and Adoptive cellular therapy (ACT) (Cancer Research UK, Types of cancer immunotherapy 2024) .

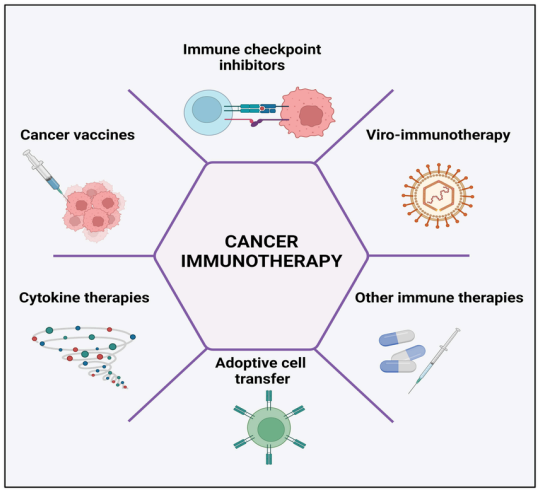

Figure 2. Types of available cancer immunotherapies (Kciuk et al., 2023).

Adoptive cellular therapy can also be classified into 2 types: genetically and non-genetically modified ACT therapies. Genetically modified therapies are Engineered T Cell Receptor (TCR) based therapy and Chimeric Antigen Receptor (CAR-T) cell therapy. On the other hand, non-genetically modified therapies included Tumor Infiltrating Lymphocyte (T-IL) therapy, NK cell therapy (Dana Farber Cancer Institute, 2024; Cancer Research Institute, 2023).

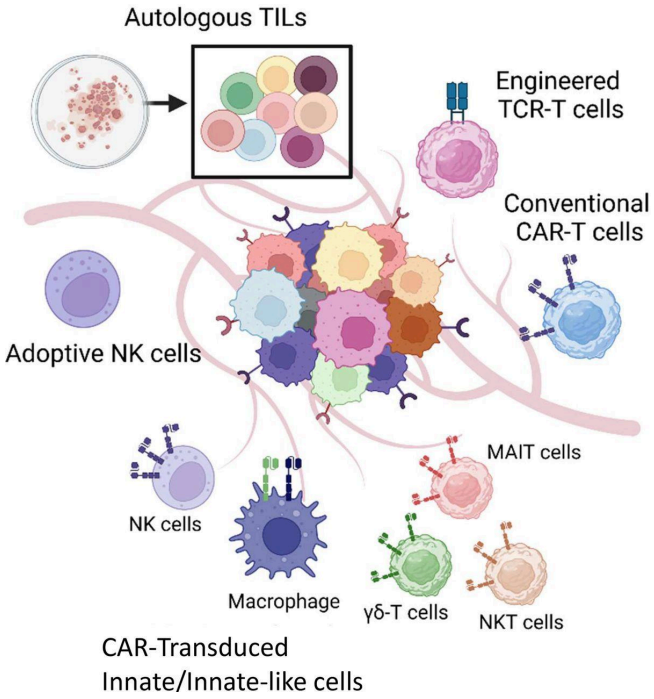

Figure 3. Available types of Adoptive Cell Therapies (Neo et al., 2023).

1.1 Background

CAR-T cell therapy is a genetic modification based cancer immunotherapy which is based on the expression of a recombinant antigen receptor used to treat mainly relapsed as well as refractory malignancies. It is generally a personalized therapy that boosts as well as redirects autologous T-cell's inherent attacking capability through expression of a specialized receptor (CAR) that can aid T-lymphocytes to actively target and selectively kill tumor cells specifically express the corresponding antigen (e.g., CD19, HER-2) (Zhang, 2022).

CAR, abbreviation for Chimeric Antigen Receptor, which is also known as Chimeric Immunoreceptor, is a type of engineered receptor designed to express artificially to provide enhanced target specificity. This technology, when used on T-lymphocytes, to boost their targeting ability to be of increased selectivity in identifying the surface tumor antigens, is known

as the CAR-T cell therapy. This is derived to support better antigen recognition that aids T-cells to readily activate in an otherwise hostile tumor microenvironment.

A major advantage of CAR-T therapy over other immunotherapies including artificially expressed endogenous TCR based T cell therapy is that they do not need the involvement of antigen presenting cells (APCs) and the major histocompatibility complexes (MHCs) on their surface to present antigen to the CAR receptor. CAR-T cells recognize and bind to targeted tumor antigen directly in a MHC-unrestricted way which brings CAR-T cells in a hugely favorable position in cases of immune evasion attempts of neoplastic tumor cells (e.g., decreased exposition MHC-class-1 proteins causing disturbance in not only antigen presentation but also processing mechanism) which is unsuccessful on CAR expressing T cell. Thus, the irrelevance of MHC-receptor pathway aids in targeting any tumor cells irrespective of their human leukocyte antigen (HLA) type. Again, Unlike TCR-T cells only targeting peptides as antigens, CAR-T cells can accept a variety of molecules-carbohydrate, glycolipid, or peptide as target antigens. Moreover, CARs, with the incorporation of varied kinds and numbers of co-stimulatory molecules can provide with diverse types of oncolytic effect meeting specific requirements like safety and persistence (Muhammad et al., 2017).

Due to all these advantages, CAR-T cell therapy has become one of the most well-accepted therapeutic technologies among scientists as well as patients with successful clinical trials leading to the approval of 6- European Medicines Agency (EMA) and U.S. Food-and-Drug-Administration (FDA) approved therapeutic agents for various leukemias, lymphoma and multiple myeloma even in the case of their relapse/refractory (R/R) stage.

1.2 Methodology

This dissertation utilized a systematic search across reputable scientific databases and high-impact journals, employing keywords like "CAR-T cell therapy," "cancer immunotherapy," and "T cell engineering." Criteria prioritized recent, relevant studies elucidating CAR-T principles, innovations, clinical applications, and challenges. Each of the 76 selected sources underwent rigorous evaluation for relevance, credibility, and scientific merit. Critical analysis of methodologies, results, and interpretations provided a comprehensive understanding of CAR-T therapy, complemented by theoretical frameworks. Insights synthesized from the literature delineated key themes, trends, and future directions in the field.

1.3 Overview

The aim for this dissertation is to provide an in-depth exploration of Chimeric Antigen Receptor T-cell (CAR-T) therapy and its pivotal role in cancer immunotherapy. Moving forward, Chapter 2 delves into the intricate domains of CAR design, elucidating the structural components crucial for target specificity and efficacy. Following this, Chapter 3 briefly shows the administration process, while Chapter 4 examines gene transfer methods. Chapter 5 explores the mechanism of action of CAR-T therapy, followed by a classification based on generation in Chapter 6. Chapter 7 delves into advanced approaches in CAR-T therapies, aiming to enhance efficacy and mitigate adverse effects. Furthermore, Chapter 8 scrutinizes accessible-approved CAR-T therapies, while Chapter 9 delves into clinical trials and their implications. Lastly, Chapter 10 provides insights into the disadvantages of CAR-T therapy, offering a balanced perspective on its clinical utility. This structured approach offers the readers a comprehensive understanding of CAR-T therapy and its transformative potential in cancer treatment paradigms.

Chapter 2

Domains of CAR Design

In cellular immunotherapy, the Chimeric Antigen Receptor (CAR) embodies four crucial domains: the Extracellular Antigen Recognition Domain, the Spacer/Hinge, the Transmembrane Domain, and the Intracellular Domain. This chapter is dedicated to the discussion of these domains.

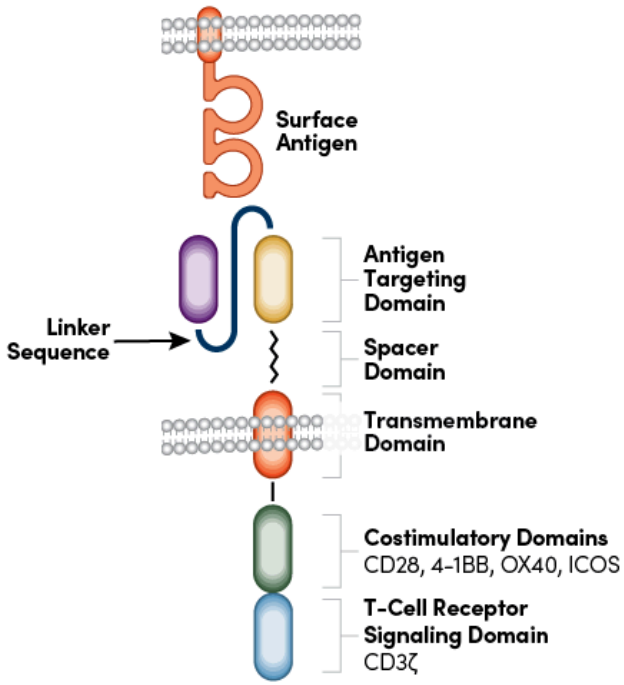

Figure 4. Structural illustration of the Chimeric Antigen Receptor (CAR) with Intracellular Signaling, Costimulatory, Transmembrane, Spacer and Antigen Recognition Domains targeting Tumor Associated Surface Antigen of the target cell (Amrik Singh, Anti-CAR linker antibodies for CAR detection in CAR-T research).

2.1 Antigen Recognition Domain (ARD)

This is the outermost part of the CAR structure that is responsible for its primary function, the specific recognition of the intentionally targeted extracellular-surface-cancer-antigens or ligands, otherwise known as tumor-associated antigens (TAA). This is also called the 'Antigen Binding Domain' along with ARD or Ligand Recognition Domain as it ensures strong bond with the pre-defined surface antigen alongside preliminary recognition. It is called both because only by binding to the complete epitope of the surface tumor antigen, it ensures recognition in the first place which makes it independent from MHC expression requirement; making it only dependent on co-receptor expression, proper processing after binding along with successful specific antigenic epitope presentation in the first place.

Now, a crucial thing to be weighed in the ARD selection is the parameters to be considered relevant to the design that primarily regulate the whole CAR function; them being affinity and specificity towards the selected antigen (TAA). Though increased specificity is always preferred, affinity should be just optimal. The elevated the specificity, the decreased the occurrence probability of the 'off-target-toxicity' that can further stimulate antigen-independent-cytotoxicity; but this heightened specificity can also set off CARs to additionally pursue non-malignant healthy cells having normal amount of TAAs. On the other hand, ARD affinity should neither be too high causing adverse effects like- toxicity and, T-cell activation caused readily death, or too low for antigen recognition or, signal instigation or, effector cells' compulsory full-activation in the first place (Oldham & Medin, 2017; R.C. Sterner & R. M. Sterner, 2021).

Regarding TAAs, type-CD19 has been able to be worked with somewhat successfully through this treatment. This CD19-based CARs have been researched about as well as run clinical trials on most substantially and, 4 of the 6 FDA approved therapies- Kymriah, Yescarta, Breyanzi and Tecartus are designed to target this very specific antigen for hematologic malignancies. Nonetheless, an increasing number of TAAs are being studied to give out novel TAAs simultaneously through exciting clinical trials and insightful results (Guedan et al., 2019). Different types of ARDs are available to construct desired CARs, which are described below

2.1.1 ScFv (Single Chain Variable Fragment)

Among all the different types of ARD, ScFv is the most common option, consisting of a variable heavy (V_H) and variable light (V_L) domain forming a joint-entity through a short flexible polypeptide chain called linker. The variable domains are generally monoclonal antibody (MAB) derived and the tiniest-component-part of the MAB is responsible for the binding ability of the ScFvs which aids in the synthesis of Bindable CAR-T structures (Guedan et al., 2019; Oldham & Medin, 2017).

Though minute, the linker of the structure is crucial for ScFv's desired flexibility, stability, folding and functionality. Its flexible characteristics keeps the ScFv oriented in either the V_H or the V_L direction that forms differentiating structures. This orientational difference works as a deciding factor of the whole CAR's express-ability in the genetically modified effector cells as well as its targetability and signaling ability. Again, linkers' stability is a direct contributor in the ScFv's stability, itself, which is dependent on its length as well as the framework of the amino acid polypeptide chain. Proteolytic stability is another factor for linkers

as when directed to increase, it intensifies the affinity of the whole ScFv structure. (Guedan et al., 2019).

Before recognition and binding, the affinity as well as specificity of ScFvs are dealt with; which are dependent on the reciprocity of the variable domain polypeptides along with the corresponding orientation of the complementarity determining regions (CDR). In spite of having indistinguishable affinity, ScFvs can have divergent effectivity. On the contrary, different affinity can be seen for the identical epitope of the TAA due to mutation of the CDRs or, the very antigen marking separate epitopes based on proven studies.

The statement that even lessened affinity can be desirable in a specific case can be established through the example of Trastuzumab-originated CAR-T option having intentionally minimized affinity; generally used for HER2+ malignancies. In a study, Its ScFv was proven to be able to degranulate only when encountered the HER2+ breast cancer cells expressing excessive HER2 protein considered to be antigen in this case; but did not while passing healthy in vivo primary cells with normal level of HER2 presentation.

All in all, position of the specific epitopes along with target-antigen density as well as deflecting antigen-independent-tonic-signal giving out ScFvs should be thoroughly contemplated for preferred bondage.

ScFv can be derived from the MABs of various origins- murine, humanized or human; which can impact CAR's fundamentally required characteristics like specificity. Murine sourced ScFvs are most common with desired specificity but cause immunogenicity and thus binding-restriction many times. This immune response against the effector CAR-expressing cells has been exhibited to result in reduced CAR-T cell tenacity in about quarter of the participants

arising from immune clearance conforming to an anti-CD19-CAR therapy based clinical trial. In this scenario, Humanized or completely human based ScFvs have come to the rescue as better alternatives. 40% of the of the murine-sourced-ScFv defiant patients from a study, showed positive results from the humanized-ScFv based CAR treatment. Nevertheless, these options are yet to be improved due to their reduced specificity; which is also a common phenomenon due to affinity-evolution and for in vitro cultured antibodies as they necessarily do not encounter the in vivo immune surveillance to the real extent. Lastly, the Human phage display library is one of the more advanced options being explored recently (Oldham & Medin, 2017).

ScFv as a part of CAR works as an initiator as well as amplifier of cytolytic characteristics of the original t-lymphocytes through better recognition and bondage. CAR-T can work on immediate terms as well as be long living memory cells for relapsed necrotic identification resulting in desired destruction (Mohanty et al., 2019).

ScFv is one of the, if not the most prominent antigen binding domain that fuels CARs of its précised targetability instead of the tactic of generally assuming the antigens/ epitope by the naturally occurring T-lymphocytes which, many a time, gets masked by the tumors' immune evading mechanisms.

2.1.2 Other ARD Domains

Though ScFv is the most-widely used ARD domain while engineering the CAR structure, there are other types of ARD available that are used in developing various types of CAR constructs.

Ligand-Receptor ARD

Various types of ligand/receptor ARD based CAR can be constructed utilizing the naturally occurring ligand-receptor interaction where either a ligand or, a receptor protein is fused to the CD3-ζ containing intracellular signaling domain that can bind to the corresponding receptor or, ligand expressed on the tumor cell resulting in direct ligand-receptor binding based CAR-T activation forming specialized physiological CAR-T cells discussed later. For example, NKG2D-receptor expressing CAR-T cell targeting NKG2D-ligand presenting lymphoma tumor cell and IL13-mutein-receptor expressing CAR-T cell targeting IL13Rα2(+)ve glioblastoma cells.

TCR-mimicking (TCRm) ARD

Though CARs' MHC-unrestricted antigen binding ability is considered as an advantage, it can sometimes restrict T-cells from targeting endogenous target molecules. Thus, CARs with TCRm-ARD can have the best of both CAR and TCR ARD domains. This approach was applied while in vitro leukemic and ovarian cell-lines targeting Wilms Tumor-1 (WT-1) protein that stayed embedded in the intracellular region of target cell; but TCRM-ScFv-based-CAR can identify a peptide-based-antigen presented by HLA-A*02:01 through the WT-1/HLA coupling and exerted anti-tumor effect against the tumor cells.

Variable Lymphocyte Receptors (VLR)

VLR antibody is an immunoglobulin-similar but varied structure as well as binding-affinity showing antigen receptor derived from jawless vertebrates' immune system; which is incorporated into the CAR structure as an ARD for its binding ability to a bigger pool of antigens

to target various antigen presenting tumor cells. Though there is a probability of possible immunogenicity, two types of VLR-ARD based-CAR-T cells have shown promising results in vitro through T cell activation based cytotoxicity (Oldham & Medin, 2017).

Lastly, various other types of ligand, nanobody, cytokine and peptide based ARDs are also constructible provided that the armor capable of expressing the specific ARD, fused to the intracellular signaling domain (Rafiq et al., 2019).

2.2 Hinge (Spacer)

The second part of the extracellular portion of the CAR structure; which is otherwise known as the Spacer. It is an amino acid chain derived from the immunoglobulins (e.g., IgG1 and IgG4), various clusters of differentiation (e.g., CD8α and CD28) or, some less popular options (e.g., IgD or, Cd7) sometimes; that works as a connection between the ARD and the embedded intracellular domain. This Hinge region has various functionalities- a) it contributes with the ARD linker in CAR's ARD flexibility enhancement to get beyond steric hindrance, b) it's length can be customized to help reach targeted TAA epitopes in order to develop structured immunological synapses and, c) it also provides the whole CAR with stability (Oldham & Medin, 2017; Mohanty et al., 2019).

Optimal length for hinges varies due to the location of the aimed epitope as well as the degree of steric hindrance the malignant cells challenge them with; which along with the constitution impacts whole CAR's expression, signaling and activation level (Sterner, R.C & Sterner, R. M., 2021). Longer hinges (e.g., mucin-1 (MUC1) hinge) are better for approaching near-membrane epitopes or antigens with glycosylation complex. Contrastingly, shorter options (e.g., CD19, or carcinoembryonic antigen CAR hinges), are generally opted for the

far-membrane epitopes being easily findable comparatively. Nevertheless, beyond this broad generalization, to be more purposeful, empirical testing is usually done to ensure ideal hinge length and is a must for targeting any novel antigen as having suitably-long-hinge adjusted to the specific ARD is a prerequisite for increasing the probability of the therapeutic outcome.

As mentioned previously, hinges can be extracted from different origins but immunoglobulins (mainly, IG1 and IG4) are the most common options due to their therapeutic effectiveness as well modularity providing options to design length diversity; and is being used in CD19-CAR based clinical trials. Unfortunately, these IG-portions also includes the Fc (Fragment Crystallization) region; still able to interact with the Fcγ-receptors (FcγRs) causing in vivo toxicities rooted from antigen-independent-signaling inducing off-target activation and effector cell elimination caused persistence issues. This region in hinges has been shown to cause off-tumor-localization and reduced engraftment in in vivo animal models. To mention, Fc region is basically made of CH2 and CH3 groups. So, these problems can be solved either by utter elimination of all the CH2s or by mutating the CH2s to Fcs unable to bind to FcγRs. Additionally, the hinge's FcγR binding ability has been shown to decrease $1x10^4$ times by replacing IG2 residues with the IG1 or IG4's ones.

Among the comparatively lesser but still moderately used options CD8α and CD28 are the most significant ones; which are also surprisingly orderly found in 2 of the FDA approved CAR options- Kymriah and Yescarta. CD8α based hinges comes together with its own transmembrane domain where CD28 based options are used with both their transmembrane and co-stimulatory domain all-together. CDs instead of IGs usage has been shown to have some prominent advantages like-the natural inaptitude in the FcγR interaction and exchanging amino acids to improve dimerization (e.g., using alanine or serine instead of cystine in CD8α resulting

in hetero as well as homo type of CAR dimerization which subsequently leads to heightened CAR expression on NK92 immune cells through forming effective dimers. For this reason, they are being studied rigorously to unlock future possibilities.

2.3 Transmembrane Domain (TMD)

It is the gateway to the intracellular segment of the CAR, primarily comprised of hydrophobic α helix amino acids ranging throughout the effector cell membrane, working as a liaison between extracellular and intracellular fragments. Along with having the only distinguishable attribute of operating as a stabilizing hook, it can also aid in augmenting the degree of CAR stability, expression as well as cell signaling. TMDs support the transmission of proper ligand identification signal to the endogenous structure through conformational alteration after initial immunological synapse formation due to the dimerization and bond formation with endogenous region in the first place, which subsequently catalyzes the downstream cell signaling cascade resulting in activation induced cell death via cell activation through the release of cytokines and direct cytotoxic molecules.

Though TMDs are routinely switched to accompany the other CAR structure, like ARDs and hinges, they also have some somewhat narrowed source of origin; like- CD3ζ being used in first generation, CD28, CD8α and CD4 in second to later generations of CARs along with CD4, CD7, FcεRIγ, OX40 and H-2K^{b} being used scarcely.

CD3ζ based TMDs can bind to intracellular CD3ζ signaling domain through heterodimer formation bringing about the decrease in the antigen binding threshold which promotes better immunological synapse establishment. This leads to the induction of proper suited cell signaling, followed by an optimum level of cell activation, finally resulting in unleashing sufficient

cytotoxic effect that ensures a comparatively better anti-neoplastic therapeutic response. The infrequently utilized TMD, FcεRIγ, in conjugation with the intracellular CD3ζ chain also operates in a similar manner along with exhibiting a comparable expression of stability. However, both of the TMDS demonstrate compromised stability compared to CD28 based domains.

On the other hand, CD8α and CD28 originated TMDs and their respective spacers, in spite of having different degree of TNF and IFNγ cytokines, resulting in making CD28 more aggressive, both TMDs are being used routinely in approved therapies as well as various preclinical and clinical trials nowadays as CARs designed with them have been shown to have the utmost extent of expression and stability on cell membrane. CD19 targeting CD8α-centered-CARs have been shown to provide full-remission to an overwhelming nine-tenth of the participating patients from their relapsed and refractory Acute Lymphoblastic Leukemia (ALL) according to a clinical study done in 2014.

Nevertheless, effective inter-domain pairing is being proven to be crucial through recent discoveries. For example, ICOS (Inducible T Cell Co-stimulator) based TMDs has been proven to work better than CD8α TMDs, to be complemented with ICOS intracellular region resulting in excellent CAR-T cell tenacity and anti-cancer implication. Again, CD4 TMDs function as a great pair with the IgG based spacer domains due to their likewise magnitude of cytokine emission coupled with the paralleled cytotoxicity in relation to the CD8α TMDs.

2.4 Endo Domain

2.4.1 Intracellular Signaling Domain (ISD)

It is the most important and distinguishing element of the CAR structure. To actively avert the cytotoxic effect of T-lymphocytes towards an intended antigen parallel to the therapy, it is crucial to commence the obligatory signaling cascade through a suitable ISD post the ARD engagement. Inherently, CD3ζ and FcRγ take on the ISD roles in the TCR of the T-cells and NK, myelocytic cells respectively where they both house the immunoreceptor tyrosine based activation motifs (ITAMs); which is an essential intracellular signal mediator as well as innate immune response regulator that activates the CAR-T cells by catalyzing downstream signal transducing reactions after initial phosphorylation of tyrosine amino acids through lymphocyte specific protein tyrosine kinase enzyme (Lck) that eventually elicits cell-mediated responses like- cell activation, proliferation and cytokine release for antitumor effects.

CD3ζ and FcRγ both are extracted to be used as the ISDs in the engineered CARs with CD3ζ being chosen as the predominant one. They both were compared comparatively where in spite of having different characteristics of CD3ζ responding to antigens with an easily achievable threshold and FcRγ being surface-expressed concentratedly, both of them showed recognition based Intraleukin-2 (IL-2) excretion causing eventual TAA expressing necrotic cell lysis. However, CD3ζ ISD based CARs showed to release cytokines as well as cause cytolysis more effectively is several in vitro and in vivo studies in spite of FcRγ having to step up in some cases of incomplete phosphorylation-based activation hindrance, causing CD3ζ to be selected as primary choice for CAR construction in most of the approved as well as preclinically and clinically studied CAR-T therapies currently.

Additionally, options like- CD3ζ similar isoform CD3η, CD3ε, cell signaling cascade consequential molecules like- tyrosine kinase Syk have also been compared studied, used along with DAP10, DAP12 which have been considered as potential choices for ISDs.

2.4.2 Costimulatory Domain (CSD)

To design an ideal endodomain, alongside the primary ISD, one to several other secondary signaling stimulants known as the costimulatory domains are added on, to amplify the preliminary signal ensuring a complete and constructive cellular response including improved activation, proliferation and cytokine release causing eventual necrotic cytolysis. They also confirm improved CAR resilience and subsequent longevity. Generally, CSDs are obtained from the CD28 and TNFR lineages, examples specifically being- (CD28, ICOS) and (CD27, 4-1BB (CD137, OX40) respectively (Guedan et al., 2019). Among these, CD28 and 4-1BB are the only ones getting FDA approval due to the increased percentage of patient therapy response rate, thus being employed most frequently (Sterner, R.C & Sterner, R. M., 2021).

When compared, in spite of being proven to be quite comparable in some hematological oncologic conditions, they have shown to have many significant differences. For example, CD28 CSD produces preliminary co-stimulation signal after initial naïve ARD T cell (thymus cell) engagement facilitating inceptive T cell activation and heightens signaling magnitude reducing the threshold for activation; where on the other hand, 4-1BB assists the CAR-Ts through secondary co-stimulation signaling to improve as well as sustain immune response for prolonged time. Functionally, after maturation into memory cells, CD28 incorporated CAR-Ts adapt as effector and 4-1BB ones as central kinds. Metabolically, the effector memory T cells rely on aerobic glycolysis (the Warburg effect) and central memory T cells on oxidative

phosphorylation; alongside their minimized and intensified mitochondrial biogenesis consecutively. To mention, CD28 heavily impacts amplified IL-2 expression through appointing the second Growth Factor receptor bound protein and Lymphocyte specific protein tyrosine kinase to CARs intracellular tail portion, associated with effector cell proliferation along with IL-4, IL-10 compared to 4-1BB that uses TNF receptor associated factors (TRAFs) alike its relative members; together with CD28 based upregulation of IL-6, IFN-γ and TNF- α cytokines in general. In addition, CD28 escalates CAR presentation on T cells along with their expansion and ensures immunological vigilance. Alternatively, 4-1BB ensures to be more advantageous in stimulating better multifunctional CD4 and CD8 based T lymphocyte response in spite of being similar in catalyzing CAR-T proliferation, counteracting co-stimulation deprived hypoactivity known as anergy and aid cytokine secretion as CD28. Regarding tenacity, 4-1BB CAR-Ts have been found to be much more resilient than CD28 CAR-Ts, making them with increased longevity. Thus, in spite of CD28 ensuring a better proliferation rate, the resilience of 4-1BB ensures an enriched and sustained T-cell assemblage (Mohanty et al., 2019). In two significant 2011 and 2018 published clinical studies regarding responsiveness, safety and resistance of CD19 based CAR-T therapies on various B-cell leukemia with several different CSDs, 4-1BB based memory T cells were found to be evident even after multiple years when tested providing protection, where CD28 based memory T cells became unidentifiable just after three months. Assumingly, it is due to 4-1BB's competence in managing T cells' exhaustion related outcome in chronic malignancy, in addition to its aptitude in triggering antigen-independent signaling (Guedan et al., 2019; Brentjens et al., 2011; Fraietta et al., 2018). All in all, CD28 CAR-Ts show repeated prompt cellular response with transient tenacity and 4-1BB CAR-Ts exhibit comparatively moderate cell-mediated response with persistent tenacity making them optimum

choices for immediate tumor clearance in treatments like-bridge therapy and consistent re-enforcing response for long-term therapies respectively. Lastly, 4-1BB has been found to be a bit more superior regarding in vivo animal models than CD28 contradicting the in vitro comparisons; but both domains are equally used in practice (Oldham & Medin, 2017).

Several other CSDs are also being considered significantly due to their recent promising preclinical results. For example, CD27 and OX40 domains being arose from the same superfamily as 4-1BB, shows similar characteristics like- aiding in persistence better than CD28 and initiating secondary co-stimulation along with improved tenacity and potency respectively. Conversely, ICOS being related to CD28, elicits a more robust PI3K stimulation causing it to transduce better signals through the PI3K based Akt signaling pathway. ICOS also catalyzes the deviation of the CD4+ T cells towards being differentiated into T_H1 and T_H17 cells as well as their sustainability that improves the helper T action, T cell grafting and overall anti-neoplastic activity through improved resilience, a bit better than CD28 and 4-1BB. To mention, CAR-Ts can be most advantageous and effective when specific T cells pair with particular CSDs. Example- ICOS influencing CD4+ T cells and 4-1BB impacting CD8+ T cells specifically in improving their response and resilience. Newer options like- NKG2D, DNAM-1 (CD226), DAP10, MYD88 (CD40), CD40L (CD154), CD244, GITR (Glucocorticoid Induced Tumor Necrosis Factor Receptor) etc. are also showing some positive results to be assessed for future prospect.

All in all, important therapeutic aspects of CAR-Ts like- cellular activity, metabolic pathways, endurance etc. heavily depend on the genre of selected CSDs in the modified CAR assemblies. Therefore, thoroughly thoughtful CSDs as well as ISDs should be incorporated based

on divergence in ARD affinities, TAA expression, risk of adverse effects and type of therapy needed for most effective therapeutic outcome.

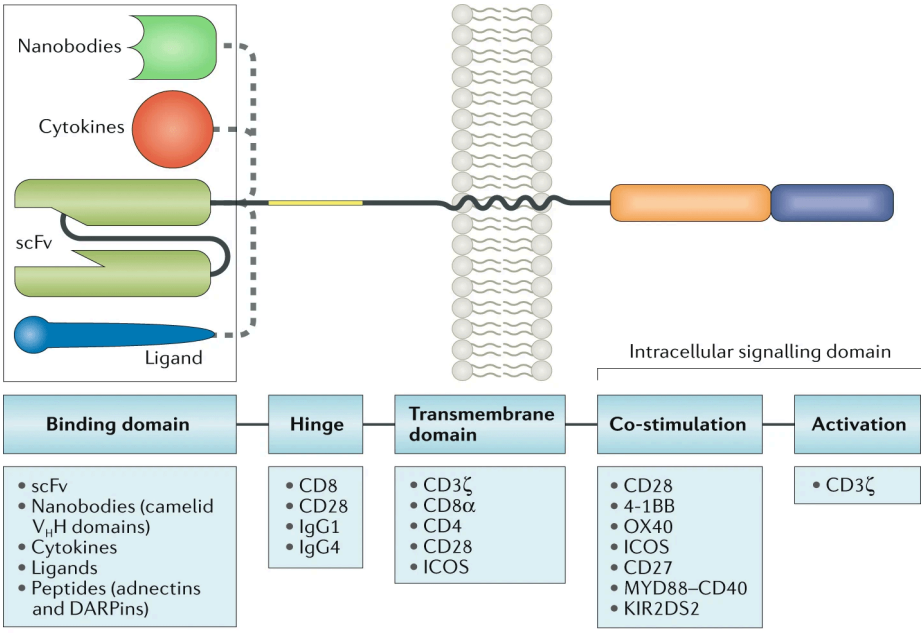

Figure 5. Domains of the CAR structure (Rafiq et al., 2019).

Chapter 3

CAR-T Production and Administration Process

For infusing CAR-T cells into patients' circulatory system, due to being an autologous approach, patients have to provide the manufacturing facility with their own T-cells which then will be modified to produce tumor associated antigen targetable CAR-T cells and administered through intravenous route.

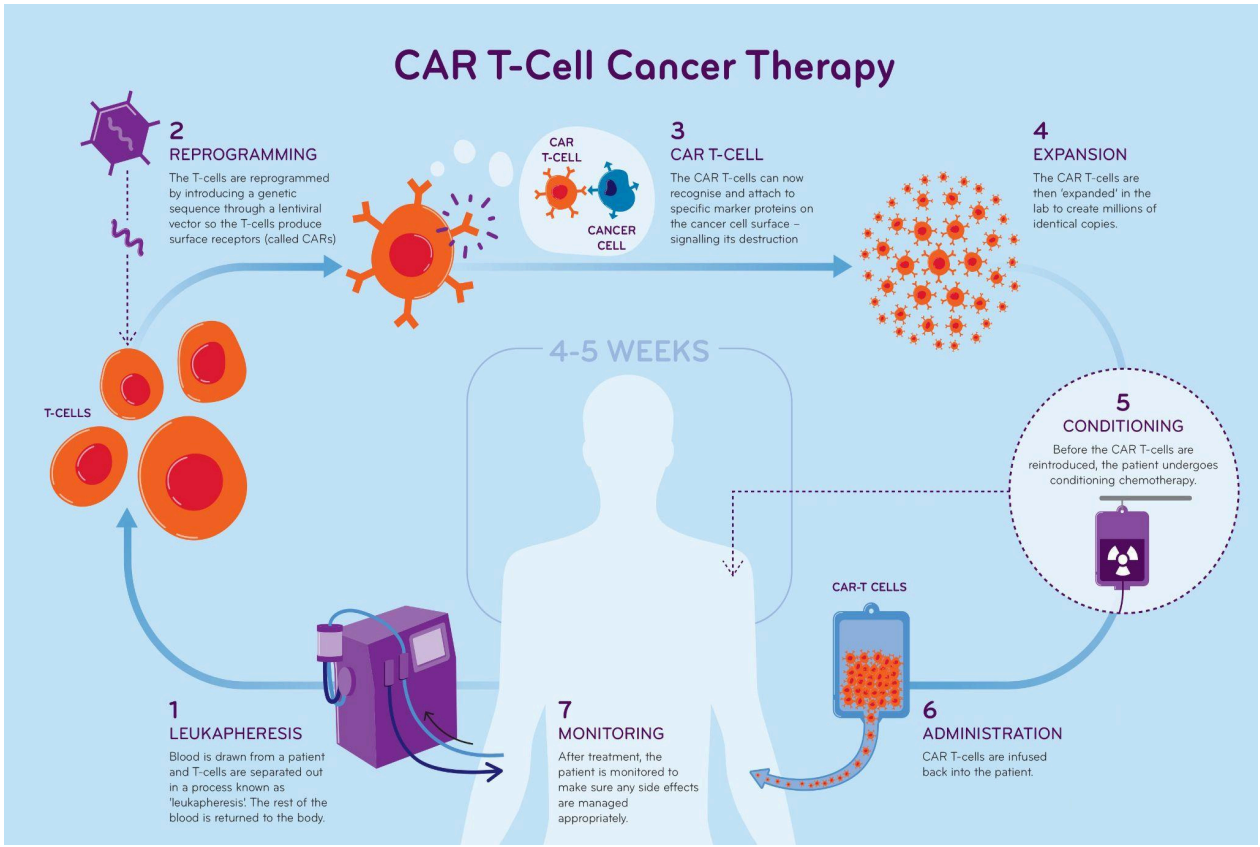

Figure 6. Complete administration process of the CAR-T cell therapy (Malaghan, CAR-T cell therapy).

The CAR-T manufacture and administering process can be divided into several significant steps:

Leukapheresis

The initial phase of CAR-T production involves collecting blood sample from the patient through phlebotomy. After that, the peripheral blood mononuclear cells (PBMC: monocytes, lymphocytes, natural killer cells, dendritic cells) are segmented alone from the other blood cells through density-gradient centrifugation mediated apheresis (a laboratory technique to separate certain blood compound) using Ficoll-Paque density gradient media. Otherwise or, afterwards, leukapheresis is directly used which is a specific type of apheresis to isolate lymphocytes (B cell, T cell and Natural Killer cell) from the whole blood or, PBMC (Tahmasebi et al., 2020; De Marco et al., 2023).

T-cell activation

The isolated CD4+ T cells , CD8+ T cells or, if per requirement, NK-cell will have to get activated. It occurs either naturally through pre-meddled PBMC presence mediated enrichment or, in presence of anti-CD3 and anti-CD28 monoclonal antibody (MAB) coated beads, plate or, nanomatrix, resulting in activation of the isolated T cells (Tahmasebi et al., 2020; De Marco et al., 2023) .

Reprogramming of T-lymphocyte gene

In this step, using any of the numerous available viral transduction or non-viral transfection methods, the DNA of T-cells is genetically reprogrammed. The CAR-transgene is incorporated to the T-cell genome resulting in expression of a complete CAR receptor. To mention, the viral gene transfer method using the lentiviral or, γ-retroviral vector is the most commonly used option due to top-notch precision and effectivity; but non-viral transposon method is also used due to its

cost-efficiency. From this very moment, immediately after CAR-expression on T cells after transcription and translation of transgene, they are readily capable of targeting the tumor associated antigen/s (TAA/s) through their ARD of specific structure (Tahmasebi et al., 2020; De Marco et al., 2023).

CAR-T cell expansion

The modified limited number of CAR-T cells are expanded in number again through anti-CD3 and anti-CD28 MAB bead mediated co-culture with the CAR-T cells and eventual transmission into bioreactor (Tahmasebi et al., 2020).

Conditioning Chemotherapy

Before infusing patients with the newly modified and expanded CAR-T cells, patients are made to go through several lymphodepleting conditioning chemotherapy session using fludarabine and cyclophosphamide to eliminate any remaining T-cell to not cause any potential immunogenicity mediated graft (though autologous but still modified CAR-T cells) rejection and be fruitful in its in vivo expansion and long term persistence. This lymphodepleting regime is also presumed to have cytotoxic effect against immunosuppressive (e.g., Tregs, myeloid derived suppressor cells) cell; thus, aiding the graft acceptance probability (Tahmasebi et al., 2020; De Marco et al., 2023).

CAR-T Administration

Lastly, a large quantity of modified and expanded CAR-T cells are infused to the patients circulatory system intravenously. It is estimated to have infused $1x10^8$ number of modified CAR-T cells as per approved CAR-T drugs' instruction (De Marco et al., 2023).

Chapter 4

Chimeric Antigen Receptor (CAR) Gene Transfer Methods

Central to the success of CAR therapy is the efficient delivery of CAR constructs into patient cells, particularly T cells, to enable them to recognize and target specific antigens expressed on the surface of tumor cells. Several methods of delivery have been developed, each with its own advantages and limitations. In this chapter, the three main methods of CAR delivery are discussed, namely, viral transduction, transposons, and CRISPR/Cas9. In the last section, a few other non-viral methods or CAR administration that have gained significant attention have also been elucidated.

4.1 Viral Transduction

Viral transduction is a pivotal method employed in gene therapy, facilitating the delivery of therapeutic genes into target cells. This section provides a comprehensive examination of viral transduction methods, focusing on γ-retrovirus and lentivirus vectors, their advantages, limitations, and current applications.

Viral vectors like γ-retrovirus, lentivirus, adenovirus, and adeno-associated virus (AAV) are fundamental in gene therapy endeavors (Miliotou & Papadopoulou, 2018). γ-retroviral vectors, derived from the Retroviridae family, including γ-(onco)retrovirus, have historically

been extensively used in gene therapy (Oldham & Medin, 2017). Lentiviral vectors, derived from human immunodeficiency virus (HIV-1), offer efficient transduction capabilities and stable integration into both dividing and non-dividing cells (Mohanty et al., 2019), making them a promising tool for gene delivery.

γ-retroviral vectors have shown effectiveness in gene therapy applications, as evidenced by their successful correction of severe combined immunodeficiency-X1 (SCID-X1) in humans. However, concerns regarding insertional mutagenesis and subsequent leukemia development have been raised. To address safety concerns, self-inactivating vectors devoid of promoter/enhancer regions have been developed, minimizing the risk of insertional mutagenesis. Despite these challenges, γ-retroviral vectors remain a prevalent choice in clinical trials, particularly in chimeric antigen receptor (CAR) T-cell therapy (Oldham & Medin, 2017).

Lentiviral vectors offer distinct advantages over γ-retroviral vectors, including reduced genotoxicity and the ability to infect both dividing and non-dividing cells. Studies have reported lower genotoxicity associated with lentiviral vectors, although concerns regarding deregulated gene expression and clonal dominance persist. Despite these limitations, lentiviral vectors have gained traction in CAR therapy due to their safety profile and efficient transduction capabilities (Oldham & Medin, 2017).

In addition to γ-retrovirus and lentivirus, adenovirus and adeno-associated virus (AAV) have been explored for gene therapy applications. Adenovirus exhibits broad tropism and transient gene expression, making it suitable for certain therapeutic interventions. Adeno-associated virus (AAV) offers a promising vector platform due to its broad serotype diversity and non-pathogenic nature. However, challenges such as episomal transgene persistence limit its utility in long-term gene expression (Miliotou & Papadopoulou, 2018).

Viral transduction involves the integration of therapeutic genes into the host genome mediated by viral vectors. Retroviral vectors utilize packaging cell lines to produce stable virus particles for gene delivery. Upon transduction, the viral genome is integrated into the host cell genome, leading to stable expression of therapeutic genes. Lentiviral vectors, derived from HIV, follow a similar mechanism, albeit with reduced genotoxicity and broader cell tropism (Guedan et al., 2019).

Safety considerations are paramount in viral transduction-based gene therapy, necessitating stringent adherence to clinical standards (Miliotou & Papadopoulou, 2018). Retroviral vectors have undergone modifications to mitigate genotoxicity, including the use of self-inactivating vectors and the deletion of oncogenic regions (Oldham & Medin, 2017). However, challenges such as insertional oncogenesis and immune-mediated toxicity persist, highlighting the need for ongoing research and development (Oldham & Medin, 2017).

Despite challenges, viral transduction remains a promising avenue for gene therapy applications, particularly in CAR T-cell therapy (Oldham & Medin, 2017). Continued advancements in vector design, safety optimization, and manufacturing scalability hold the potential to overcome current limitations and expand the therapeutic utility of viral transduction methods (Miliotou & Papadopoulou, 2018).

4.2 Transposons

Transposons, or jumping genes, have emerged as promising tools for gene therapy, offering an alternative to viral vectors. This section focuses on the mechanisms, advantages, limitations, and current applications of two prominent transposon systems: Sleeping Beauty (SB) and piggyBac (PB).

Transposon-based gene transfer is a non-viral process that utilizes transposon DNA and a transposase enzyme for stable gene delivery (Mohanty et al., 2019). Two key transposons, piggyBac (PB) and Sleeping Beauty (SB), employ a cut-and-paste mechanism to facilitate genomic integration. The process involves the recognition and binding of the transposase enzyme to the transposon, formation of a synaptic complex, excision of the genetic element, and subsequent reintegration into the target location. Both SB and PB transposases comprise DNA-binding, transposable catalytic, and nuclear localization domains, facilitating efficient gene transfer (Mohanty et al., 2019).

Transposon-based systems offer several advantages over viral vectors, including reduced genotoxicity and enhanced safety profiles (Oldham & Medin, 2017). Sleeping Beauty (SB) is particularly attractive due to its low enhancer activity, non-pathogenic source, and minimal epigenetic alterations at integration sites. Conversely, piggyBac (PB) exhibits a larger cargo capacity and higher transposition efficiency, making it a preferred choice for certain applications. However, concerns regarding insertional mutagenesis and integration site preferences necessitate further investigation (Oldham & Medin, 2017).

Transposon-based gene transfer systems have gained prominence in chimeric antigen receptor (CAR) T-cell therapy (Miliotou & Papadopoulou, 2018). Sleeping Beauty (SB) and piggyBac (PB) transposons enable stable integration of CAR genes into T cells, facilitating long-term transgene expression. SB-modified CAR T cells have demonstrated efficacy in preclinical and clinical studies, offering a safe and effective therapeutic option for cancer treatment. Similarly, PB-mediated CAR T therapy shows promise in generating functional CAR T cells, albeit with limited clinical evaluation.

Efforts to improve transposon-based gene transfer methods focus on enhancing integration efficiency and minimizing genotoxicity (Guedan et al., 2019). Strategies such as utilizing minicircle vectors for SB-mediated transposition offer safer integration profiles and reduced risk of insertional mutagenesis. Similarly, advancements in PB transposon technology aim to optimize gene transfer efficiency while mitigating integration site preferences.

Despite their potential, transposon-based gene transfer methods face challenges, including inefficient integration and limited clinical translation (Guedan et al., 2019). Addressing these challenges requires further research into enhancing integration efficiency, optimizing safety profiles, and expanding clinical applications. Minimizing off-target effects and improving scalability are critical for the widespread adoption of transposon-based gene therapy approaches. Transposons represent promising tools for gene therapy, offering safer and more versatile alternatives to viral vectors. Sleeping Beauty (SB) and piggyBac (PB) transposons enable stable integration of therapeutic genes, particularly in CAR T-cell therapy. Despite challenges, ongoing research efforts aim to optimize transposon-based gene transfer methods for enhanced efficacy and safety, paving the way for their broader clinical application in the treatment of genetic disorders and cancer.

4.3 CRISPR/Cas9

The introduction of CRISPR/Cas9 technology marked a significant advancement in CAR therapy delivery. This innovative approach utilizes the potential of Cas9, a type II CRISPR protein, which can be precisely directed to target virtually any part of the genome with the assistance of a short RNA guide (gRNA), effectively functioning as an endonuclease. Cas9 can be delivered into cells using various techniques, such as liposome-mediated transfection, electroporation, chemical

transduction, or integration into a viral genome, either in the form of Cas9 protein/gRNA ribonucleoprotein (RNP) complexes or as a plasmid. Upon delivery, a donor template, typically in plasmid form, aids in integrating the desired transgene into the genome through homology-directed repair (HDR).

Utilizing CRISPR technology holds significant promise for enhancing the uniformity and survival rates of CAR T cells in experimental settings. Specifically, incorporating CAR sequences into the "alpha constant"-TRAC endogenous T-cell receptor locus has been found to boost the effectiveness of CAR T cells in combating targets. However, the effectiveness of genetic editing to integrate CAR remains relatively modest, with current success rates peaking at around 20%, and concerns linger about unintended genetic alterations. Despite these hurdles, ongoing investigations strive to refine CRISPR-based gene editing methods to amplify both the accuracy and efficacy of CAR integration within T cells.

Clinical trials utilizing CRISPR/Cas9 have begun to explore the knockout of inhibitory signals, such as PD-1 and the endogenous TCR, in T cells of patients with various cancers. In these trials, the primary objective is to eliminate the random integration of viral delivery systems while exerting control over CAR integration. However, the precise consequences of removing inhibitory signals from T cells remain unclear, raising concerns about the potential for uncontrolled cell proliferation or severe autoimmunity. Nonetheless, these trials represent crucial steps toward advancing the clinical application of CRISPR/Cas9-mediated CAR therapy and addressing challenges associated with viral vector delivery systems (Miliotou & Papadopoulou, 2018).

4.4 Other Non-viral Methods

Various non-viral techniques have surfaced as favorable strategies for delivering chimeric antigen receptor (CAR) therapy, presenting benefits like diminished genotoxicity and improved safety characteristics in contrast to viral vectors. This section explores the latest advancements in non-viral CAR therapy administration processes, focusing on electroporation and RNA-based delivery systems.

Innovative approaches utilizing nanotechnology offer promising solutions for the targeted delivery of CAR genes, addressing challenges in tumor targeting and therapy cost-effectiveness. Smith et al. demonstrated the efficacy of polymeric nanocarriers in delivering leukemia-specific CAR genes to host T cells, targeting specific ligands in situ. Notably, magnetic nanoparticles, such as Fe3O4, have been investigated for gene delivery, exhibiting stable cell transfection and plasmid transfection, along with gene silencing and splice correction capabilities. Recent studies, including research by Smith et al. at the Fred Hutchinson Cancer Research Center, Seattle, underscore the effectiveness of polymeric nanoparticles in introducing CAR genes into T cell nuclei and selectively recognizing leukemia cells. Utilizing nanoparticles for gene delivery presents advantages over viral vectors, such as minimizing nonspecific inflammatory or proliferative effects, thus holding significant potential for enhancing CAR T cell therapy efficacy.

Electroporation involves exposing target cells to electric fields, which transiently disrupt their cell membranes, allowing the absorption of charged molecules (Mohanty et al., 2019). Advancements in this technique include the introduction of square-wave and pulse-based electroporation systems, showcasing novel strategies in this domain. For instance, the Lonza Nucleofector II Electroporator has demonstrated efficacy in genetically modifying T cells by

employing specific electric parameters and tailored electroporation buffers. Studies indicate that electroporation of human T cells leads to approximately 40-60% gene expression and maintains 80% cell viability. However, a notable drawback of electroporation lies in its suboptimal transfection efficiency and the potential for excessive cellular damage (Mohanty et al., 2019).

Nevertheless, RNA-based electroporation offers a non-integrative, transient method for CAR expression, reducing the risk of insertional mutagenesis (Oldham & Medin, 2017). Clinical-grade mRNA is cost-effective and can be efficiently delivered into T cells using electroporation, with gene transfer efficiencies exceeding 75% and minimal effects on cell viability. The transient expression of CARs mediated by RNA-based electroporation allows for the assessment of on-target and off-tumor effects of CAR therapy, crucial for predicting toxicities in humans (Oldham & Medin, 2017).

RNA-based electroporation enables rapid changes in CAR design and offers safety advantages over long-term, integrating viral expression systems (Miliotou & Papadopoulou, 2018). The temporary presence of CARs, facilitated through RNA-mediated mechanisms, guarantees the eventual elimination of CARs from the patient's system without necessitating the incorporation of suicide genes. Additionally, systems reliant on RNA-based transfection offer streamlined integration into good manufacturing practice (GMP)-compliant frameworks, potentially resulting in cost reductions and the streamlining of release testing procedures.

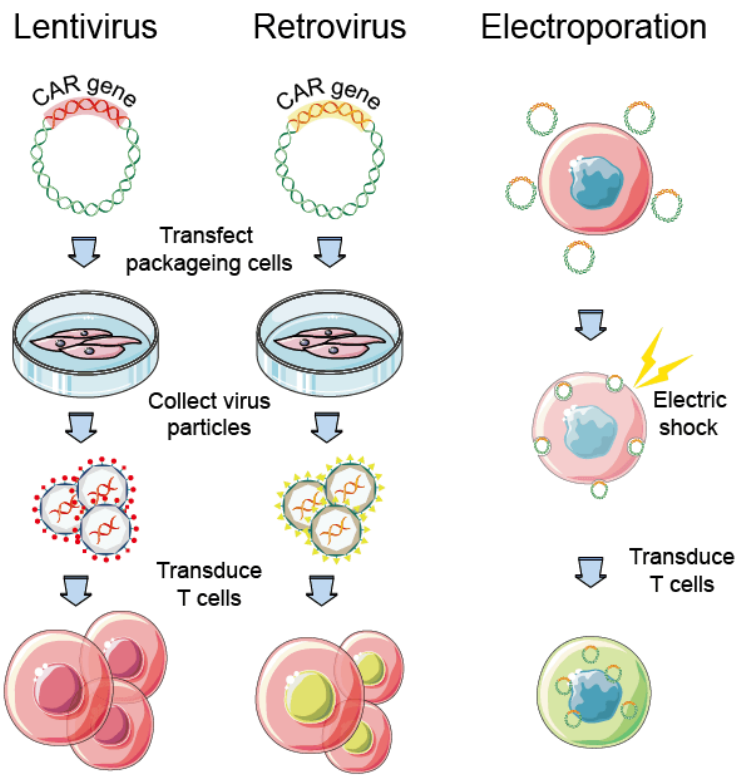

Figure 7. Two viral (Lentiviral and Retroviral) and one Non-viral (Electroporation) CAR gene delivery methods to the T cell (Creative Biolabs, n.d.).

RNA-based electroporation technology is being investigated in early clinical trials for CAR therapy, demonstrating promising results in preclinical models of B cell malignancies. Multiple administrations of RNA-based CAR T cells may be required to achieve long-lasting responses, highlighting the need for further optimization and clinical evaluation. Despite challenges such as electroporation-related apoptosis, RNA-based electroporation holds significant potential for enhancing the safety and efficacy of CAR therapy administration (Miliotou & Papadopoulou, 2018).

Furthermore, advancements in gene editing tools have ushered in a new era for CAR T cell therapy (Guedan et al., 2019). While much attention has been focused on developing

off-the-shelf universal CAR T cells from allogeneic healthy donors, the predominance of ongoing clinical trials employs autologous CAR T cells. This preference stems from concerns that allogeneic CAR T cells could provoke graft-versus-host disease (GVHD) due to the recognition of recipient alloantigens by the T cell receptor (TCR). To mitigate the risk of GVHD, strategies have been devised to eliminate the TCR from allogeneic CAR T cells via genome editing techniques.

Notably, the infusion of gene-edited universal CD19-CAR T cells in pediatric patients with acute lymphoblastic leukemia (ALL) yielded complete responses, affirming the feasibility of this approach. Efforts to prevent CAR T cell rejection by the recipient also include strategies such as editing the HLA class I expression genes, namely HLA-A or β2-microglobulin. Moreover, gene editing endeavors aim to selectively inhibit the expression of T cell-suppressive genes, with particular focus on targeting the checkpoint inhibitor PD-1. Knockout anti-MUC1 CAR T cells lacking PD-1 are entering clinical trials, reflecting the prominence of this gene as a therapeutic target.

Endonucleases with target-specificity have been leveraged to insert transgenes into loci relevant for T cell biology, offering a more precise approach compared to random insertion strategies. For instance, targeting CAR insertion into the TCR locus has been shown to result in more uniform expression and increased potency of CAR T cells (Guedan et al., 2019).

In summary, non-viral methods hold great potential for improving the delivery and effectiveness of chimeric antigen receptor (CAR) therapy. They offer benefits such as decreased genotoxicity and enhanced safety profiles when contrasted with viral vectors. This overview highlights the recent advancements in non-viral CAR therapy administration, encompassing innovative methods such as electroporation, RNA-based delivery systems, and

nanotechnology-driven approaches. Electroporation, despite its limitations in transfection efficiency and cellular damage, offers a transient yet effective means of CAR expression. RNA-based electroporation emerges as a non-integrative, transient method with significant potential for enhancing CAR therapy safety and efficacy. Moreover, gene editing technologies provide opportunities for fine-tuning CAR T cell properties and improving therapeutic outcomes. The ongoing exploration of non-viral methods underscores their importance in advancing CAR therapy towards broader clinical applications, necessitating further optimization and clinical evaluation to realize their full potential.

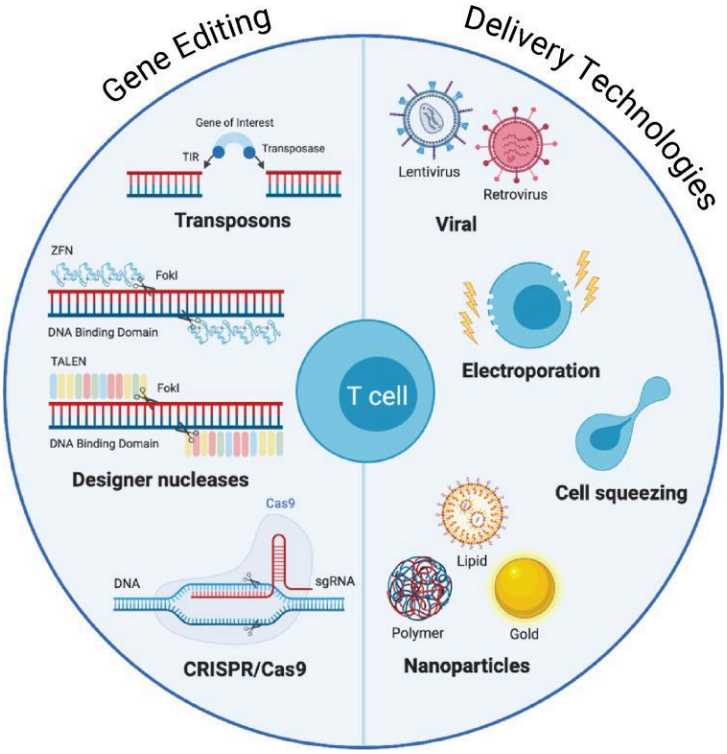

Figure 8. Every type of gene transfer methods applicable in CAR-T generation through CAR gene transfer (Atsavapranee et al., 2021).

Chapter 5

Mechanism of Action

In this chapter, we delve into the intricate mechanism of action underlying Chimeric Antigen Receptor (CAR) therapy. We examine immune synapse formation, CAR activation, and the delivery of cytotoxic loaded vesicles. Additionally, we explore apoptosis induction, elucidating how CARs trigger programmed cell death, and immune response, including mechanisms like phagocytosis.

5.1 Immune Synapse Development

Alike T-cell receptors (TCRs) on endogenous as well as modified cytolytic T lymphocytes (CTL), CAR infused T cells also proceed to execute their primary cytotoxic elimination process through immune synapse (IS) formation. IS is a precisely-arranged, particularly-distinguishable composition that works as a distinctive junction formed between the immune cells like CTLs and target cells like- antigen-presenting-cells (Macrophages, Dendritic and B-cells), pathogen-laden or, malignant cells as a result of TCRs-APC (Antigen-MHC Complex on the APC surface specifically)/ CARs-TAA binding in order to enhance communication for specific immune response. Nevertheless, there is some degree of difference between the two making CAR's IS to be a non-classical conformation instead.

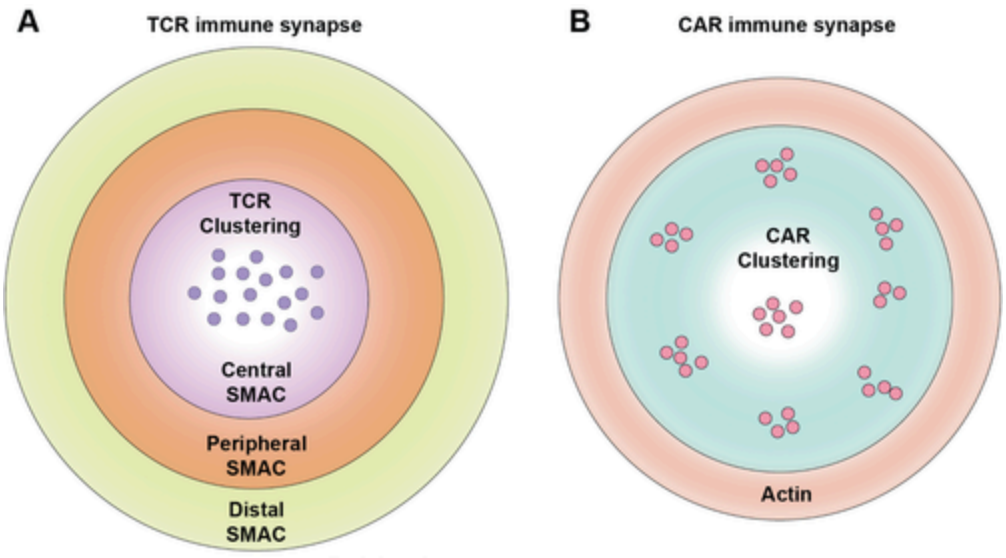

Figure 9. Classical versus Non-classical immune synapse formation in TCR and CAR respectively (Li et al., 2020).

To begin with, the TCR based 'Classical' structure is a systematically ordered framework with 3 strictly divided concentric rings known as the Super Molecular Activating Clusters (SMACs)- central (cSMAC), peripheral (pSMAC) and distal (dSMAC) completing a bull's eye layout and delivering particularized functions; but CAR IS is rather a disorganized structure with not so strictly located components. For example, the cSMAC in the classical IS specifically accommodates assembly of TCR and Lck orderly that specifically assembles effector T cell activation signals from the (antigen-MHC complex)-receptor engagement and causes phosphorylation of the distinct tyrosine fragments of the signaling proteins (e.g., ITAMs from the CD3ζ of TCRs) excreted due to the signaling cascade being triggered, further resulting in appointment of other signaling proteins like- ZAP70 lineage proteins that continues the cascade activating MAP kinase pathway and Nuclear factor of activated T lymphocyte (NFAT) transcription molecules respectively for complete activation of the CTLs, eventually resulting in absolute T cell effector function. On the other hand, CAR based ISs' CARs and Lcks especially

are far more scattered as submicroscopic fragments, not being present strictly in the cSMAC region. Again, The LFA-1 adhesive protein, though being crucial for the strengthening and stabilization of the IS structure, is much less coherent in CAR IS compared to its fixed pSMAC placement in the TCR IS. Moreover, the actin protein made ring shaped complex network in the dSMAC area is much more minimized along with the same actin constructed cytoskeleton in the cSMAC in CAR IS comparatively. Secondly, CAR IS's radius is notably compact facilitating subsequent prompt detachment. To add, through both Reverse-Phase-Protein-Array (RPPA) technology and observation, it has been seen that CAR IS takes significantly less time (less than 2 minutes) in eliciting sufficient proximal signals due to the protein-kinase-C-delta (PKCδ) proximal signaling protein repression and the unnecessity of a bull's eye structure requirement to function respectively resulting in an overall accelerated complete signaling period which further aids in rapid transmission of the lytic granules containing perforin, granzyme and cytokines that eventually causes quicker target cell apoptosis (Benmebarek et al., 2019).

5.2 Cytotoxic Payload Carriage

After the IS formation, T cells continue their cytotoxic journey through either outflux of secretory granules (e.g., cytokine, perforin, granzymes etc.) through the IS or exhibition of Tumor Necrosis Factor (TNF) superfamily-based Ligands (e.g., FasL (CD95L), TNF-Related-Apoptosis-Inducing-Ligand (TRAIL) etc.) causing target cell apoptosis through speedy degranulation or, gradual TNF based signaling events correspondingly. Though after some preliminary steps, both of the pathways end up merging through activating the same caspase cascade causing eventual programmed cell death (apoptosis). However, the

degranulation method ensures both enhanced rapidity and precision in execution compared to the much incremental TNFL-TNFR approach; making it the more desirable MOA.

5.3 Apoptosis Pathways

Apoptosis being a systematized process of a cell's demise, is a rather incredibly refined and intricate ATP driven array of biochemical changes, that mainly has two pathways-Extrinsic and Intrinsic; along with which the Perforin-Granzyme Degranulation pathway is also present being again subdivided into Granzyme A and Granzyme B pathways; which is the primary mechanism of apoptosis in the CAR-T based therapy, even though the extrinsic FasL/FasR pathway is the leading approach otherwise (Benmebarek et al., 2019). To mention, the extrinsic and intrinsic pathways are also known as the death receptor and mitochondrial pathway consequently; which along with the Granzyme B route integrate after some initial differences at the execution lane resulting in the same caspase-run apoptotic changes ultimately; leaving the Granzyme A route to be the only caspase-independent pathway through eventual single-stranded DNA damage (Elmore, 2007) .

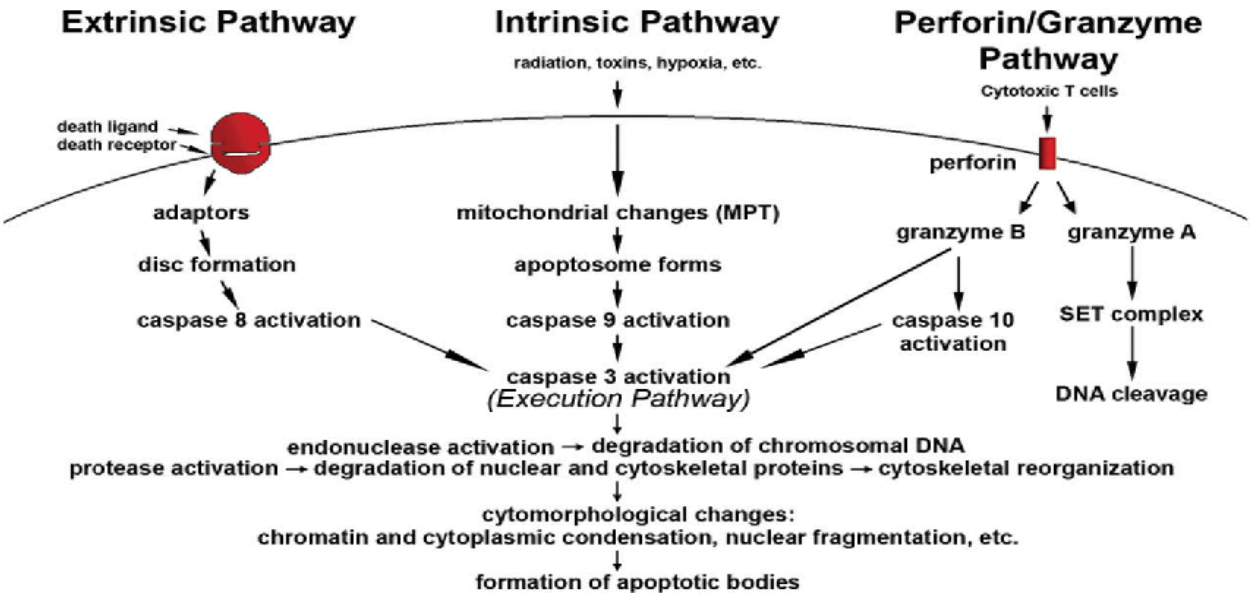

Figure 10. Cascade dependent apoptotic mechanism through intrinsic, perforin/granzyme and extrinsic pathway (Elmore, 2007).

5.3.1 Perforin-Granzyme Degranulation Pathway

CTLs along with other effector cells (e.g., NK cells), many a times, have cytolytic granules readily attached to their microtubules which are essentially excretory lysosomes containing various cytotoxic proteins like perforin and granzymes. These granules head for the centrals SMAC shifting near the conjunction point of the cell membrane after the IS is formed initially. Afterwards, these granules get discharged into the IS intersection gap area, known as the 'synaptic cleft' where the actual degranulation occurs which means the vesicle bound cytotoxic biomolecules secrete out and induce damage to the IS bound target cell. To mention, immediately after the IS formation, there is an increment of the calcium ions in CTLs which consequently cause the ion dependent signaling resulting in initial granule exocytosis.

After degranulation, perforin causes perforation into the cell membrane of the designated tumor cell which provides access to the serine protease enzymes, named granzymes among which Granzyme A and B are the most crucial. Calcium ions also play a great role here in activating the granzymes after entering through the pore from the CTLs that induce apoptosis through different pathways.

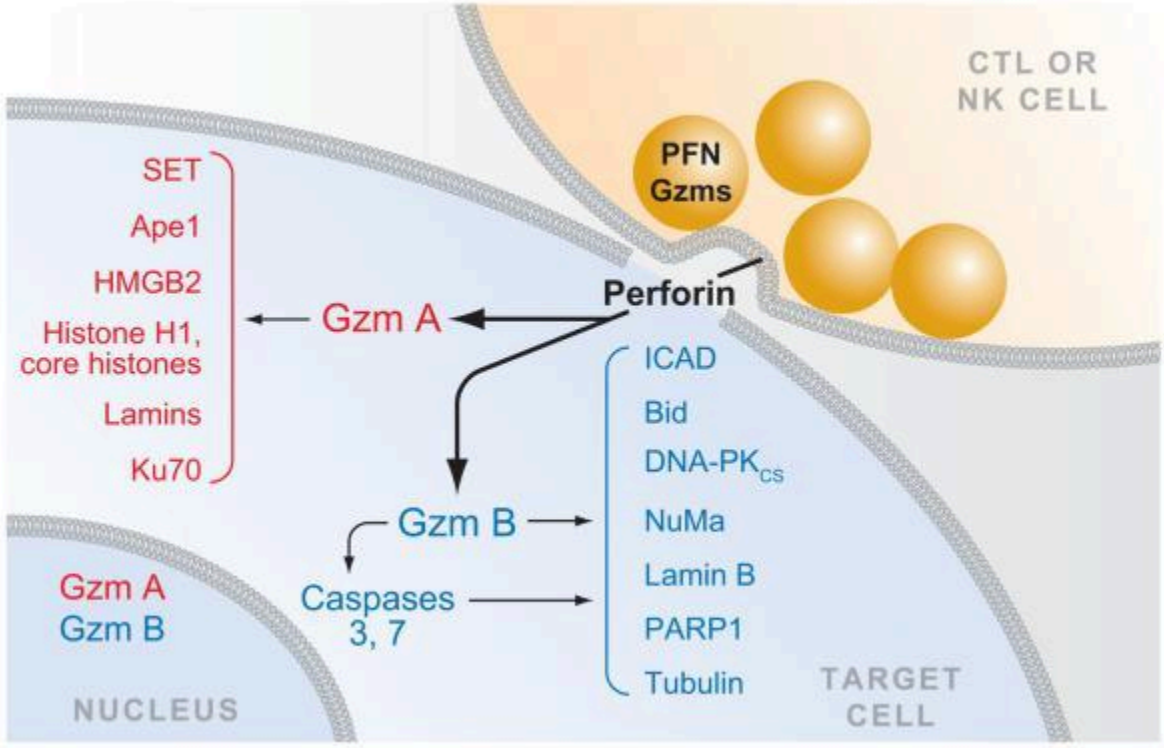

Figure 11. Granzyme A and B secretion to the target cell through perforin mediated degranulation (Chowdhury & Lieberman, 2008).

5.3.1.1 Granzyme B Pathway

Activated Granzyme B (AGB) has been shown to proteolytically target >300 protein substrates where they cleave specific sites containing aspartic amino acid at their C-end of the peptide bond with the proximate amino acid; but only a small number of them have any significance in

initiating apoptosis; among which the procaspases are the most important ones. To note, though AGB can function in a caspase independent way like Activated Granzyme A (AGA), it mainly works through directly or indirectly activating the caspase cascade in various ways.

One of the ways being that AGB can directly activate the executioner caspase-3 (along with caspase -6 and -7 sometimes too) from its procaspase form through the same aspartic acid aimed cleaving which induces the 'common execution pathway' circumventing the previous signaling stages (Elmore, 2007).

In another way, AGB can trigger the caspase cascade indirectly through its proteolytic activity targeting the initiator caspases like- caspase-10/ -8/ -9/ -2 which instigates the entire cascade from beginning to end in various ways. Nevertheless, it should be known that AGB can strictly cleave the initiator caspases which needs further activation through homodimerization in the involved multiprotein complexes like- DISC (Death Inducing Signaling Complex), PIDDsome (P-53 Induced Death Domain Protein Complex) and apoptosome (Thermo Fisher Scientific - US, P53 pathway for apoptosis signaling).

Another way of this AGB mediated apoptosis which intersects with the intrinsic mitochondrial pathway is through the cleavage of the pro-apoptotic elements of the BCL-2 family, the BH3- only member, 'BH3 interacting Death Domain Antagonist (BID)' into its active truncated phase, tBID; which, after proximal relocation, in presence of pro-apoptotic BAX and BAK causes mitochondrial outer membrane permeabilization (MOMP) through transmembrane potential disruption, outer membrane (OM) modification and causing eventual integrity issue. MOMP causes release of the first pro-apoptotic protein group- Cytochrome-C (Cyt-C), SMAC (Second Mitochondria derived Activator of Caspases) which is also known as DIABLO (Direct IAP Binding protein with Low pl) and High-Temperature Requirement A2 (HTR-A2)/ Omi

serine peptidase; along with the second group consisting- Apoptosis Inducing Factor (AIF), Endonuclease-G (Endo-G) and Caspase Activated DNase (CAD)/ DNA fragmentation Factor (DFF)/ DNA Fragmentation Factor 40 kDa subunit (DFF 40). Thereafter, the first group triggers a caspase dependent intrinsic apoptosis where the Cyt-C catalyzes formation of the macromolecular complex, apoptosome comprising of Cyt-C, dATP (Deoxy-Adenosine Tri-Phosphate), Apaf-1 (Apoptosis Protease Activating Factor-1) and procaspase-9 that activates the caspase-9 with the consequent onset of caspase-3, -6 and -7. On the other hand, Inhibitors of Apoptosis Proteins (IAPs) are suppressed using SMACs and HTR-A2 which ironically removed restrictions on apoptosis.

AGB can also can sabotage the anti-apoptotic Mcl-1.Bim Complex present on the outer membrane (OM); into unleashing another BCL-2 lineage protein, BIM which causes the same MOMP releasing Cyt-C and other proteins triggering the same pathway as BID's (Thermo Fisher Scientific - US, P53 pathway for apoptosis signaling; Rousalova & Krepela, 2010). Alongside, a similar kind of potential disruption (depolarization) induced MOMP can be incited by AGB through splitting HAX-1 present on the OM.

AGB can also act without instigating the caspase-cascade by directly cleaving the important effector-caspase-substrates like BID mentioned above along with the other options like- ICAD, DNA-PK_{CS}, NuMa, Lamin B, PARP-1, Tubulin etc.

5.3.1.2 Granzyme A Pathway

Unlike AGB, AGA based cell death function strictly in a caspase-independent manner through impairing the mitochondrial and resultant nucleolar stability using an unique pathway resulting in identical morphological attributes of apoptosis like- Plasma membrane disruption through bleb

(bulge) formation, mitochondrial activity deterioration through transmembrane depolarization due to loss of mitochondrial electrochemical and resultant membrane potential, Chromatin Condensation, DNA Damage through nick formation and so on (Chowdhury & Lieberman, 2008); where it targets substrates like the SET Complex and its components. Through perforin based perforation and consequent Ca^{2+} based activation, there is an influx of Granzyme A into the cytosol which follows its transportation into the mitochondria using the Tim/ Tom/ Pam pathway; where Tom (Translocase of the Outer Membrane) works in recognition of the mitochondrial destined proteins with specific pre-sequence or, signaling sequence as well as an access gateway of their translocation through the outer membrane, Tim (Translocase of the Inner Membrane) as a transporting gateway of the proteins into the mitochondrial matrix and Pam (Pre-sequence Translocase associated Motor) being the mechano-enzyme complex instigating protein (in this case, Granzyme A) translocation into the matrix through TIM, transforming biochemical energy (released through ATP hydrolysis) to mechanical action (Chacinska et al., 2009; Bolender et al., 2008; Lucken-Ardjomande & Martinou, 2008).

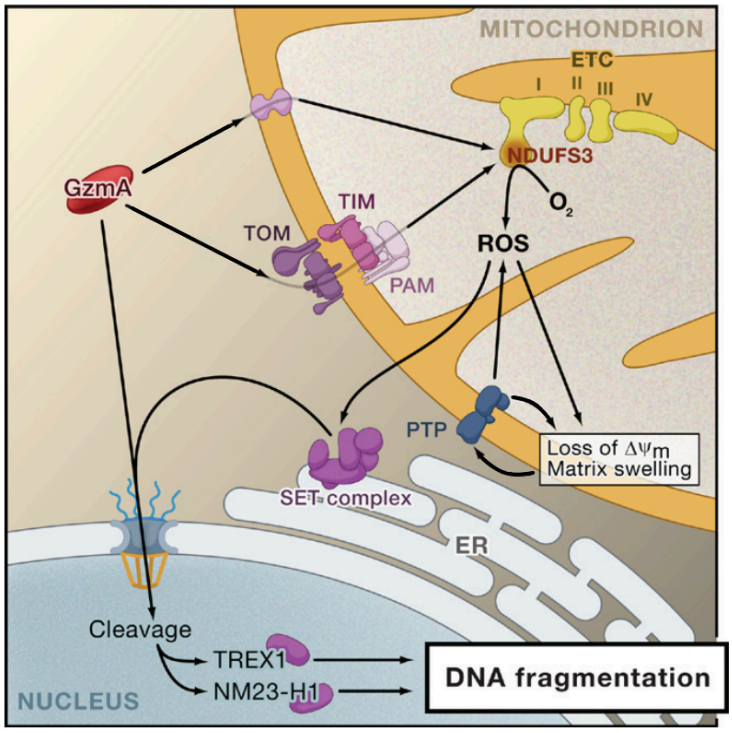

Figure 12. Granzyme A mediated tim/ tom/ pam pathway (Lucken-Ardjomande & Martinou, 2008).

Upon gaining access, AGA directly cleaves the NADH: Ubiquinone Oxidoreductase Core Subunit S3 which is actually the 'NADH Dehydrogenase Iron-Sulfur Protein-3 (NDUFS3) (U.S. National Library of Medicine., NDUFS3 NADH:ubiquinone oxidoreductase core subunit S3 [Homo Sapiens (human)] - gene - NCBI), a crucial component of the first Electron Transport Chain complex (ETC complex-1), otherwise known as the NADH: Ubiquinone Oxidoreductase. The cleavage of NDUFS3 hampers the stability of the ETC complex-1 causing interruption in the oxidative phosphorylation process containing crucial redox reactions, resultantly forming excessive Reactive Oxygen Species (ROS) which is generally considered the primary indication of assured apoptosis without any preliminary MOMP by AGA (Thermo Fisher Scientific - US, P53 pathway for apoptosis signaling; Lucken-Ardjomande & Martinou, 2008; Chowdhury &

Lieberman, 2008). This phenomenon results in extreme oxidative stress on cells and especially on mitochondria; negatively impacting ETC and resultant interruption in electrochemical potential gradient conservancy along with the eventual ATP production (Thermo Fisher Scientific - US, P53 pathway for apoptosis signaling; Chowdhury & Lieberman, 2008). One of the ROS, the 'superoxide anions', when excreted out in the cytosol, catalyzes the transfer of the 'SET Complex', an endoplasmic reticulum-coupled DNA restoring assembly into the nucleus (Yan et al., 2009). Though being a chromatin stability and DNA repair entity with its one significant job being the negation of the problematic AP (apurinic/ apyrimidinic) sites formed due to the profuse oxidative stress in DNA, the SET complex, which is comprised of several noteworthy components like- the three DNase nucleases- endonuclease NM23-H1, a base excision repair (BER) endonuclease, APE-1 and a 3'-5' exonuclease, TREX-1; an NM23-H1 inhibitor, histone methyltransferase and resultant chromatin as well as gene expression altering protein known as SET, transcription and cell cycle regulator pp32 protein (both being multifunctional and PP2A repressors) and HMGB2 that get attach to deformed DNA; transforms into a DNA annihilating contraption. (Chowdhury & Lieberman, 2008; Elmore, 2007; Biosyn, *What are Abasic or AP sites*). This is done so when, followed by the SET complex, AGA also enters the nuclear space and starts cleaving its components like- the SET protein (the NM23-H1 restrainer) and thus, freeing the DNase, transforming the complex into a genomic impairment tool. The activated NM23-H1 makes single-stranded DNA incisions which are then again expanded from the incised 3'-OH pole by trex-1 through consequently dislodging nucleotides continuously; to nullify any attempt of the cell's own DNA repair mechanism using BER method. Additionally, the Ape1, that catalyzes the BER process in the first place gets cleaved and disabled by AGA concurrently along with HMGB-2; it, itself being a noteworthy addition

(Chowdhury et al., 2006). AGA also helps in eventual chromatin relaxation consequently through the protein catabolism of the H1 linker histones along with the core histone's tail elimination process, turning that a more convenient structure for the DNases; at the end, forming single stranded mega-base segments unlike the AGB catalyzed comparatively significantly shorter double stranded oligonucleosome bits (Thermo Fisher Scientific - US, P53 pathway for apoptosis signaling; Chowdhury & Lieberman, 2008; Chowdhury et al., 2006). Thus, by cleaving 3 of the most crucial elements of the SET complex, the DNA, being the whole genetic blueprint, gets disrupted. Overall, by inflicting irreversible damage to the bodies of mitochondria and nucleus as well as their functions, the CAR-T modified cells can specifically target and consequently cause apoptosis through a caspase-independent Granzyme A based perforin/ granzyme pathway.

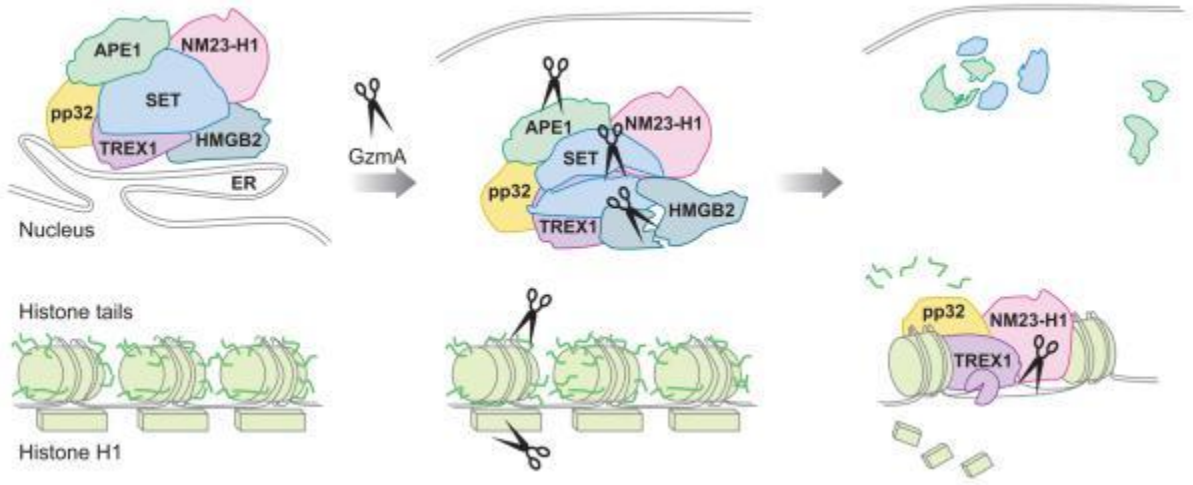

Figure 13. Granzyme A conducted DNA damage pathway of target cell (Chowdhury & Lieberman, 2008).

5.3.2 Fas/ FasL Pathway

Fas/FasL extrinsic death-receptor/death-ligand pathway is the second but comparatively less used pathway of CAR-T mechanism of action. Though, generally, in healthy bodies, it is recognized for its equilibrium maintaining immunoregulatory function, this pathway can also lead to T cell mediated cytolysis in a non-calcium-mediated way with the aid of calcium-reliant secretion of granules which is utilized by CAR-T cells tumor eradication.

Similar to perforin-granzymeB pathway, Fas/FasL is also a caspase dependent pathway where the trimeric Fas-Ligand expressed on CAR-T cell can form a complex by binding to the Fas-receptor presented on the tumor cell. This Fas/FasL complex initiates a downstream signaling cascade in the Fas receptor expressing malignant cell through pro-caspase-8 and Fas-associated death domain (FADD) dimerization sequential death inducing signaling complex (DISC) formation; that eventually, fully activates caspase-8 which then again activates pro-caspase-3 into mature caspase-3. Caspase-3 activates the same 'common execution pathway' like the previously discussed granzyme-B pathway that results in schism of cellular components and eventual apoptosis mediated cell death of the tumor cell.

To mention, Fas/FasL pathway can also facilitate cytolysis in an antigen negative tumor cell population; given that antigen-positive tumor cell population is present nearby that aids this non-antigen-mediated cell-cell interaction based trans-lysis. This statement was verified by Hong and team whose experiment with anti-CD19 and anti-CD30 CAR-T cells showed even positive cytolysis of antigen-negative cells that are in a co-cultured tumor microenvironment with the antigen positive cells. The Fas/FasL pathway initiated cell lysis was augmented in the antigen negative cells once the ligand-receptor complex formed. The importance of this pathway was

again established when the CD95 gene encoding the Fas-receptor protein was experimentally knocked out from the CAR-T-cognate cells which showed significantly diminished activation of caspase-3 resulting in reduced level of lysis in the target cell population.

All in all, the Fas/FasL pathway another apoptosis inducing pathway of T-lymphocytes as well as CAR-T cells, which is though secondary but is an irreplaceable pathway operating in an antigen-negative manner in cases of tumor heterogeneity (Benmebarek et al., 2019).

5.3.3 Cytokine Pathway

The third crucial mechanism of CAR-T driven tumor cell eradication is through the generation of cytokines which results in supplementary-cytotoxicity of the targeted cells in addition to the primary apoptotic pathways through receptor-antigen (granzyme A and B mediated pathway) and/or, receptor-ligand (Fas/FasL pathway). Cytokine expression can be an effective way of dealing with tumor cells, especially of solid tumors that in spite having a varied degree of targetable proteins, becomes obstacle to deal with due to the loss/ escape of tumor antigens and FAs receptor which is an immune escape mechanism that also downregulates ways of initiating any direct interacting based killing mechanism of CAR-T cells. In this context, unconditional cytokine secretion becomes the primary process of eliciting anti-tumor activity against the tumor cells.

Textor and team showed that anti-HER-2-CAR-T cell that is also capable of excreting cytokines irrespectively; this cytokine secretion could sensitize the xenogeneic tumor stroma of a solid tumor and trigger the augmented expression of IFN-γ receptor that can cyclically give opportunity to the pre-secreted cytokine, IFN-γ to bind to the stromal receptors and thus eliminating the stromal cells in an antigen-independent fashion. The anti-HER2-CAR-T also aids

redirection of the tumor-associated-macrophages towards anti-tumor-M1-macrophage through polarization. Implementation of this type of CAR-T fulfilling these 2 conditions of-sensitizing tumor stroma through predetermined cytokine secretion as well as M1-macrophage production showed to be an invincible way of reenforcing tumor cytotoxicity through CAR-T cells. Fourth generation TRUCK-CAR-T cells with its redirected T cells capable of this cytokine-induced killing mechanism in an antigen unrestricted way that utilizes this cytokine-based stromal-killing mechanism through unconditional secretion of cytokine, IL-2 or, IL-12; along with direct ARD-TAA interaction simultaneously (Benmebarek et al., 2019).

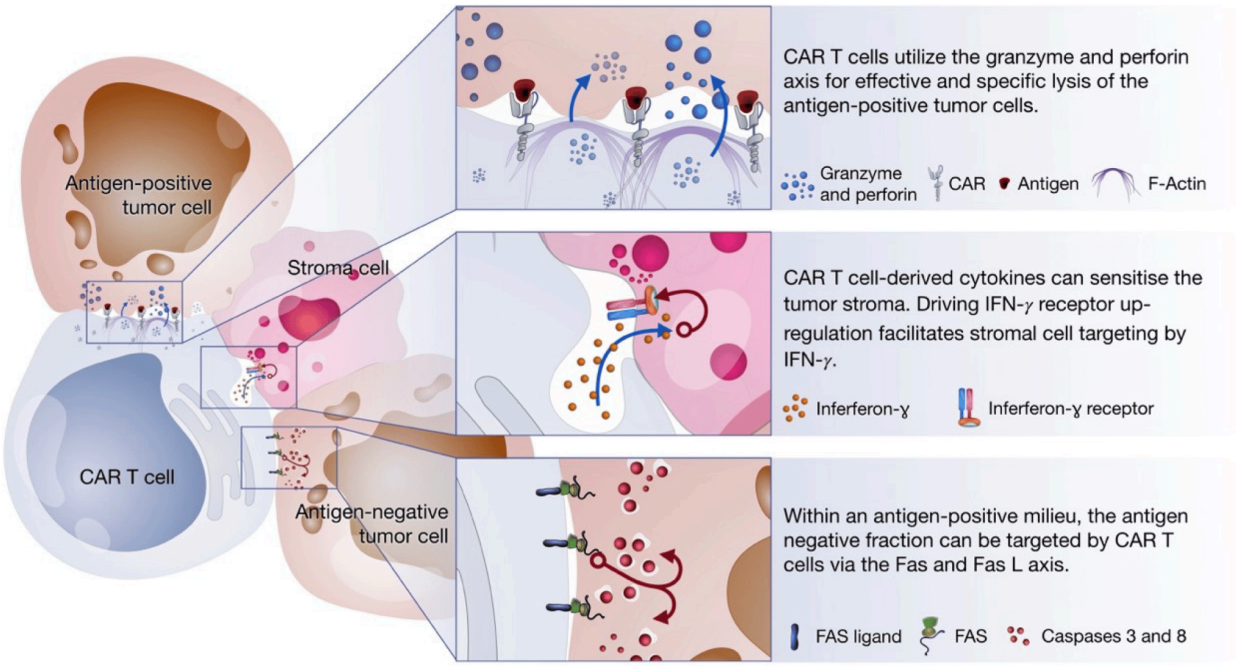

Figure 14. CAR-T driven tumor cytotoxicity and cytolysis through granzyme/perforin, cytokine secretory and Fas/FasL pathway (Benmebarek et al., 2019).

Chapter 6

Generation Classification

As per the preliminary breakthrough in 1989, the CARs can be classified into four distinct generations from first to fourth; based on the tally of costimulatory domains (CSD) conjoined to the intracellular signaling domain (ISD) in their physical structure; in hope to synthesize engineered receptor bodies containing the most ideal endodomain (Muhammad et al., 2017; Miliotou & Papadopoulou, 2018).

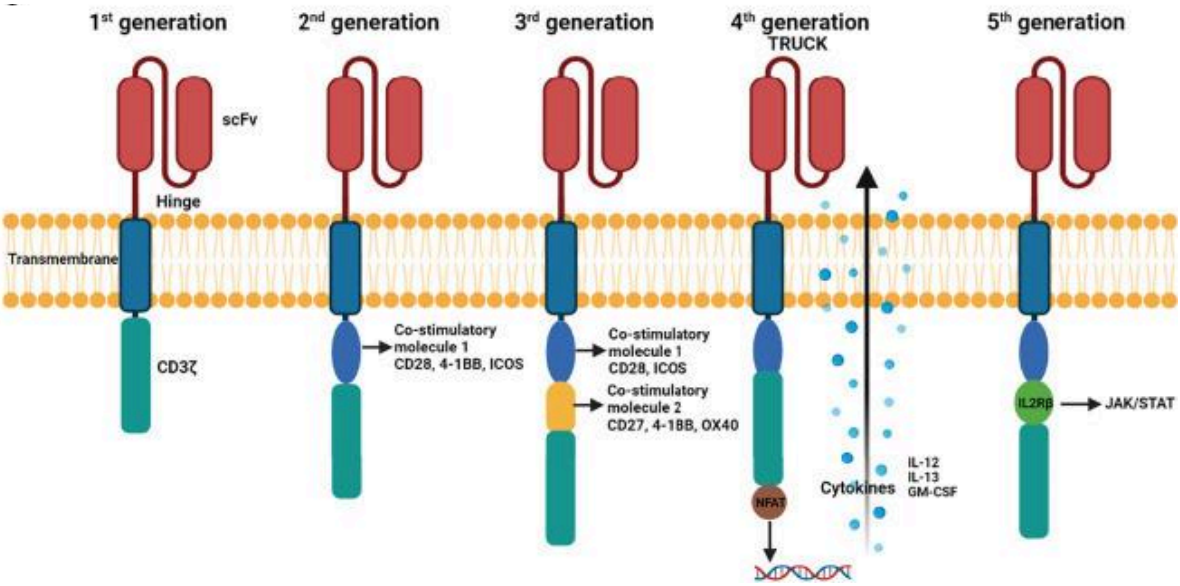

Figure 15. Detailed structure of five generations of CAR-T cell (Kandra et al., 2022).

6.1 First Generation CAR-T Cell

The first-generation CARs, officially established at the end of the 1990s, contain no additional CSDs, making ISD the solely responsible signal conducting entity for generating activation

signals from T-cells after initial recognition through ARD. Here, though both CD3ζ and FcRγ were used to be chosen as ISD initially, for the huge popularity of the CAR-T modified cells especially, CD3ζ was comparatively the more favored option, which catalyzed T-cell activation in an MHC-independent pathway through ITAMs (Sterner, R. C. & Sterner, R. M., 2021). The pioneering 'true CAR', which was initially known as the first 'T-body-approach' was simply modeled using a ScFv and CD3ζ incorporation, which eventually became the first lab-made CAR molecule (Mohanty et al., 2019). CARs of this generation showed promising results in preclinical trials making them eligible for the initial phase of clinical trials against hematological malignancies like- leukemia and lymphoma; and solid tumors like- neuroblastoma and ovarian cancers. But due to the absence of any CSDs, these ITAM signal based responses, singularly, were not impactful enough causing issues regarding immediate sensitivity, longevity and robustness of CAR modified T-cells in vitro and surely enough, did not perform up to the mark in the later phases of clinical studies (Sterner, R. C. & Sterner, R. M., 2021). Nevertheless, somewhat beneficial anti-cancer responses have been observed using α-CD20-CD3ζ and ScFv-CD3ζ CAR-T therapies of this generation against B-cell lymphoma and neuroblastoma respectively through recurrent introduction to the neoplastic microenvironment that paved the path for next generations of CAR development (Mohanty et al., 2019).

6.2 Second Generation CAR-T Cell

Second generation CAR receptors were developed by adding one single CSD to the already existing CAR molecule, behind the ISD, at its endodomain end; and, they effectively verified the generation of a supplemental and overall magnified transmittable signal compared to the first generation CARs making them a superior choice for treatment. Malignant cells expressing TAA,

CD-19 were targeted with CD3ζ based CAR-T's with an additional signaling molecule, CSD; like- CD-28 or, 4-1BB (CD137) in clinical trials inspecting different types of B-cell neoplasia. One of them regarding relapsed as well as refractory B-cell ALL successfully passed the phase-I study providing patients with overall better anti-leukemic results along with complete remission (CR) in almost nine-tenths of the participants which proved the significance of CSDs for additional transmittable signals alongside the dominant one of ISD for elevated proliferation rate and cytokine production (Sterner, R. C. & Sterner, R. M., 2021; Mohanty et al., 2019). After this establishment, these second generation CARs have also been studied in context of other blood cancers like- B-cell chronic lymphocytic leukemia (B-CLL), diffuse large B-cell lymphoma (also known as the non-Hodgkin lymphoma) and multiple myeloma along with previously studied B-ALL; all of which consistently showed a substantial therapeutic outcome (Sterner, R. C. & Sterner, R. M., 2021). Accordingly, due to the promising results, potential of anti-cancer effects against solid neoplastic bodies like- glioblastoma, mesothelioma, metastatic sarcoma, metastatic liver, ovarian and pancreatic cancer are also being explored using these generation of CARs. On top of that, various other substitutes like- CD27, CD40, CD244, ICOS, OX40, DAP10, MYD88 of the two above mentioned FDA approved CSDs are currently being researched about after their success in preclinical trials which unfolds the possibility of developing an ampler pool of qualified selective picks to choose from (Sterner, R. C. & Sterner, R. M., 2021).

6.3 Third Generation CAR-T cell

Like second generation CARs, the third generation of CARs were constructed by further including one extra CSD behind the first, at its cytosolic caudal appendage; with the expectation of unleashing the full potential of CAR-T cell activation as it there was still some skepticism

about previous CAR-T generations' activation aptitude (Sterner, R. C. & Sterner, R. M., 2021). Receptors like- CD3ζ-CD28-4-1BB, CD3ζ-CD28-OX40 were assembled for that purpose to examine the capacity of T-cells regarding enhanced activation, rapid as well as sustained proliferation, heightened cytokine release for overall augmented anticancer outcome (Mohanty et al., 2019). However, the preclinical trials showed heterogeneous outcome; like- α-CD19-CD3ζ-CD28-4-1BB CAR-T's documented tumor infiltrating lymphocyte (TIL) mediated cell lysis in tumor microenvironment resulting in 'CR' in a significant portion of the B-CLL patients along with relapse negating improved memory T-cell formation. Again, the same CARs in some other trials improved cytokine release showcasing benefitted antitumor result in lymphoma and advanced lung cancer patients; but did not appear to have done any better job than second generation CARs for some other cancers like- pancreatic cancer. Moreover, some showed a surge in uncontrollable cytokine production, further increasing chances of Cytokine Release Syndrome resulting in multifold-organ-failure and death (Sterner, R. C. & Sterner, R. M., 2021; Mohanty et al., 2019).

6.4 Fourth and Above Generation of CAR-T Cell

Fourth generation CARs were started to be experimented on due to the excess co-stimulation related side effects of third generation CARs, the development of which was rather opted for, modifying the second generation CAR ISD with the incorporation of transgenic promoter of cytokines like- IL-12 and IFNγ for an universal cytokine mediated killing; making fourth generation CARs known as the TRUCKs (T-lymphocytes Redirected for antigen Unrestricted Cytokine conveyed Killing) or, the armored CARs generally (Miliotou & Papadopoulou, 2018; De Marco et al., 2023). Fused to the second generation CAR-ISD, there is an immune modifying

molecules like- IL-2, IL-5, IL-12 and IFNγ cytokine promoting transgene housing in the 'nuclear factor of the activated T-cell (NFAT) sensitive cassette' which activates when the TAA induced CAR is already activated and, the NFAT cassette containing transgene excretes cytokine in the tumor microenvironment. This CAR is also useful in causing cytokine mediated cytotoxicity to the antigen-lost tumor cells in heterogeneous tumor microenvironment through trans-killing. LIke, cytokine transgene expressing CAR-T, other molecules (e.g., chemokines, peptides, ligands, receptors, nanobodies etc.) can also be expressed if their corresponding Armour is fused with the CAR-ISD.

Lastly, fifth generation CAR which is also known as the next-generation CAR, where like the fourth generation CAR, another structure is fused to the ISD which is JAK/STAT signaling pathway inducing IL2Rβ domain which works in an antigen-dependent manner as a precondition; showing improved perseverance with robust anti-tumor effect. Along with that advanced CAR-T therapy incorporating technologically enhanced CARs that are discussed in the next chapter, can also be considered fifth generation CAR- On/Off switch CAR, Logic-gated CAR etc. (Asmamaw Dejenie et al., 2022).

Chapter 7

Sophisticated Engineering Approaches in CAR-T Cell Therapy

In addition to conventional CAR-T structure with varied generations of expression, numerous other sophisticated approaches of CAR-T cell design have been developed in laboratories and is being worked on to ensure enhanced precision, precaution and performance through either minimizing on-target/off-tumor (OT/OT) effect which is one of the most prominent adverse effects of CAR-T cell therapy or, increasing range of targetable antigens as per requirement. Some of them are based on newly innovative strategies like- on/off switches, suicide genes, inhibition in presence of healthy cell antigens (iCAR), gene alteration through CRISPR/CAS9 etc. in the expression of the CAR receptor, itself and others are considered high-tech, manifold-targetable and reconfigurable CAR-Ts like- Dual CAR-T, Tandem CAR-T (Tan-CAR-T), Physiological CAR-T, Universal CAR-T, SynNotch CAR-T, Switchable and Programmable Universal CAR-T (SUPRA CAR-T), Split CAR-T, TRUCK CAR-T etc. These CARs can again be divided based on Boolean Logic Gated operations applied like- OR Gate, AND Gate, NOT Gate, IF-THEN Gate, IF-BETTER Gate etc. Some of these advanced CAR-T cells will be discussed in the following sections.

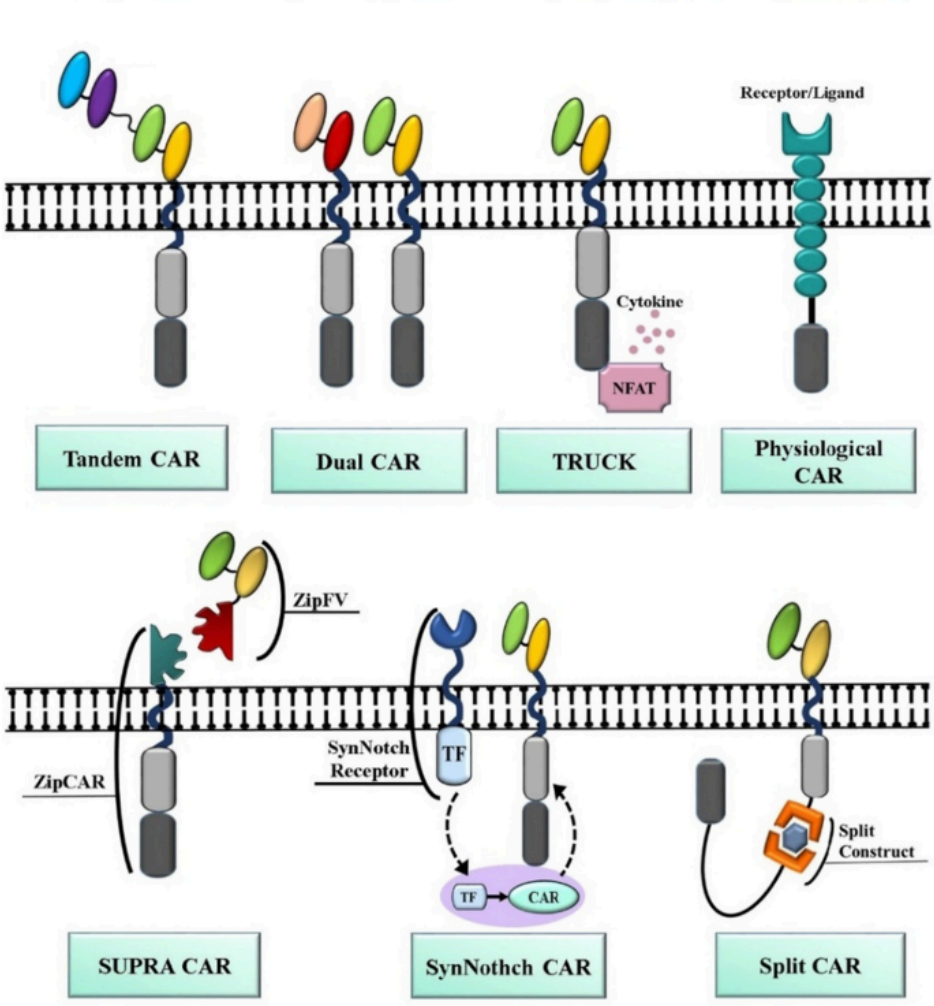

Figure 16. Some of the advanced types of CAR-T cells (Tahmasebi et al., 2020).

7.1 Dual CAR-T Cell Therapy

This special CAR-T structure is constructed through incorporation of two individually unique ARD, expressed separately in the form of two distinct CARs in an individual T cell instead of one single CAR like standard CAR-Ts; in order to target two different antigens (TAA) concurrently on one specific malignant cell. Just like traditional CARs, both of the CARs are also assembled as ISD (e.g., CD3ζ), one or, multiple CSDs, hinge and TMD along with the highlighted structure of 2 ARDs (e.g., ScFv or, VHH) that can increase the safeguarding

probability for non-malignant cells through subdued OT/OT effect using the AND logic gate or, in general, improve the potency through a broader targetable antigen pool to bind to working in OR gate manner (Tahmasebi et al., 2020). In case of AND gate program, binding to both of the antigens in unison can cumulatively amplify the activation signal resulting in cell lysis. On the other hand, OR gate works efficiently in case of evasion of immune recognition through mutation or loss of TAA where either of the Antigens' susceptibility towards its respective CAR is enough to pull off a total T cell activation (Mohanty et al., 2019). The bispecific-CD19-CD20 and CD19-CD22 are two of the most prominent dual-targeting CAR-T agents being studied on, in multiple clinical trials against various leukemias like R/R B-ALL and CLL as well as several lymphomas including small lymphoblastic lymphoma (SLL) and non-Hodgkin lymphoma (NHL). The bispecific CD19-CD22 CAR-T therapy has exhibited robust oncolytic outcome against R/R B-ALL patients through efficiently targeting both the CD19 and CD22 antigen positive malignant cells. Another first phase clinical study produced 28.57% CR+ stature in a B-ALL or, DLBCL positive study population of 7. In context of solid tumors, AND gated dual CAR-Ts were made to target against the CEA+ and MSLN+ TAA of the human pancreatic adenocarcinoma (AsPC-1) cell lines in xenogeneic model; again, bringing about robust oncolytic outcome that did not get replicated in any of the monospecific TAA+ healthy cells reassuring reduced OT/OT based precision-focused-safety; in comparison to standard singular antigen targeting CAR-Ts. Again, numerous other solid tumor originating cancers; like- breast, pancreatic, prostate, ovarian and glioblastoma multiforme cancers were also experimentally targeted with- ERB2-MUC1, MUC1-PSCA, PSMA-CD19/PSCA, Meso-Fra and EGFR-EGFRvIII targeting dual positive CAR-T cells respectively in various preclinical studies always resulting in prominent tumor-cytolytic action through augmented level of cytokine (e.g.,

IL-2, IFN-gamma etc.) production with a diminished OT/OT effect, improved T cell proliferation, persistence and immune-vigilance in general; thus, considered to be an advanced technology to be further developed on for improved therapeutic outcome (Tahmasebi et al., 2020).

7.2 Tandem CAR-T Cell Therapy

Similar to Dual CAR-T structure, Tandem CAR-T cell development is another approach of bispecific CAR development for simultaneous and synergistic signal transduction consequential antigen binding; where the main difference from the Dual CAR-T is the involvement of both of the ARD (e.g., ScFv) on a single CAR structure in a tandemly ordered manner through amino acid (e.g., serine, glycine etc.) made linker sequence of varied lengths and composition for flexibility in antigen recognition. Nevertheless, it is quite similar to Dual CAR-T design in action but is an alternative approach due to realistic factors of convenience; like- a more economical and streamlined manufacturing.

The ARDs of the Tandem CAR-T (Tan-CAR-T) cells can function both as an ‘AND’ gate where recognition of both the pre-targeted antigens simultaneously can yield as a safety net by ensuring high precision and reduced chances of ‘OT/OT’ toxicity; or, as an ‘OR’ gate where either of the antigens is enough to cause complete T-cell activation based on the solution requirement for specific patients’ issues like- tumor’s immune escape mechanism through heterogeneity, subsidence or, even diminishment of antigen.

Many successful Tandem CAR-T cells have been developed till now that has shown prominent therapeutic success against various cancers including hematological malignancies

like- leukemias and lymphomas; as well as solid tumors like- ovarian cancer, breast cancer, glioblastoma, multiple myeloma and so on.

CD19-HER2 Tan-CAR-T cell is one of the pioneering Tandem CAR-T cell therapy studied against 3 types of tumor cells- only CD19 expressing Raji Burkitt lymphoma cells, only HER2 expressing Daoy medulloblastoma cells and neither antigen expressing MDA-MB-468 cells by Grada and team; that showed effective antigen detection as well as cytotoxicity in both lymphoma and medulloblastoma cells separately (effective OR gated T cell activation); and, cytokines like- IFN-γ and IL-2 release in the co-culture of both cells (AND gate based activation); but no significant action in presence of both-antigen-negative MDA-MB-468 (Grada et al., 2013).

CD19-CD20 targeting Tan-CAR-T cell is another example of Tandem CAR studied by Schneider-and-team as well as Zah-and-team separately; in vitro and in vivo against leukemic cells; showing parallelly equivalent outcome of prominently improved 'AND' logic based oncolysis compared to single antigen targeting conventional CAR-T cell; through heightened degree of- IFN-γ, TNF-α, IL-2 and GM-CSF expression.

Similarly, CD19-CD22 Tan-CAR-T, CD19-CD133 Tan-CAR-T, CD19-CD79a Tan-CAR-T, CD70-B7H3 Tan-CAR-T, Her2-IL13Rα2-Tan-CAR-T, EGFRvIII-IL13Rα2-Tan-CAR-T and many more were and is being studied pre-clinically as well as clinically; against various cancers like- B-ALL, mixed phenotype acute leukemia (MPAL), lymphoma, breast cancer and glioblastoma (by the last two CARs mentioned); mostly always resulting in lessened degree of antigen escape, specifically targeting double antigen positive malignant cells, high degree of cytokine release that has almost always been accumulated as a

better safety-efficacy-complete response providing, long term and persistent anti-tumor agent compared to standard one-antigen targeting CAR-T cell therapy both in vitro and xenogeneic in vivo mouse models (Mohanty et al., 2019; Tahmasebi et al., 2020; Hamieh et al., 2023; Zhang et al., 2022; Schmidts et al., 2022).

7.3 SynNotch CAR-T Cell Therapy

SynNotch (Synthetic Notch) was the original 'AND' gated CAR that was developed by Leonardo Morsut and Kole Roybal in 2016; as a synthetically configurable CAR-T cell therapy to target assorted number of antigens in a collaborative antigen recognition manner which can also function as 'IF-THEN' gated approach if needed. Wild type endogenous notch receptor proteins are substantially sized transmembrane proteins; critically involved in intercellular signaling consequential to genetic modulatory action that greatly impacts cellular prospect of embryonic morphogenesis and mature tissue equilibrium through regenerative mechanism (Mao & Ito, 2017). Unaltered notch is a 2 subunit (EGFs and rest) based heterodimeric complex; made of 3 components- an extracellular domain (NECD) made up of multiple identical EGF copies targeting Delta and Jagged ancestral proteins as ligands along with a negative recognition domain (NRR) made up of Lin-12 notch repeats (LNR) and heterodimerization domain (HD) as a fail-safe; a transmembrane domain that is an integral structure for motorized commencement of NECD and one intracellular domain (NICD) working as the transcriptional factor (TF) that gets split from the original notch structure after ligand-receptor engagement through the action of gamma-secretase enzyme at the cleavage site and consequential nucleus-translocation results in transcription and eventual expression of the translated target gene (Khamaisi et al., 2022; Steinbuck & Winandy, 2018).

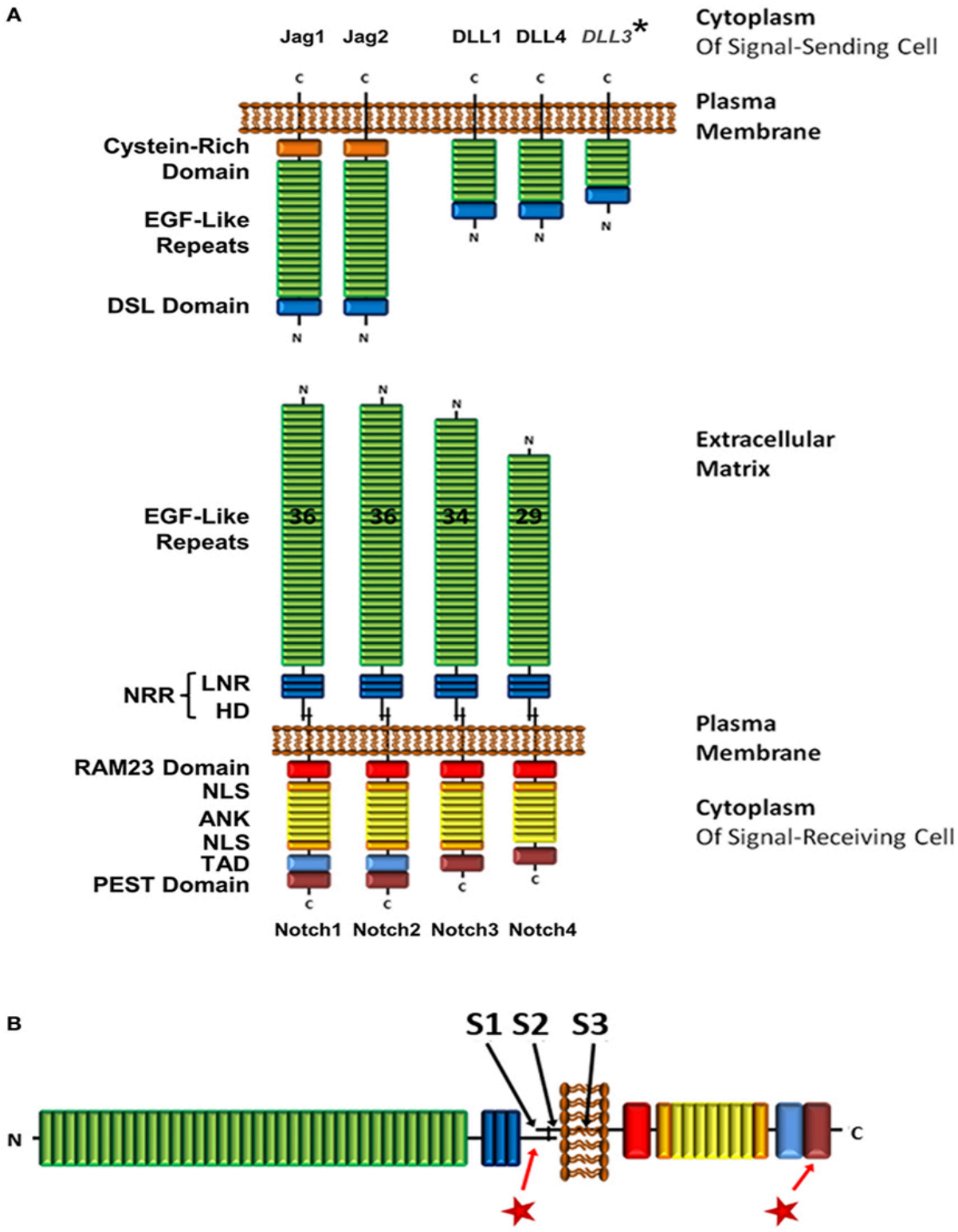

Figure 17. Detailed structure (A) and cleavage sites (B) of the endogenous notch receptor (Steinbuck & Winandy, 2018).

Based on the original structure, chimeric synthetic notch, SynNotch is engineered to express on T-cells where both the extracellular and intracellular region can be altered to target specific antigen on tumor cell which in turn can cleave the pre-modified or, synthetic TF (e.g., Gal4-VP64, tetR-VP64, ZFHD1-VP64, Gal4-KRAB etc.) encouraging expression of a CAR targeting another (the second) antigen. When both the SynNotch receptor and newly expressed CAR form bondage with both of the antigens, only then, the T cell becomes fully activated and cis-lyses the tumor cell (kills the very cell expressing both antigens) exempting patients from OT/OT effect through 'AND' gate mechanism (Morsut et al., 2016).

Figure 18. Basic structure comparison between Wild-type and Synthetic-Notch receptors (Morsut et al., 2016).

SynNotch CAR-T has shown degrees of effectivity in various solid tumors having antigen heterogeneity issue like- lung adenocarcinoma, mesothelioma, ovarian-breast-colon-pancreatic cancer etc. along with various leukemias (ALL and CLL) through interaction with numerous priming antigens like- ALPPL2 or, receptor tyrosine kinase Axl through SynNotch receptor.

Roybal and team has developed various types of SynNotch CAR-T cells since its invention; for example- CD19-SynNotch receptor stimulated anti-mesothelin/4-1BBζ Jurkat

CAR-T cells, GFP-SynNotch receptor stimulated anti-CD19/4-1BBζ CD4+ as well as CD8+ CAR-T cells, GFP nanobody-SynNotch receptor stimulated anti-HER2-CD19 bispecific CAR-T cells and so on; working in an 'AND'-logic gated fashion yielding enhanced specificity and reduced chances of OT/OT toxicity. Additionally, Srivastava and team from Stanley Riddell's lab has also developed 'AND' gate based EpCAM- or, B7H3- SynNotch receptor triggered anti-ROR1/4-1BBζ CAR-T cells for breast cancer xenogeneic model showing high efficiency in tumor eradication through disregarding antigen forfeiture based immune evasion (Tahmasebi et al., 2020; Abbott et al., 2022).

Again, instead of or, along with the TF activation-based CAR expression for targeting the second antigen, Roybal and team has shown to even activate cytokine release or, monoclonal antibody expression through incorporation of SynNotch receptors in T cells; which was again experimentally shown through IL-10 cytokine release along with CAR expression by means of previously mentioned Axl-SynNotch interaction at first, in case of lung, breast, pancreatic and colon cancers by Cho and team.

In addition, to target antigen negative malignant cells, which is another of theirs' immune escape mechanism, 'IF-THEN' gated SynNotch CAR-T cell therapy is a wise choice as it can induce an accommodating trans-killing of the TAA (-)ve cells adjacent to the TAA (+)ve tumor cells in the tumor-microenvironment without affecting far-flung healthy cells (Simon et al., 2022).

An ideal TAA should be both tumor-specific and homogeneously expressed on all tumor cells which definitely does not happen most of the time. In glioblastoma, EGFRvIII antigen is incredibly-specialized but quite heterogeneous in nature and MOG is not strictly glioblastoma specific but central nervous system specific in general; so experimentally, a 'prime and kill'

approach was taken through 'IF-THEN' gated SynNotch CAR-T development where the SynNotch receptor is engineered to target EGFRvIII or, MOG as the prime antigen to 'prime' (prepare) the T cell for activation which in turn causes expression of Tandem 'OR' gated CAR targeting the comparatively ambiguous but evenly homogenous EphA2 or, IL13Rα2 killing antigens; that could cytolyze cells expressing either of the antigens in an 'OR' gate targeting manner given that the T-cell has been already primed by SynNotch receptor presence of the priming antigen on the same malignant cell (cis-killing) or, at least in the near vicinity (trans-killing) resulting in a remarkably complete clearance of glioblastoma along with long-term persistence (Choe et al., 2021).

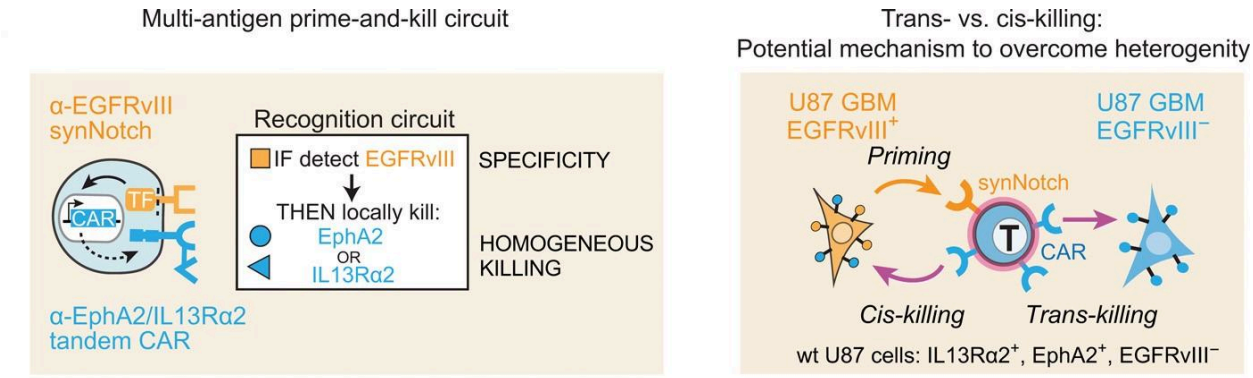

Figure 19. Multi-antigen targeting through Syn-Notch CAR-T based priming and a second tandem CAR-T based cis-and-trans killing (Choe et al., 2021).

Lastly, SynNotch approach of CAR expression has been shown to have helped to retain T cell health through saving T cells from tonic signaling, exempting them from T-cell exhaustion and maintaining a comparatively naïve less differentiated status aiding longer survival and persistence.

7.4 Universal CAR-T Cell Therapy

Due to problems regarding structural rigidity as well as inability to be flexible regarding antigen targeting after primary CAR-T synthesis, Universal CAR (Uni-CAR) structure has been developed which is rooted in the original CAR structure but also is quite different due to adaptation of a split structure; where a soluble tumor-antigen-targeting ligand (TL) works as a mediating intermediary that binds to the TAA directly; and, on the other hand, an universal immune receptor (UIR) consisting of an extracellular adapter domain (EAD) and a parallel ISD binds indirectly to the antigen through TL using the EAD as a linker bridge between TL and ISD. Here, generally, the EAD binds to the specific binding domain, known as the 'tag' that is present on the TL and causes full T cell activation for malignant cytolysis. Till now, 4 kinds of Universal CAR-T have been developed with 4 unique UIRs- a) Tag specific Universal Immune Receptor, b) Anti-Tag specific Universal Immune Receptor, c)Bi-specific Immune Receptor and d)Antibody Dependent Cellular Cytotoxicity (ADCC) based Immune Receptor (Minutolo et al., 2019; Tahmasebi et al., 2020).

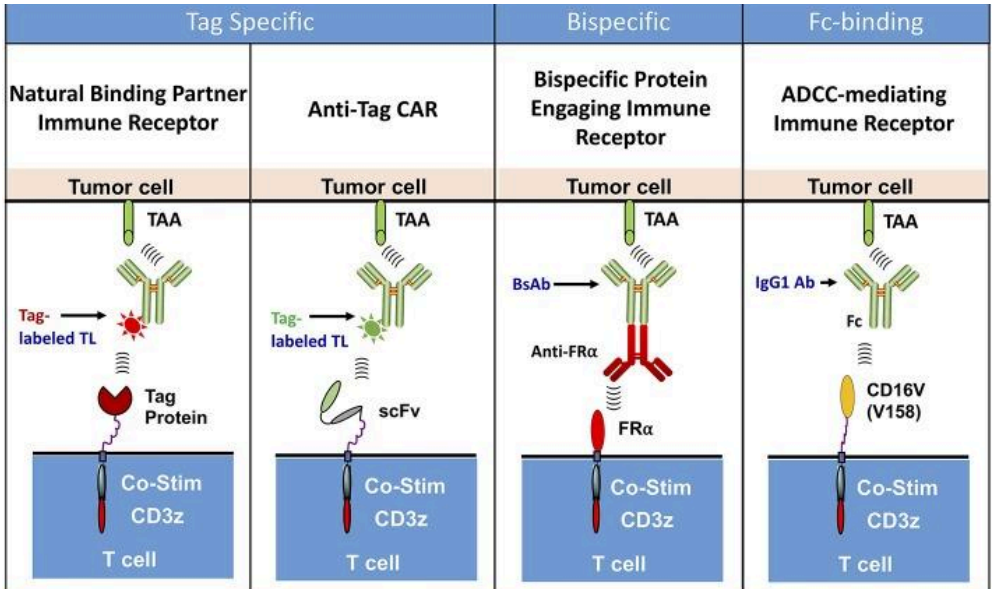

Figure 20. Tag, Anti-Tag, Bi-specific and ADCC mediated universal CAR-T cell structures and how they work in presence of TAA and bridging molecule (Minutolo et al., 2019).

7.4.1 Tag specific Universal Immune Receptor

The design-niche of this very type of immune receptor (IR) is founded on the naturally occurring ligand-ligand interaction which is the foundation of the IR-TL coupling system. One of the first UIR of this type is based on the foreknown avidin-biotin binding nature, where the dimeric avidin (dcAv) is engineered to be integrated as the EAD to the biotin binding immune receptor (BBIR) along with the ISD, forming a completely modified UIR. The dcAv EAD is capable of binding to the infused biotinylated antigen-specific molecule (e.g., ScFv, monoclonal antibodies (mAbs) or, any other TL) pre-bound to the TAA; which becomes biotinylated due to being engineered to be fused with biotin that works as the 'tag' where the counterpart UIR through avidin EAD (Zhao et al., 2018).

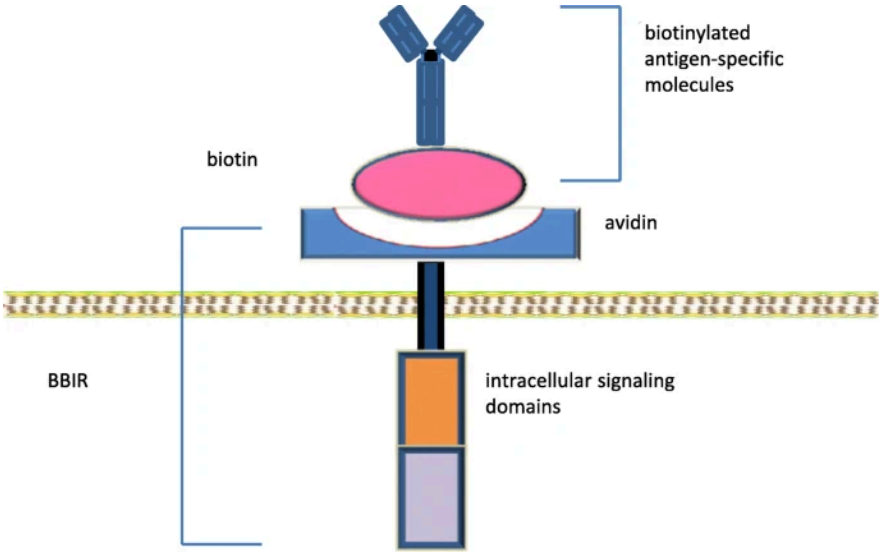

Figure 21. The structure of biotin-avidin based BBIR tag-specific universal immune receptor (Zhao et al., 2018).

The main advantage of this UIR based Uni-CAR-T cell therapy is that-

They can ensure precision and safety by being active and providing cytotoxicity only to tumor cells already bound to only biotinylated tumor specific molecules disregarding non-biotinylated ones and thus, exempting healthy cells.

They can also be engineered to work in a concurrent or, consecutive manner in cases of multi-targeting to mitigate antigen evasion issue or, targeting a diverse array of tumor cells presented with various antigens using only one type of CAR-T cell given that additional complementary biotinylate-able antigen-specific ligand/ antibodies are also infused.

Although in experimental phase, this specific BBIR based universal CAR-T has shown successful control of tumor growth against ovarian cancer in vivo xenogeneic mouse model in preclinical studies. Other types of tag specific UIRs are also in experimental phases of development like- BBIR with monomeric streptavidin as the EAD and leucine amino acid based split zipper CAR system; having a TAA targeting ScFv attached leucine zip and its complementary UIR counterpart having another leucine zip connected to the ISD known as the SUPRA CAR which promises to bring better therapeutic effect and efficacy through the CAR-T cell therapy.

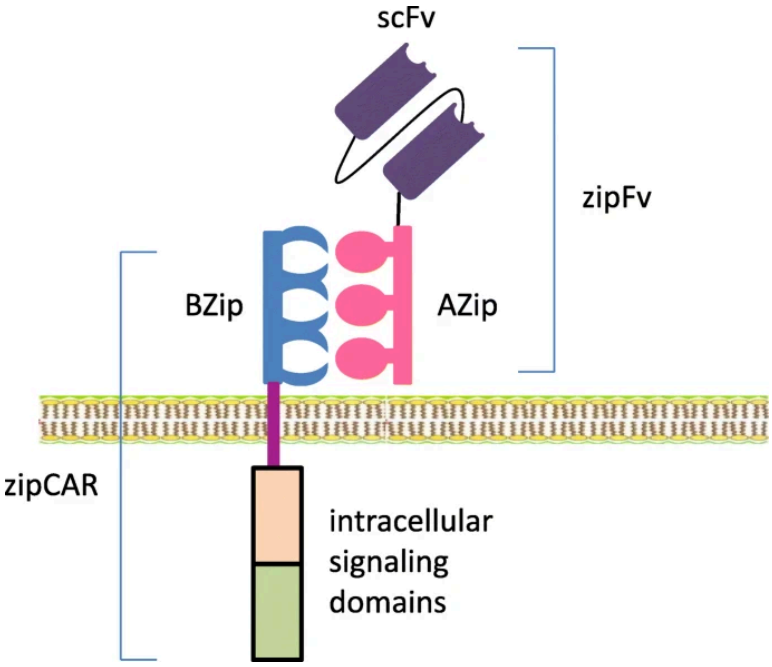

Figure 22. The structure of leucine-zipper based SUPRA CAR (Zhao et al., 2018).

7.4.2 Anti-Tag specific Universal Immune Receptor

Where tag-specific UIR is based on utilizing the naturally pre-existing ligand-ligand interaction, the anti-tag UIR is based on developing an immune receptor (IR) directly against a cognate peptide tag that is targeted through an ARD like standard CAR (e.g., ScFV, Fab, nanobodies or, diverse small molecules); where the tag, itself is genetically or, chemically modulated to express on or, with the predetermined TL capable of forming a direct bond with the foreordained TAA; and thus, forming an indirect bond with the anti-tag UIR embedded modified T-lymphocyte.

One of the prominent anti-tag UIR was developed by Tamada and team, where they developed an anti-FITC ScFv based UIR expressed Uni-CAR-T cell against FITC (Fluorescein isothiocyanate) which is already frequently employed as a labeling tag for antibodies. In this context, FITC gene is engineered to express FITC as a biomarker tag on the infused TL

complementary to the aimed TAA; and, the anti-FITC-CAR-T gets activated and cytolyses cancer cells through an indirect synapse formation. Using this foundation, many of the already clinically approved monoclonal antibodies like- Cetuximab, rituximab and trastuzumab are labeled with FITC-tag that can help target CAR-T cells through anti-FITC ScFv containing UIR, the complementary TAA- EGFR, CD20 and HER2 respectively.

Alongside FITC, GCN4 transcription modulator of yeast derived peptide neoantigen epitope (PNE) and E5B9 originated nuclear antigen LASSB are the 2 other tags, who are being experimentally targeted with their corresponding anti-tag CAR-Ts. Till now, this CAR-T cell therapy has been pre-clinically studied using SW480 cellular lineage that has shown to successfully repurpose the pre-infused cetuximab in cancer patients with Kras (Kirsten Rat Sarcoma virus caused) mutation and also against HER2-(+)ve tumor cell through FITC-tagged Trastuzumab using anti-FITC Uni-CAR-T showing complete eradication but with higher adverse effects which is not ideal but leaves room for improvement (Sutherland et al., 2020).

7.4.3 Bispecific Immune Receptor

When the TAA and the EAD of an UIR is connected through a soluble bispecific antibody directly as an intermediary without the necessity of any conditional ligand or, tag based binding between the UIR and the mediator antibody, the UIR is known as the bispecific immune receptor. Here, the bispecific antibody's both ARMS are capable of orthogonally binding to both TAA and EAD without the need of any additional help. The original bispecific UIR was constructed by Urbanska and team with folate receptor-α (FRα) EAD and CD28/CD3ζ endo-domain that worked exclusively in eradicating CD20+ tumor cells in presence of an anti- FRα-anti-CD20 bispecific antibody that works as a soluble linking bridge with the inclusion of CD28 CSD

presence leading to improved degree of cytokine (e.g., TNFa, IFNg and IL2) release. Soluble CD19 antibody fusion protein is another recently developed bispecific antibody containing an arm of CD19 protein that is capable of directly binding to the CD19-receptor specific CAR-T on one-end and other end containing an ScFv ARD that can directly bind to TAAs that are not always CD19 presented on the tumor cells; thus, also repurposing the original CD19-specific CAR-T to target supplementary TAAs ensuring precise-rerouted cytotoxicity. In practice, one of this kind, 'CD19-CD20ScFv antibody fusion protein' is used to target CD20 TAA on tumor cells in case of CD19-antigen-negative relapse in previously CD19 susceptive patients pre-infused with CD19 specific CAR-T cells which becomes an efficient way of reutilizing pre-administered engineered T-cells (Tahmasebi et al., 2020; Minutolo et al., 2019).

7.4.4 ADCC based Chimeric Immune Receptor

ADCC is a specific type of immune reaction where the antigen presented cell is surrounded by the susceptible-cognate-antibodies that bind to the antigen with its Fab (fragmented-antigen binding) end and again, bind to the Fc receptor (FcR) expressed on the effector white blood cells (e.g., macrophage, neutrophils, natural killer cells and eosinophil) with the Fc (fragment crystallizable) portion on its other end; which, through this bridged connection of effector cell and antigen though antibody results in antigen-dependent cellular cytotoxicity. One of the prominent naturally occurring examples is the Fc receptor- FcγRIIIa (CD16) expressing a natural killer (NK) cell that induces ADCC to the neighboring antigen presenting target cell through IgG antibody.

Based on this mechanism, Clémenceau and team has developed an ADCC based UIR where FcγRIIIa-158V (VV) which can also be expressed as CD16(VV) with valine at location, 158 is expressed as the EAD on top of CD3ζ-based-ISD forming FcIR that can form bondage of high affinity to the Fc region of the IgG isotypes- IgG1 and IgG3. They infused TAA-CD20 specific IgG1 antibody called 'Rituximab' that can work as a bridging molecule between the FcIR expressing Uni-CAR-T cell and CD20 presenting leukemic cells in vitro showing effective and precise antibody-dependent-cellular-cytotoxic (ADCC) and eventual cytolytic response.

After that, they again applied this chimeric CD16 (VV) EAD based UIR structure expressed on NK-92 cell; against HER2+ breast cancer expressed in vivo human subcutaneous preclinical models which resulted in tumor inhibition following the intraperitoneal infusion of TAA-HER2-specific-IgG1 antibody, 'Trastuzumab'.

Correspondingly, Ochi and team also developed FcIR based Chimeric Uni-CAR-T cells; capable targeting CD20, HER2 and CCR4 through cognately-specific-IgG antibodies, Rituximab, Trastuzumab and Mogamulizumab correspondingly in vitro; resulting in successful T cell redirection and precise cellular cytotoxicity.

Like previously discussed, various types of ADCC mediated FcIR based CAR-T, due to the success of its established technology, is now various under clinical trials for HER2+ cancers like- breast, bladder, pancreatic, ovarian and stomach cancers, CD20+ non-Hodgkin lymphoma (NHL) and BCMA+ multiple myeloma. Also, another CD16 (VV) FcIR containing universal CAR-T therapy, Unum (ACTR087) against NHL; being accompanied by Rituximab in a minimal dose of 0.5 million CAR-T cells/kg showed 33.33% CR+ and 16.67% PR+ response in a Rituximab-refractory study population of 6. Lastly, these FcIR based Uni-CAR-T can be

improved by additionally incorporating Tan-4-1BB/CD3ζ CAR has also demonstrated remarkable outcome in vitro (Minutolo et al., 2019).

7.5 Inhibitory CAR-T (I-CAR-T) Cell Therapy

To immune healthy patient cells from CAR-T cytotoxicity and help CAR-T cells to distinguish between malignant and non-malignant cells, inhibitory CAR-T therapy has been developed. Here, a bicistronic Dual-CAR approach is taken by expressing 2 independent CARs on a single T cell. The first CAR structure is of conventional build that targets TAA and capable of causing standard CAR-mediated cytotoxicity; conversely, the second CAR is of inhibitory nature, rooted in the function of immunoregulatory molecules like- naturally occurring immune checkpoints like- PD-1 (programmed cell death receptor-1), PDL-1 (PD-1 ligand), CTLA-4 (cytotoxic T lymphocyte associated molecule-4) that works as a fail-safe break against the T cell activation. This inhibitory CAR's ISD is developed by specially incorporating the endogenous signaling region of the immunoregulatory molecules along with an exocellular recognition domain with the modified specificity against an 'exclusive' antigen only expressed on healthy cells and thus putting a stoppage in T cell activation even a ARD-TAA bondage forms between the first mentioned standard CAR and the target cell (Hashem Boroojerdi et al., 2020; Mohanty et al., 2019). Prominent examples of I-CAR-T cells that are under clinical study:

iHLA-A2-HER2-CAR-T cell therapy (an anti-HER2-CAR-T cell containing HLA-A2 targeting I-CAR) in vitro in vivo immunodeficient mouse model showing specifically potent cytotoxicity against HLA-A2 knock out gene expressing, HLA-A2 protein-negative imitated-tumor cells; but, no response in HLA-A2 protein expressed healthy cells (Bassan et al., 2022).

iKP-19-CAR-T cell therapy (Anti-CD19-CAR-T cell additionally containing HLA-C1 targeting KIR/PD-1 based 'iKP CAR'); where, KIR is the killer-inhibitory-receptor functioning as an ARD; derived from the 2DL2 receptor of the NK cell or, T cell's transmembrane glycoprotein, KIR- (killer-cell immunoglobulin like receptor) which is also known as KIR2DL2 and PD-1 represents the ISD derived from the original immune checkpoint structure) to subdue B-cell aplasia through inhibiting OT/OT in CD19(+)ve.HLA-C1(+)ve normal B cells and only targeting CD19(+)ve.HLA-C1(-)ve Burkitt's lymphoma cells in both in vitro and xenogeneic in vivo model (Tao et al., 2020).

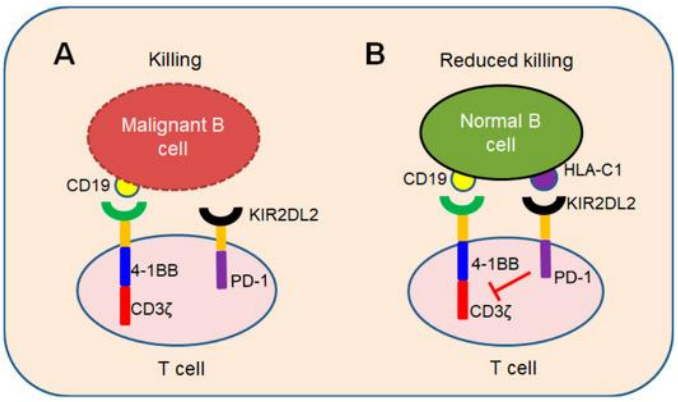

Figure 23. Mechanism of action of inhibitory CAR against malignant versus healthy normal cells (Tao et al., 2020).

7.6 Physiological CAR-T Cell Therapy

The preliminary development of physiological CAR-T cell was done due to the issue of immunogenicity (which can eventually lead to anaphylaxis) occurring against the murine-derived-ARD containing CARs expressed on the human-T-cells. Immunogenicity is the active immune response inducing ability of a cell or tissue against the pre-detected align entity;

which is the murine originated ScFv in this case. This issue results in reduced long-term perseverance of the modified CAR-T cells which minimizes its long-term effectiveness.

Physiological CAR structure is based on the ligand-receptor relationship in the endogenous context, which at times, has proven to be more robust and straightforward post-interactional shorter signaling cascade in T-lymphocyte-activation as well as eventual cytotoxic-cytolytic effect. The main difference between physiological and standard CAR is the incorporation of a receptor or, a ligand instead of any conventional ARD (e.g., ScFv) as the ectodomain ARD with the very same, conventional ISD (e.g., CD3ζ) forming the CAR structure (Mohanty et al., 2019; Tahmasebi et al., 2020) .

Till now, quite a few numbers of physiological CAR-T therapies have been studied on. Shaffer and team has developed a CAR using the naturally occurring CD70-CD27 ligand-receptor bond; with a CAR expressing the complete CD27 receptor as its ARD to target CD70 ligand presenting tumor cells resulting in fast cytolysis of CD70(+)ve tumor cell through immediate T-cell activation along with a defense mechanism against immunogenicity induced activation induced cell death (AICD) of the CAR-T cell.

Zhang and team developed Physiological CAR using human-NKG2D (natural killer group 2 member) receptor as the ARD against numerous NKG2D-Ligand(+)ve lymphoma malignant cells showing immediate CAR-T activation resulting in significantly better tumor eradication through improved cytokine production. Additional examples include- Muniappan and team developed CAR using HER3 as well as HER4 receptors against ligand, heregulin present on breast tumor cell, Kahlon and team developed IL13-zetakine-Receptor expressing CAR-T cells working against IL13Rα2(+)ve glioblastoma cells, Niederman and team developed VEGF

(vascular endothelial growth factor) ligand expressing CAR-T cells targeting VEGF-Receptor(+)ve tumor cells and again previously mentioned Zhang and team developed NK_p30 receptor targeting B7-H6 ligand(+)ve tumor cells; all of which showing efficient tumor eradication through robust cytotoxicity and elevated degree of cytokine production (Tahmasebi et al., 2020).

7.7 Other Specialized CAR-T Cell Therapies

In addition to these detailed above-explained CARs, there are myriads of diverse types of CAR-T cells that have been as well as are being developed by scientists; and are under preclinical and clinical trials with a huge fragment of the population showing promising results through ensuring improved efficiency, efficacy and safety for still unmanageable cancers in the future.Some of these CARs are:

A. Trivalent CAR-T cells expressing three independent CARs on a single cell or including three Antigen recognition domains through tandem linking.

B. Boolean logic gated CAR-Ts like- AND gate, OR gate, NOT gate, IF-NOT gate, IF-BETTER gate and so on with numerous expressed CARs working in an multi-targeting and programmable logic gated manner for effective and efficient antigen targeting.

C. Chimeric Switch Receptor (CSR) CAR that works in the reverse way of inhibitory CAR, where instead of the ISD, the extracellular domain of the immune checkpoint (e.g., PD-1, CTLA-4) is designed with the conventional intracellular domain (CD28-TM/CD28-ISD) capable of manipulating the checkpoint's immunosuppressing signal into the T cell activation signal.

D. Synthetic T cell receptor and antigen receptor (STAR) CAR developed combining both the intracellular and extracellular structure of TCR and CAR construct respectively into a novel structure that has both the TCR's strong signal transduction and CAR's antigen recognition capability.

E. On/Off Switch CARs designed as a split structure where the intracellular signaling domain and the costimulatoey domain stays separate that can only assemble in vivo in presence of a special drug functioning as the 'On-switch' which can further cause T cell activation in presence of TAA. Though the 'On-switch' CAR uses the same development process, 'Off-Switch' CARs work through the infusion of an off-switch molecule resulting in eventual CAR protein degradation.

Photon or, ultrasound-switchable CAR, Suicide gene (e.g., HSV-tk, iCAsp9, EGFRt etc. induced target cell apoptosis) based CAR, Natural Killer based NK-CAR, CRISPR CAR etc. and so on are the additional examples of sophisticated approach applied 'next generation' CAR-T therapies; that exhibit promising result in dealing with the various issues that occur as potential solution along with improving the antigen targeting as well as the antitumor activity (Miao et al., 2022).

Chapter 8

Accessible-approved CAR-T Therapies

Among the 4 established as well as newer emerging type of adoptive cell therapies, up until 2022, only 6 of the CAR-T therapy preparations have gotten marketing authorization from FDA and EMA, the regulatory bodies of USA and European Union (EU) along with the European Economic Area (EEA) respectively; only starting from the approval of Tisagenlecleucel in 2017. Till now, most of these products have been constructed to target CD19, one of the most prominent pan-B-cell markers with all 6 of them being used against 7 B-cell-hematologic malignancies with admirably remarkable outcome. It is because, this marker is almost exclusively expressed in every developmental stage of B lymphocytes from progenitor to memory as well as plasma B cell level, which henceforth, significantly decreases on-target-off-tumor toxicity of CAR-T cells in case of B cell leukemias and lymphomas. Having said that, though restricted, they end up aiming for the elimination of the whole B cell population resulting in B lymphocyte aplasia causing immunodeficiency due to acquired hypo-gamma-globulinemia; but this resultantly decreased clinically low immunoglobulin (e.g., IgG, IgM, IgA, IgD and IgE) level can comparatively easily be managed though intravenous immunoglobulin (IVIg) infusion therapy.

B -cell maturation antigen (BCMA) is also the base of two of the approved therapies with antigens like- CD20, CD22, HER2, EGFRvIII, IL-13Rα2 and many more are being studied for both hematological and solid tumors.

To mention, all of the approved CAR-T therapies have been indicated for blood-related neoplasia because they have been found to be exceptionally superior in getting an easier hold of the targets due to the absence of any solid extracellular matrix which solid tumors lack especially.

Table 1. Summary of CAR-T Therapy: Manufacturers, Targets, Indications, and Approvals (European Medicines Agency, 2024; Drugs.com; De Marco et al., 2023; Hamieh et al., 2023).

CAR-T therapy (Brand name and respective Generic name)	**Manufacturer company**	**Aimed Target, Endo-domain and used Vector**	**Indications For**	**Approved By and First Approval Date**
Kymriah (Tisagenlecleucel)	Novartis AG (Switzerland)	Cluster of Differentiation - 19 (CD-19); **ISD:** CD3ζ **CSD:** 4-1BB; **Vector:** Lentiviral vector	• Acute B-cell Lymphoblastic leukemia (B-ALL) • Large B-Cell Lymphoma (LBCL) • High-Grade B-Cell	Both FDA and EMA; Except HGBCL (FDA only) **Since:** FDA: August 30, 2017 EMA: August 23, 2018

			Lymphoma (HGBCL) ● Follicular Lymphoma (FL)	
Yescarta (Axicabtagene ciloleucel)	Kite Pharma, Inc. (USA)	CD-19; **ISD:** CD3ζ **CSD:** CD28; **Vector:** γ-retroviral vector	● Primary Mediastinal Large B-Cell Lymphoma (PMBCL) ● LBCL ● HGBCL ● FL	Both FDA and EMA; Except HGBCL (FDA only) **Since:** FDA: October 18, 2017 EMA: August 23, 2018
Tecartus (Brexucabtagene autoleucel)	Kite Pharma, Inc. (USA)	CD-19; **ISD:** CD3ζ **CSD:** CD28;	● Mantle Cell Lymphoma (MCL)	Both FDA and EMA; Except B-ALL (FDA only)

		Vector: γ-retroviral vector	• B-ALL	**Since:** FDA: July 24, 2020 EMA: December 14, 2020
Breyanzi (Lisocabtagene maraleucel)	Juno Therapeutics, Inc. (USA)	CD-19; **ISD:** CD3ζ **CSD:** 4-1BB; **Vector:** Lentiviral vector	• Follicular Lymphoma Grade-3B (FL3B) • LBCL • HGBCL • PMBCL	Both FDA and EMA; Except HGBCL (FDA only) **Since:** FDA: February 5, 2021 EMA: April 4, 2022
Abecma (Idecabtagene vicleucel)	Calgene Corporation (USA)	B- Cell Maturation Antigen (BCMA);	• Multiple Myeloma (MM)	Both FDA and EMA; **Since:**

		ISD: CD3ζ **CSD:** 4-1BB; **Vector:** Lentiviral vector		FDA: March 26, 2021 EMA: August 18, 2021
Carvykti (Ciltacabtagene autoleucel)	Johnson and Johnson Innovative Medicine (Belgium)	BCMA; **ISD:** CD3ζ **CSD:** 4-1BB; **Vector:** Lentiviral vector	• MM	Both FDA and EMA; **Since:** FDA: February 28, 2022 EMA: May 25, 2022

Chapter 9

Clinical Trials

The first historically prominent phase-1 clinical trial, NCT01029366 occurred in which, CAR-T pioneer, Carl H. June with David L. Porter and team initially experimented on 3 refractory B-CLL diagnosed patients at the UPenn Medicine Center, where Bill Ludwig and Doug Olson became the first and second participants respectively. Among them, 1996's stage-1 preliminarily diagnosed Doug Olson's case report was elaborately published who was administered with a significant sub-minimal dose ($1.5x10^5$ / kg) of lentiviral transduced, autologous second-generation (4-1BB/ CD3ζ) CD-19 targeted CAR-T cells on July, 2010 after a decade-long treatment of multiple rounds of monoclonal antibody and antimetabolite or, alkylating agent combinations for his refractory condition. Where 40% of his bone marrow cells had become saturated with leukemic cells (LC) pre-treatment, this treatment evidently improved his overall condition which was concluded through flow-cytometry that analyzed absence of any LC achieving complete remission (CR) by day 23 and any detectable lymphadenopathy by the day 28 post-infusion which was again analyzed on the thirty-first through CT scan reassuring diminished adenopathy. In addition, the presence of CAR-T modified cells was also detected in the bone marrow by day 23 that ended up having prevalence of almost half a year indicating a longer-termed persistence due to the 4-1BB than CD28 as CSD. Moreover, the 3 and 6 month follow-up assessments showed negative for any apparent swelling of the lymph nodes and any presence of LC using karyotypic, flow-cytometric as well as morphological analysis along with continued sustained remission for the 10 consecutive months. Alongside, the subject was devoid

of any acute toxicity and the tumor lysis syndrome emergence deferred significantly comparative to the previously adapted therapies (Porter et al., 2011). Simultaneously, another pioneer, Michel Sadelain along with Renier Brentjens were also designing several clinical trials parallelly to study the safety and persistence of CAR-T therapy at the Memorial Sloan Kettering Cancer Center, one of which is NCT01044069, where 5 adult patients in the first cohort, with relapsed B-ALL diagnosis were administered with CD19 targeting, second generation (CD28/ CD3ζ) autologous CAR-Ts named 19-28z^{+} T lymphocytes in a 1.5-3 x 10^{6} cells/kg manner after infusion of a high dose (1.5-3 gm/m^{2}) of alkylating chemotherapeutic agent, Cyclophosphamide (CY) as a conditioning therapy for lymphodepletion which is essential for modified T-cells' optimized engraftment, amplification along with overall oncological proficiency (Wang et al., 2023). Following the protocol, all of the participants were chosen based on at least their first complete remission (CR01) subsequent relapse-positive, allogeneic hematopoietic stem cell transplantation (allo-HSCT) -negative and chemotherapy refractory status; irrespective of the degree of remission they could achieve post-salvage therapy. Among them, MSK-ALL04 and MSK-ALL05 were even unsuitable for allo-HSCT and MSK-ALL01 and MSk-ALL06 were clinically CR+ but also minimal residue disease positive (MRD+), expectedly making them to have substantially inferior therapeutic outcome than MSK-ALL03 with MRD- status. Nevertheless, in unison, the whole study population attained accelerated elimination of neoplasia with MRD- CR+ status; the longest case of MSK-ALL06 being 4 months and 2/7 weeks; which was accompanied by long-term molecular remissions in 80% of the patients through making them eligible and responsive towards sequential allo-HSCT (Brentjens et al., 2013).

The specific trials for the different products are discussed in the following sections.

9.1 Kymriah (Tisagenlecleucel)

The first prominent supportive clinical trial, NCT01626495, done using the future to be named, Kymriah (Tisagenlecleucel; then, 4-1BB/ CD3ζ CTL019) was funded by University of Pennsylvania and executed in Children's Hospital of Philadelphia as a phase 1/2a single-center study where initially 30 participants (children and young adults combined) with relapsed or refractory (chemotherapy and/or, allo-HSCT) (R/R) B-ALL were injected with CTL019 CAR-Ts in a 0.76 - 20.6 x 10^6 cells/ kg manner from 2012-2014. As an outcome, 90% of the cohort (27 recipients) got evaluated to have obtained CR+ status morphologically (mCR+) including 66.67% of the Blinatumomab resistant triad subgroup; along with 73.33% (22) recipients attaining MRD- just after 4 weeks. Crossing the 26 week benchmark, The CAR-T persistence prospect was found to be about 68% with overall survival (OS) being 78% including 67% event-free-survival (EFS) indicating sustained remission. Cumulatively, in a biennium period of monitoring, 70.37% (19) and 55.56% (15) subjects of the preliminary CR+ group, were found to be remissive and decided to be devoid of any additional treatment respectively (Maude et al., 2014).

The second significantly supportive trial NCT02228096, ENSIGN was conducted as the first multi-center (9) single arm, open level, phase 2 study experimenting on 29 R/R B-ALL patients (children and young adults combined) with an average of 68.2% blast presence who were infused with the same CTL019 Tisagenlecleucel in a 2-5 x 10^6 or, 1-2.5 x 10^8 modified CAR-T cells/ kg dose manner for ≤ / > 50kg patients respectively. It was the first prominent multi-center study to triumphantly work with a comparatively diverse heterogeneous group with a large refractory subgroup. In a median of 6.4 months of follow-up, primary end-point was met

through achieving the overall remission rate (ORR) of 69% (20) via an attainment of CR/CRi status with at least 4 weeks of sustenance including 2/5 recipients that had been infused with a suboptimal dosage. Minimal residual disease negative (MRD-) status, relapse free survival (RFS) and overall survival (OS) in the same timeframe was found to be 62.1%, 66.4% and 75.7% respectively. Concurrently, CRS was found to develop in 89.7% (26) of the population including grade 3 or 4 CRS in 37.9% (11) patients; all of which were transient and reversible leading to 0% CRS caused demise in the follow-up.

As a continuation, another singular cohort, phase 2 study NCT02435849 conducted devoid of the ideal randomized-control standard, named ELIANA was conducted using Tisagenlecleucel that was proven to be pivotal for gaining approval of international pharmaceutical regulatory bodies' (FDA, EMA and so on). 75 refractory and/or, recurrent B-ALL subjects of ≥3-21≤ age range, from 2015-2017, in numerous locations worldwide were infused with 0.2-5.4 x 10^6 cells/ kg patient mass whose circulatory systems were never exposed to any anti-CD19+ treatment formerly and followed-up for a median of 13.1 months. A minimum of quarter-yearly follow up exhibited total blood count normalization positive and negative status with common CR+ (CR/CRi) and MRD- report in 60% (45) and 21% (16) recipients respectively making up to 81% (61) of overall remission rate (ORR) meeting the primary endpoint goal; with its 95% (58) achieving MRD- by the exact end of fourth week. On the other hand, half and a full yearly evaluation of relapse-free (RFS), event-free (EFS) as well as overall survival (OS) set of the responders of any degree was found to be of 80%, 73%, 90% and 59%, 50%, 76% respectively. Additionally, prolonged persistence of as long as 1.67 years (617 days) was found to have been maintained with a median of 0.46 year (168 days) ensuring an overall stable remission accompanied by controllable and revertible cytokine release syndrome

(CRS) (Maude et al., 2018). In November 2022, a revised 38.8 months of median follow-up report of ELIANA was published with an addition of 4 subjects (n=79) to re-evaluate sustained performance and safety profile of Tisagenlecleucel. This time, the quarter-yearly result showed up to be of about 82% ORR with a cumulative of 66 recipients attaining CR/CRi status. Additional factors like RFS, EFS, duration of remission (DOR) and OS at a biennium and triennium were also measured; where at 2- and 3-years end benchmarks, RFS was found out to be of 58% versus 52.3%; and 52% versus 47.8% (in a supplementary allo-HSCT or other therapy censored versus uncensored approach), EFS to be of 49.4% (almost median) and 44.4%, DOR of 57.9% and 52.2% and OS of about 67.7% and 62.8% on an average respectively. To mention more specifically, RFS was discovered to be of 81% and 76% in an intersected subset of subjects proceeding without any follow-up treatment after achieving CR/CRi status at 2- and 3- years end. On the other hand, In a 1.5-year follow-up, RFS was seen in 100% of the inquired patients (n=8) of the 17 (22%) subjects undergoing allo-HSCT including 16.67% (11) of the CR/CRi patients. Lastly, B-lymphocyte aplasia on year 1 and 2 benchmarks were sequentially found to be of 71% and 59% which is an established biomarker of CAR-T persistence re-ensuring prolonged persistence (Laetsch et al., 2023).

9.2 Yescarta (Axicabtagene Ciloleucel)

Yescarta (Axicabtagene Ciloleucel), an FDA-approved chimeric antigen receptor (CAR) T-cell therapy developed by Kite Pharma and now under Gilead Sciences (Immuno-oncology news, Yescarta (Axicabtagene CILOLEUCEL) 2019), has demonstrated notable efficacy in treating relapsed or refractory (R/R) large B-cell non-Hodgkin lymphoma (NHL) (Inacio, 2020). Derived from the patient's own T-cells, Yescarta is engineered to express a chimeric antigen receptor

(CAR) targeting CD19, a protein commonly found on NHL cells, facilitating their destruction (Immuno-oncology news, Yescarta (Axicabtagene CILOLEUCEL) 2019). Clinical trials, particularly the ZUMA-1 trial, underscore Yescarta's significant therapeutic potential (Inacio, 2020). Following lymphodepleting chemotherapy, patients receiving Yescarta exhibited high overall response rates (ORRs) of 82%, with 54% achieving complete response (CR). Long-term follow-up data revealed sustained responses and durable remissions, with a median overall survival (OS) exceeding two years and a well-tolerated safety profile. Notably, real-world data from the US Lymphoma CART consortium corroborate the efficacy and safety findings observed in clinical trials (Inacio, 2020).

Expanding its therapeutic reach, Yescarta received additional FDA approvals for the treatment of relapsed or refractory follicular lymphoma (FL) and marginal zone lymphoma (MZL) (Inacio, 2020). The ZUMA-5 study, comprising patients with R/R FL or MZL, demonstrated an impressive overall response rate of 92%, with 76% of patients still in continued remission after 18 months. The median duration of response had not been reached yet. Grades 3 or higher cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) were 8% and 21%, respectively.

9.3 Tecartus (Brexucabtagene Autoleucel)

Tecartus (Brexucabtagene Autoleucel) emerges as a pivotal therapeutic option for adults with relapsed or refractory mantle cell lymphoma (MCL) (Immuno-oncology news, Yescarta (Axicabtagene CILOLEUCEL) 2019). This chimeric antigen receptor (CAR) T-cell therapy, developed by Kite, a Gilead Company, has garnered attention for its promising efficacy in this challenging patient population (Inacio, 2020). The pivotal Phase 2 ZUMA-2 trial, comprising

105 MCL patients who had received multiple prior therapies, including chemotherapy and a Bruton's tyrosine kinase inhibitor (BTKi), demonstrated compelling results. Notably, 87% of patients exhibited a response to a single dose of Tecartus, with 62% achieving complete cancer eradication. These response rates notably surpassed those typically observed in MCL patients progressing on BTKi therapies. Moreover, at one year post-treatment, 83% of patients remained alive, with 61% displaying no signs of disease progression (Immuno-oncology news, Yescarta (Axicabtagene CILOLEUCEL) 2019).

The mechanism of action of Tecartus involves genetically modifying autologous T-cells to express a chimeric antigen receptor (CAR) targeting CD19, a protein expressed on malignant B-cells (Inacio, 2020). This innovative immunotherapy harnesses the patient's immune system to recognize and eliminate cancer cells while sparing healthy tissues. The manufacturing process, known as XLP™, incorporates T-cell enrichment, a crucial step in diseases like MCL where circulating lymphoblasts are prevalent. Preclinical studies elucidate how Tecartus engages CD28 and CD3-zeta co-stimulatory domains to activate T-cells, leading to their proliferation, acquisition of effector functions, and subsequent eradication of CD19-expressing tumor cells. This mechanistic insight underscores the precision and potency of Tecartus in combating MCL (Inacio, 2020).

While Tecartus demonstrates notable efficacy, its safety profile remains a critical consideration (Immuno-oncology news, Yescarta (Axicabtagene CILOLEUCEL) 2019). In the ZUMA-2 trial, manageable adverse events were observed, with notable occurrences of neurologic toxicity (37%) and cytokine release syndrome (18%). Notably, these adverse events were consistent with those observed in previous CAR-T cell therapies. Additionally,

thrombocytopenia, anemia, and neutropenia were among the common Grade 3 or higher adverse reactions observed during the trial (Inacio, 2020).

9.4 Breyanzi (Lisocabtagene Maraleucel)

Liso-cel secured its position as the third FDA-approved CD19-targeted CART product for R/R HGBCL on February 5, 2021. This milestone was achieved based on findings from the TRANSCEND trial, a multicenter study focusing on patients with relapsed or refractory large B cell lymphoma who had undergone two or more lines of therapy. The trial encompassed 192 participants, showcasing an overall response rate (ORR) of 73%, coupled with a commendable complete response (CR) rate of 54%. Notably, among those achieving CR, approximately two-thirds sustained remissions spanning 6 to 9 months. Adverse events of Grade 3 or higher cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) were noted in 4% and 12% of patients, respectively. Additionally, significant occurrences of cytopenias and infections were observed (Sengsayadeth et al., 2021).

9.5 Abecma (Idecabtagene Vicleucel)

Abecma (Idecabtagene Vicleucel) marks a significant advancement in the realm of CAR T-cell therapy, particularly in the treatment landscape of multiple myeloma (MM). FDA's accelerated approval of Abecma on March 29, 2021, has paved the way for addressing the unmet medical needs of patients with relapsed or refractory MM who have undergone four or more prior lines of therapy, including key agents such as a proteasome inhibitor, an immunomodulatory agent, and an anti-CD38 monoclonal antibody (Sengsayadeth et al., 2021). The pivotal KarMMa study, comprising 128 evaluable patients, showcased encouraging outcomes, with an overall response

rate (ORR) of 73% and a commendable complete response (CR) rate of 33%. Furthermore, patients exhibited promising progression-free survival (PFS) and overall survival (OS) rates of 8.8 and 19.4 months, respectively. Notably, a substantial proportion of patients achieving CR or better demonstrated minimal residual disease (MRD) negativity, with rates reaching 79%. The incidence of Grade 3 or higher cytokine release syndrome (CRS) remained low at 5%, while immune effector cell-associated neurotoxicity syndrome (ICANS) was observed in 3% of patients, with no cases surpassing Grade 3 severity. These findings underscore the efficacy of ide-cel in eliciting responses, particularly in a heavily treated patient population, where over a quarter of patients attained MRD status (Sengsayadeth et al., 2021).

9.6 Carvykti (Ciltacabtagene Autoleucel)

Carvykti (Ciltacabtagene Autoleucel), also referred to as cilta-cel or JNJ-68284528, represents a gene-edited autologous chimeric antigen receptor (CAR) T-cell therapy designed to target BCMA-expressing cancer cells in relapsed or refractory multiple myeloma (MM) patients (Chekol Abebe et al., 2022). CARTITUDE-1 and CARTITUDE-2 trials reveal high response rates, including a stringent complete response (sCR) rate of 80.4% and >VGPR of 94.8% in CARTITUDE-1. In CARTITUDE-2, a 12-month progression-free survival (PFS) rate of 90% is observed, while the median duration of response (DoR) is not reached. These statistics underscore the efficacy of cilta-cel in advanced MM patients.. It is the sixth CAR T-cell product authorized by the FDA for B cell malignancies and the second approved BCMA-targeting CAR T-cell therapy specifically for MM. Manufactured by the Pharmaceutical Companies of Janssen and Legend Biotech, Carvykti employs synthetic receptors bearing features of both monoclonal antibodies (mAbs) and T-cell receptors (TCRs).

Notably, Carvykti's efficacy has been demonstrated in clinical trials, with high overall response rates (ORRs), stringent complete response rates (sCRs), and sustained minimal residual disease (MRD) negativity observed in heavily pretreated MM patients. Despite its therapeutic potential, Carvykti treatment may lead to manageable adverse effects, including cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS), necessitating careful monitoring and timely management (Chekol Abebe et al., 2022).

Chapter 10

CAR-T Disadvantages

CAR-T therapy in cancer treatment presents significant challenges and potential toxicities. Issues such as CAR-T cell exhaustion, antigen escape due to tumor heterogeneity, and difficulties in trafficking and infiltration into tumor sites are prominent. Moreover, toxicities like Cytokine Release Syndrome (CRS), Tumor Lysis Syndrome (TLS), neurotoxicity, and immunogenicity require careful management. This chapter aims to examine these complexities, offering insights into strategies for optimizing CAR-T therapy's efficacy while minimizing associated risks.

10.1 Challenges in CAR-T Application

Some of the obstacles in the application of CAR-T therapy are briefly discussed in this section.

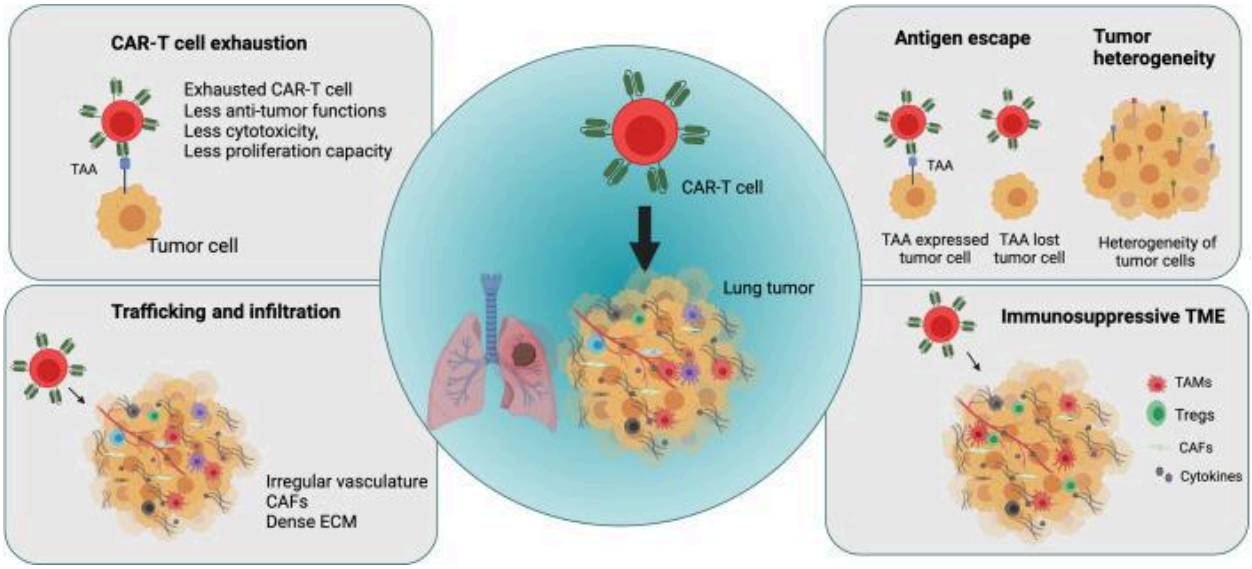

Figure 24. Principal challenges in CAR-T therapy usage (Kandra et al., 2022).

CAR-T cell exhaustion: CAR-T cell therapy faces a significant obstacle known as CAR-T cell exhaustion, hindering its effectiveness, particularly in solid tumors like lung cancer. This exhaustion arises within the tumor microenvironment due to continuous antigen exposure, elevated levels of inhibitory receptors, and the presence of suppressive immune cells and cytokines. Notably, the NR4A transcription factor family is implicated in driving T-cell exhaustion in solid tumors, thereby curtailing CAR-T cell functionality within this context (Kandra et al., 2022).

Antigen escape: Antigen escape poses a significant challenge in CAR-T cell therapy; one of the reasons of which is a prominently occurring issue- antigen heterogeneity (Kandra et al., 2022; R.C. Sterner & R. M. Sterner, 2021). In solid tumors like lung cancer, tumor antigen escape and the emergence of multiple resistance mechanisms contribute to disease relapse (Kandra et al., 2022). This phenomenon occurs as cancer cells either partially or completely lose target antigen expression post-treatment, leading to reduced CAR-T cell efficacy (R.C. Sterner & R. M. Sterner, 2021). For instance, CD19-targeted CAR-T cell therapy in ALL and BCMA targeted CAR-T cell treatment in multiple myeloma patients resulted in disease recurrence due to antigen escape mechanisms (Kandra et al., 2022). Similarly, IL13Ra2-targeted CAR-T cell therapy in glioblastoma patients led to relapse because of reduced IL-13Ra2 expression in tumors (Kandra et al., 2022). To address this challenge, strategies such as targeting multiple antigens simultaneously with dual CAR constructs or tandem CARs are being explored (R.C. Sterner & R. M. Sterner, 2021).

Trafficking and Infiltration: Effective trafficking and infiltration of CAR-T cells into solid tumors, such as lung cancer, presents a formidable challenge (Kandra et al., 2022; R.C. Sterner & R. M. Sterner, 2021). Tumor infiltration by T cells is predominantly influenced by factors such as

chemokines, chemokine receptors, adhesion molecules, and the complex tumor microenvironment (TME) (Kandra et al., 2022). Moreover, the dense fibrotic environment in lung cancer, characterized by abnormal collagen deposition and activation of cancer-associated fibroblasts (CAFs), acts as a physical barrier hindering immune cell infiltration and therapeutic efficacy (Kandra et al., 2022). To overcome these limitations, alternative delivery routes such as local administration have been proposed (R.C. Sterner & R. M. Sterner, 2021). Preclinical studies have demonstrated the superiority of intraventricular injection in breast cancer brain metastases and glioblastoma, showing enhanced therapeutic efficacy (R.C. Sterner & R. M. Sterner, 2021). Additionally, strategies involving the expression of chemokine receptors on CAR-T cells matching tumor-derived chemokines have shown promise in improving trafficking and antitumor efficacy (R.C. Sterner & R. M. Sterner, 2021). Innovative approaches, including the development of CAR-T cells expressing enzymes to degrade extracellular matrix components and target fibroblasts, hold potential for enhancing tumor penetration and therapeutic outcomes in solid tumors (R.C. Sterner & R. M. Sterner, 2021).

Immunosuppressive tumor microenvironment: The tumor microenvironment (TME) in solid tumors is characterized by a complex interplay of immunosuppressive elements (Kandra et al., 2022; De Marco et al., 2023; R.C. Sterner & R. M. Sterner, 2021). Various cell types, including myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T cells (Tregs), contribute to the immunosuppressive milieu (Kandra et al., 2022; R.C. Sterner & R. M. Sterner, 2021). These cells secrete factors such as TGF-b, IL-10, ARG-1, inducible nitric oxide synthase (iNOS), COX2, PGE2, FAP, and PD-L1, which regulate metabolism, cytokine networks, and immune checkpoints within the TME (Kandra et al., 2022). Moreover, the TME is often oxidative, hypoxic, acidic, and nutrient-starved, further promoting

an immunologically detrimental environment (De Marco et al., 2023). Immune checkpoint pathways, including PD-1 and CTLA-4, serve to diminish antitumor immunity within the TME. The presence of these immunosuppressive factors and cell types not only inhibits CAR-T cell functionality but also fosters an environment conducive to tumor growth and progression. Strategies to overcome this immunosuppression include combination immunotherapy with CAR-T cells and checkpoint blockade, aimed at enhancing T cell persistence and function. Additionally, efforts to engineer CAR-T cells resistant to immunosuppressive signals or capable of providing immunostimulatory signals hold promise for improving therapeutic outcomes in the hostile TME (R.C. Sterner & R. M. Sterner, 2021).

10.2 Toxicities

Enhancing the effectiveness of T cells through CAR genetic modification for cancer treatment also heightens associated toxicities, highlighting the importance of categorizing these diverse adverse events into on-target on-tumor, on-target off-tumor, off-target, neurotoxicity, and other toxicities for clearer assessment in clinical trials. In this section, we will briefly explore some of the toxicities that may arise due to administration of CAR-T cell therapy.

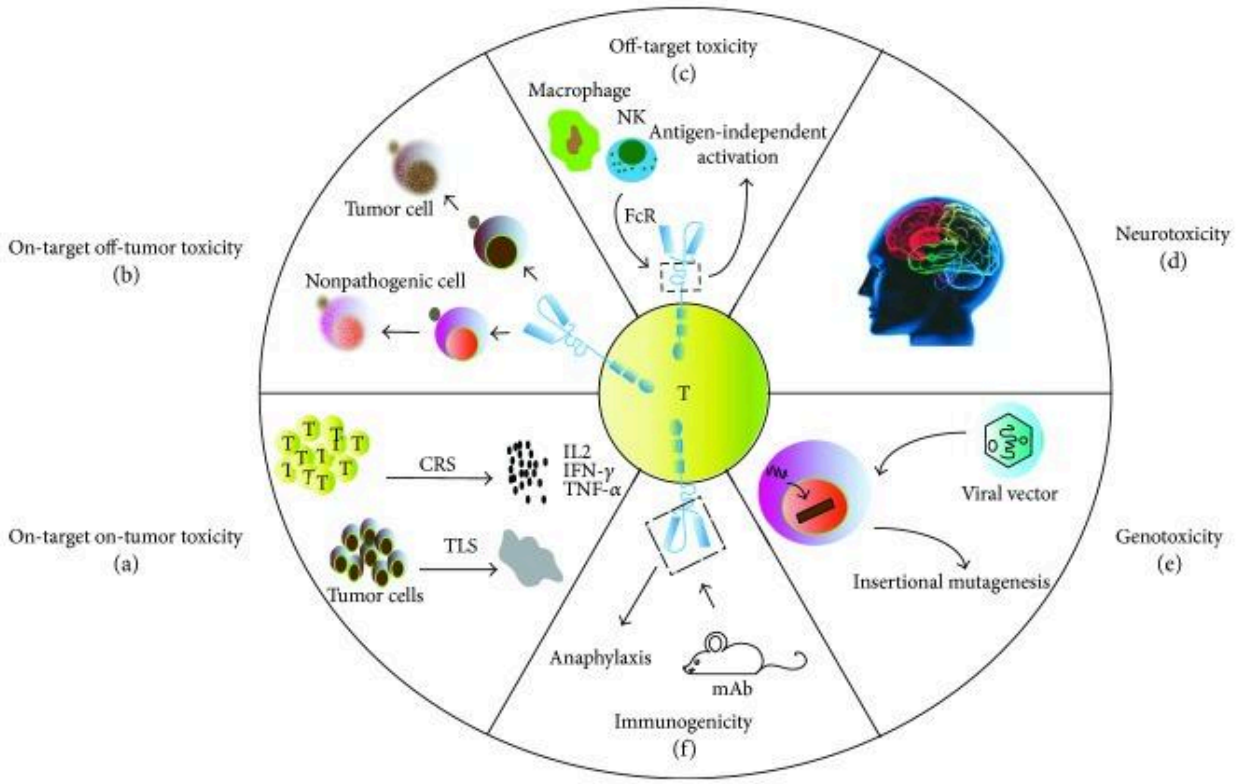

Figure 25. Probable toxicities of CAR-T therapies (Sun et al., 2018).

On-target-on-tumor toxicity: Two main types of toxicities fall under this category, namely, Cytokine release Syndrome (CRS) and Tumor lysis Syndrome (TLS).

Cytokine release syndrome (CRS) is a common toxicity post CAR-T cell therapy, marked by fever, nausea, fatigue, and cardiac dysfunction (Kandra et al., 2022; De Marco et al., 2023). Elevated IL-6 levels in serum correlate with CRS severity, indicating CAR-T cell activation and cytokine release (Kandra et al., 2022). While mild cases present flu-like symptoms, severe CRS can progress to circulatory shock and multi-organ failure, necessitating prompt medical intervention (De Marco et al., 2023). Management includes high-dose corticosteroids and IL-6 receptor antagonist mAbs like tocilizumab to temper immune reaction and cytokine release (Sun et al., 2018; De Marco et al., 2023).

Tumor lysis syndrome (TLS) is a potential toxicity arising from CAR-T cell-induced tumor cell lysis (Kandra et al., 2022). TLS leads to metabolic abnormalities, organ damage, and renal failure. Rapid tumor cell destruction releases intracellular contents, causing systemic disturbances reflected in elevated serum phosphate, potassium, and uric acid levels (Sun et al., 2018). Disease burden influences TLS severity, underscoring the need for electrolyte balance monitoring and management to prevent complications. Hydration, uric acid-lowering agents, and renal function monitoring are key management strategies to mitigate TLS-related risks (Sun et al., 2018).

On-target-off-tumor toxicity: On-target off-tumor toxicity arises when CAR T cells attack normal tissues expressing the targeted antigen (Sun et al., 2018; Kandra et al., 2022; De Marco et al., 2023). For instance, CAR T cells targeting carbonic anhydrase IX in renal cell carcinoma patients led to cholestasis due to antigen expression on bile duct epithelial cells (Sun et al., 2018; Muhammad et al., 2017). Similarly, HER2/neu-specific CAR T cells caused respiratory failure by recognizing pulmonary tissue antigens (Kandra et al., 2022; Muhammad et al., 2017). B-cell aplasia, though being comparatively quite easily manageable, occurs following CD19-specific CAR T cell therapy, necessitating gamma globulin infusion to reverse the adverse reaction (De Marco et al., 2023; Muhammad et al., 2017). This toxicity underscores the importance of selecting antigens with minimal expression in nonpathogenic tissues to avoid harmful effects (De Marco et al., 2023; R.C. Sterner & R. M. Sterner, 2021).

Off-target-off-tumor toxicity: Off-target toxicity in CAR T cell therapy arises when engineered T cells mistakenly attack antigens other than the intended target or self-activate independently. This occurs due to interactions between CAR constructs and Fc receptors on innate immune cells, leading to antigen-independent activation. While cross-reactive antigen recognition has not

been observed in CAR T cell trials, fatal cardiac toxicity resulted from autologous T cells engineered with an enhanced affinity TCR, highlighting the need for caution in targeting novel tumor-associated antigens (Sun et al., 2018).

Neurotoxicity: Neurotoxicity is a significant concern in CD19-specific CAR-T cell therapy, characterized by a range of symptoms from confusion to seizures (Sun et al., 2018). The pathophysiology remains unclear, with potential involvement of cytokine-mediated inflammation and blood-brain barrier dysfunction (Kandra et al., 2022). Evidence suggests that CAR T cells may infiltrate the cerebrospinal fluid, contributing to neurotoxicity (Sun et al., 2018). Clinical manifestations include encephalopathy, memory loss, seizures, and impaired speech (De Marco et al., 2023). Management strategies include corticosteroids, although the efficacy of tocilizumab remains uncertain (De Marco et al., 2023). Additionally, GM-CSF neutralization and IL-1 receptor antagonists show promise in mitigating neurotoxicity associated with CAR-T cell therapy (R.C. Sterner & R. M. Sterner, 2021).

Immunogenicity: Immunogenicity is the physiological phenomenon of inducing an immune response in a subject when any foreign organism or molecule is identified. Immunogenicity in CAR T-cell therapy, often stemming from mouse monoclonal antibodies (mAbs) in the antigen recognition region (Sun et al., 2018), can lead to severe anaphylaxis, as seen in mesothelin-specific CAR T-cell therapy. Humanizing single-chain variable fragments (scFvs) and utilizing human or humanized antibody fragments help mitigate this risk (Sun et al., 2018; Hashem Boroojerdi et al., 2020). Modification of CAR construct domains also reduces immunogenicity and improves CAR-T cell persistence. Prompt recognition and treatment are crucial for managing potential life-threatening immunogenic reactions.

Genotoxicity- insertional mutagenesis: Genotoxicity in CAR T-cell therapy is a concern due to the use of integrating viral vectors, which may lead to oncogenic insertional mutagenesis (Sun et al., 2018; Muhammad et al., 2017). Although no such toxicity has been reported in CAR therapy to date, the risk of insertional mutagenesis remains a consideration for the future, especially considering the potential lifetime persistence of CAR T cells in treated patients (Sun et al., 2018). Additionally, while anaphylaxis is a recognized adverse reaction in CAR T-cell therapy, no cases of insertional mutagenesis have been reported (Muhammad et al., 2017).

Graft-Versus-Host-Disease: Graft-versus-host disease (GVHD) is an immune response in transplant recipients that can harm vital organs, often necessitating immunosuppressive drugs, increasing infection risks. Early CAR T-cell therapy for ALL has shown minimal GVHD incidence. However, a case documented chronic GVHD post-therapy, managed with corticosteroids. Donor-derived CAR T cells can induce GVHD, typically appearing 3-4 weeks after infusion. GVHD severity was addressed using genome-editing strategies to disable αβ T-cell receptor expression (De Marco et al., 2023).

Chapter 11

Future Prominent Targetable Antigens

Like mentioned above in the approved therapeutic agent chapter, CD-19 and BCMA are two of the most prominent targetable antigens with 4 of them successfully working against CD19-presenting tumor cells which are mostly leukemia and lymphomas of various kinds; and 2 of them against BCMA presenting multiple myeloma cells. Among all the discovered TAA, CD19 and BCMA are the most ones; but a lot more new antigens have been identified till now; a lot of which are under preclinical as well as clinical trials exhibiting notable clinical outcome.

Table 2. Leading target specific CAR-T therapies in hematologic cancers and inquiring clinical trials (Asmamaw Dejenie et al., 2022; De Marco et al., 2023).

Target specific CAR-T	**Targeted Cancer**	**Prominent Clinical Trials**	**Trial Phase and Study Outcome**
CD20-CAR	Leukemias and Lymphomas (e.g., R/R B-NHL)	NCT03277729, NCT04007029, NCT04697940, NCT01735604	Phase 1/2; NCT03277729: High CR+, ORR+ and safety profile; NCT01735604: 54.55% CR+, 18.18% stable disease (SD)+, 180 days median PFS
CD22-CAR	Leukemias and Lymphomas (e.g., R/R B-ALL)	NCT03262298, NCT05470777, NCT055078227, NCT04088890	Phase 1/2; NCT03262298: Dose-reliant

			anti-tumor effect; NCT04088890: 100% CR+ effect in CD19-treated recu
CD30-CAR	R/R Hodgkin's Lymphoma	NCT02690545, NCT02917083	Phase 1/2; 59% CR+; 72% ORR; high safety profile
NKG2D-ligand based CAR;	R/R Acute Myeloid Leukemia;	NCT02203825;	Phase 1; NCT02203825: High antitumor efficacy
NKG2D-Ligand based CAR	R/R- (T cell Acute Lymphocytic Leukemia; Acute Myeloid Leukemia, Myelo-dysplastic-syndrome)	NCT02203825	Phase 1; High antitumor efficacy
CD33-CAR	R/R Acute Myeloid Leukemia	NCT03971799	Phase 1/2; Not complete
CD123-CAR	R/R Acute Myeloid Leukemia	NCT04014881	Phase 1; Not complete
CLL-1.CD33 and/or, CD123-CAR	R/R Acute Myeloid Leukemia	NCT04010877	Phase 1/2; Not complete
FLT3-CAR	R/R Acute Myeloid Leukemia	NCT05023707	Phase 1/2; Strong anti-tumor effect and low OT/OT effect
IL-1RAP-CAR	Chronic Myelogenous Leukemia	NCT02842320	Phase 1; anti-leukemic

			cytotoxicity against antigen expressing cells
CD7-CAR	R/R T cell Acute Lymphocytic Leukemia	NCT04689659	Phase 1/2; effective in vivo CAR-T cell expansion, High CR+, feasible safety profile
CD4-CAR	R/R T-cell Lymphoma	NCT04712864	Phase 1; powerful anti-tumor cytotoxicity
CD38-CAR	R/R Acute Myeloid Leukemia	NCT04351022	Phase 1/2; 66.7% CR+, 240 days overall survival (OS)
CD5-CAR	R/R T cell Non Hodgkin's Lymphoma; T cell Acute Lymphocytic Leukemia	NCT03081910; NCT04594135	Phase 1; moderate response and safety profile; robust tumor-elimination and controlled disease progression

Table 3. Leading target specific CAR-T therapies in solid cancers and inquiring clinical trials (Asmamaw Dejenie et al., 2022; De Marco et al., 2023).

Target specific CAR-T	**Targeted Cancer**	**Prominent Clinical Trials**	**Trial Phase and Study Outcome**
HER2-CAR	Central Nervous System tumor	NCT03500991	Phase 1; specially localized immune response
HER2-CAR	HER2+ expressing Breast, gastric, pancreatic cancer	NCT04650451, NCT04430595	Phase 1/2; NCT01935843: 4.5 month of PR+, 45.45% SD+, 146 days median PFS+
Allogenic NKG2D-CAR (CYAD-101)	Metastatic Colorectal Cancer	NCT03692429, NCT04991948	Phase 1; NCT03692429: 13.33% CR+, 60% stable disease (SD), 46.67% with quarterly SD and 119 day median PFS
IL13Rα2-CAR	Glioblastoma	NCT04003649, NCT04661384, NCT02208362, NCT01935843	Phase 1; NCT01935843: tumor shrinkage maintenance for 7.49 months; but antigen downgrading based tumor re-occurance
GPC3-CAR	Hepatocellular carcinoma	NCT03198546; NCT02395250, NCT03146234	Phase 1/2 ; NCT02395250 and NCT03146234: 18.18% PR+, Highest 3.68 year OS+, 50.3% and 10.5%

			OS+ rate in 6 and 36 months
EGFR806-CAR	Advanced Solid tumors	NCT03618381	Phase 1; Not complete
MUC1/PD-1-CAR	Non-small-cell lung cancer	NCT03525782	Phase 1/2 ; Not complete
GD2-CAR	Brain Cancer	NCT04099797	Phase 1; Enrolling participants
Mesothelin-CAR	Lung cancer, Pancreatic cancer, Overian cancer, Cervical cancer	NCT01583686	Phase 1/2; Enrolling participants
CEA-CAR	Lung, Colorectal, breast, stomach, pancreatic	NCT02349724	Phase 1; Enrolling participants
CD19.B7H3-CAR	Advanced Solid tumors	NCT04483778	Phase 1; Enrolling participants
TnMUC1-CAR	R/R solid tumors	NCT04025216	Phase 1; Not complete

Chapter 12

Conclusion

To sum up, CAR-T cell therapy stands as a testament to the power of innovative engineering in cancer immunotherapy. Its transformative potential offers hope for patients with refractory and relapsed malignancies, marking a paradigm shift in the oncological landscape. The success of CAR-T therapy lies in its ability to harness the body's immune system to selectively target and destroy cancer cells, thereby overcoming the evasion mechanisms employed by neoplastic cells.

The evolution of CAR-T technology, from first-generation to advanced generations, underscores continuous refinement aimed at optimizing efficacy and minimizing adverse effects. Additionally, sophisticated approaches such as dual CAR-T cell therapy, tandem CAR-T cell therapy, and universal CAR-T cell therapy demonstrate innovative strategies to further enhance the therapeutic potential of CAR-T cells.

Moving forward, ongoing research endeavors will undoubtedly further elucidate the intricacies of CAR-T therapy and pave the way for continued advancements in the field of cancer treatment. Despite its remarkable success, CAR-T therapy is not without challenges, and continued research efforts are necessary to optimize safety profiles and expand its applicability across different cancer types.

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