Biological context of CAR therapy in cancer treatment

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ABRREVIATIONS

CAR – CHIMERIC ANTIGEN RECEPTOR

TCR- T CELL RECEPTOR

FDA- FEDERAL DRUG ADMINISTRATION

MHC – MAJOR HISTOCOMPATIBILITY COMPLEX

FAS – CD95 RECEPTOR

CD - CLUSTER OF DIFFERENTIATION

SCFV – SINGLE CHAIN VARIABLE FRAGMENT

TM – TRANSMEMBRANE DOMAIN

ITAM – IMMUNORECEPTOR BASED TYROSINE ACTIVATION MOTIFS

CRS – CYTOKINE RELEASE SYNDROME

ICANS- IMMUNE EFFECTOR CELL ASSOCIATED NEUROTOXICITY SYNDROME

CPAP - CONSTANT POSITIVE AIRWAY PRESSURE

BIPAP – BILEVEL POSITIVE AIRWAY PRESSURE

TRUCKS – T CELLS REDIRECTED FOR UNRESTRICTED CYTOKINE INTIATED KILLING

CAE – CARDIOVASCULAR ADVERSE EFFECTS

CTCAE – COMMON TERMINOLOGY CRITERIA FOR ADVERSE EVENTS

ASTCT – AMERICAN SOCIETY OF TRANSPLANTATION AND CELLULAR THERAPY

LCMV – LYMPHOCYTIC CHORIOMENINGITIC VIRUS

APC – ANTIGEN PRESENTING CELL

GVHD – GRAFT VERSUS HOST DISEASE

LVEF – LEFT VENTRICULAR EJECTION FRACTION

VF – VENTRICULAR FIBIRILLATION

NICE – NATIONAL INSTITUTE FOR HEALTH AND CARE EXCELLENCE

SUPRA T CAR – SPLIT, UNIVERSAL AND PROGRAMMABLE CAR-T SYSTEM

NHL – NON-HODGKIN LYMPHOMA

CLL – CHRONIC LYMPHOCYTIC LEUKEMIA

ALL – ACUTE LYMPHOBLASTIC LEUKEMIA / ACUTE LYMPHOCYTIC LYMPHOMA

DIC – DISSEMINATED INTRAVASCULAR COAGULATION

SNP – SINGLE NUCLEOTIDE POLYMORPHISM

PBMC – PERIPHERAL BLOOD MONONUCLEAR CELL

UCB – UMBILICAL CORD BLOOD

IPSC – INDUCED PLURIPOTENT STEM CELLS

NK – NATURAL KILLER

HLH – HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

MAS – MACROPHAGE ACTIVATION SYNDROME

LDH – LACTATE DEHYDROGENASE

MUGA – MULTIGATED AQUISITION

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ABSTRACT

CAR-T therapy in malignant diseases

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Malignant diseases have been prevalent in people since recorded history. The etiologies are numerous but usually cancer is driven by the transformation of normal cell into a pre-cancerous state due to mutations. It is known that these cells emerge every day due to errors in DNA replication, however most of them are eliminated either through apoptosis or via the immune system during immune surveillance. The issue arises how a pre-cancerous cell manages to proliferate while evading those mechanisms and consequently gives rise to cancer.

Various therapies exist to treat malignancies, from classical chemotherapy and radiation therapy to more novel therapies, including "biologicals" where monoclonal antibodies directed at a specific antigen on the surface of malignant cells are used. New advances in genetics have allowed the advent of the adoptive cellular therapies combined with gene editing in genes of immune cells in order to alter the protein structure of their receptors and by that the molecular conditions required for their activation.

One such therapy is the chimeric antigen receptor (CAR) T-cell therapy. This therapy utilizes a viral vector for gene editing of both CD4+ and CD8+ T cell receptor (TCR) to change intracellular signalling components thus enabling T cells to operate without a supporting environment, one which is usually lacking around malignant cells. CAR-T cell therapy was first developed in the 1980's and since then massive strides have been achieved in transformation of this experimental tool to a recognized and FDA approved therapy, and as a third line/ treatment for refractory haematological malignancies since 2017. Since then, in several years it has led to a significant increase in remission rates with a substantial adverse effect profile and less successful lasting of remission. The treatment is performed in specialized centres and is currently only available in some countries due to the difficulty of therapy preparation as well as high costs.

The goal of this review is to collate the various sources, trials, reviews, and meta-analysis and form a coherent review of the CAR-T cellular therapy. The principle behind its conception, the various

generations and FDA approval process to the current therapy, its indications and - adverse effects have been presented and even as well as the conceptualization of the future of the therapy.

CAR-T cells are CD4+ and/or CD8+ T cells that have been genetically engineered to produce chimeric (artificial) antigen receptors (CAR) on their surface.

SAŽETAK

CAR-T terapija u malignim bolestima

Autor: Yaniv Izhaki Kotchinsky

Maligne bolesti u ljudi dokazane su još u dalekoj prošlosti. Mogu biti brojnih etiologija no obično je kancerogeneza pokrenuta transformacijom normalne stanice u pretkancerogeno stanje, najčešće zbog mutacija. Zna se da takve stanice nastaju svakodnevno zbog pogrešaka u replikaciji DNA, međutim većina ih se eliminira ili apoptozom ili putem imunološkog sustava tijekom imunološkog nadzora. Pitanje koje se postavlja jest kako se pretkancerogena stanica uspijeva dijeliti izbjegavajući navedene mehanizme i posljedično tome vodi do razvoja maligne bolesti.

Postoje različite terapije za liječenje malignih bolesti, od klasične kemoterapije, terapije zračenjem do novijih terapija, uključujući "biološke" – gdje se koriste monoklonska protutijela usmjerena na specifični antigen na površini malignih stanica. No napredak u genetici omogućio je pojavu adaptivnih staničnih terapija u kombinaciji s uređivanjem gena (engl. *gene-editing*) imunosnih stanica kako bi se mogla izmijeniti proteinska struktura njihovih receptora a time i molekularni uvjeti potrebni za njihovo usmjerenje ka uništavanju tumorskih stanica.

Jedna od takvih terapija je terapija putem kimernog antigenskog receptora T-stanicama (CAR). Ova terapija koristi virusni vektor za uređivanje gena za receptor na T stanicama (TCR), radi promjene njegovih unutarstaničnih signalnih komponenti, kako bi omogućila T stanicama da rade bez suportivne okoline, one koje obično nedostaje oko malignih stanica. Terapija CAR-T stanicama prvi je put razvijena 1980-ih godina prošlog stoljeća i od tada je postignut veliki napredak u transformaciji eksperimentalnog alata u priznatu i odobrenu FDA terapiju kao treću liniju / tretman za refraktorne hematološke maligne bolesti od 2017. Odtad je u nekoliko godina pokazala značajan porast u postotku remisije no i sa bitnim štetnim učincima i manje uspješnom trajanju remisije. Liječenje se provodi u specijaliziranim centrima i trenutno je dostupno samo u nekim zemljama zbog poteškoća u pripremi terapije, kao i visokih troškova.

Cilj ovog pregleda je iz različitih izvora, kliničkih ispitivanja, preglednih članaka, te meta-analize izložiti koherentan pregled CAR-T stanične terapije. Prikazan je princip koji stoji iza njegova koncepta, različitih generacija CAR receptora i postupka odobrenja FDA, do trenutne terapije, njezinih indikacija, štetnih učinaka, kao i konceptualizacija budućnosti terapije.

1. INTRODUCTION

In order to understand how why CAR-T cell therapy has become attractive anti-cancer tool but also how it works, one must first understand the mechanism of anergy utilized in vivo in order to prevent autoimmunity which is the way how malignant cells subvert the mechanism of anergy to adopt one of the hallmarks of cancer- avoidance of immune destruction. Every cell in our body (apart from erythrocytes) displays parts of internally produced proteins of a major histocompatibility complex type I (MHC I), which allows CD8+ cells to monitor cell health and if need exist, to eliminate infected or malignant cells. CD8+ cells must be first activated by the dual signal system but also maintained by cytokine release from CD4+ cells. In the dual signal system costimulatory signals come from antigen presenting cells (APC), especially dendritic cells[1]. When activated, CD8+ cells find the infected target and initiate cellular apoptosis by one of two main mechanisms. First, FAS-FASL interaction occurs when a CD-8+ T cell (Tc) is activated. The Tc expresses a ligand termed FAS ligand (CD95L). When a Tc attached to an infected cell it also attaches FASL receptor to the cell's FAS (CD95) receptor. This process activates downstream caspases and promotes apoptosis of the cell (Figure 1). Cytokine induced apoptosis is a second mechanism. When activated, a T cell will circulate and seek out applicable MHC I presenting cells. Once found, the activated cytotoxic T cell will attach to the MHC I and begin releasing cytokines such as perforins, granzymes and granulysins. These cytokines promote apoptosis via a similar mechanism to FAS activated apoptosis. This mechanism is not limited to Tc cells only. Recent evidence shows that when circumventing MHC II activation restrictions via monoclonal antibody blockade CD4+ cells may also release perforins and granzymes[2] and by thus participate in cytokine induced apoptosis.

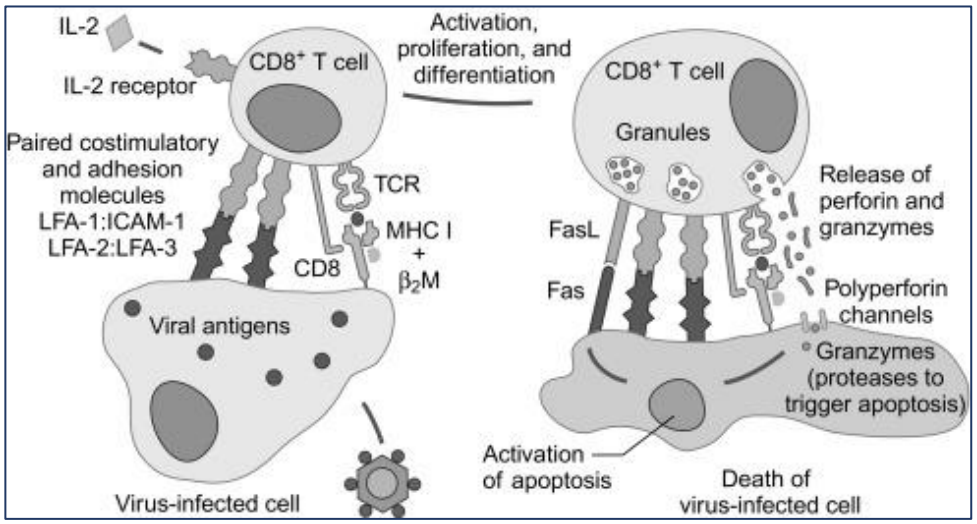

Figure 1. The two main mechanisms of CD8+ T cell induced cellular apoptosis. Taken from: [3].

Malignant cells can achieve avoidance of immune destruction by several mechanisms, most of which inhibit the activation of Tc cells. They do so by reducing the expression of MHC-I molecules, which leads to a decrease in immune surveillance[4] and an overall worse prognosis, especially in solid tumors[5]. They also release decoy molecules which inhibit either T cell activation or activate other immune cells, especially macrophages[6]. Moreover, an expression of cytokines and various surface molecules are found on cancer cells suppress immune reactivity and promote a switch to Th2 or Treg subtypes[7]. Lastly, by production of intracellular survival signals, overriding apoptotic signals and production of a poorly vascularized environment, cancer cell precludes access to most immune cells. This mechanism is especially prevalent in solid tumours.

CAR-T therapy was designed to block the mechanisms listed above. It acts by ensuring CD8+ and CD4+ T cells to circumvent the requirement for the dual signal co-stimulation, allowing them to be activated by just one signal. In addition, recent generations of CAR-T cells have more related activities which aid them in removal of malignant cells. The aim of this thesis is to form a coherent review of the CAR-T cellular therapy, where molecular basis behind its conception, the structure of CAR-T in various generations and FDA approval process up to the current therapy, its indications and-, adverse effects will be presented together with the conceptualization of the future of this therapy.

2. CAR RECEPTOR STRUCTURE (ECTO-, ENDO- AND TRANSMEMBRANE DOMAINS)

CAR-T receptors have been first constructed in 1987[8]. Since then, the procedure has developed immensely, by re-designing it in order to enable prolonged survival in vivo, and to increase the efficacy while reducing adverse side effects. The general structure of CAR-T construct has remained relatively similar throughout the years with 4 distinct generations and is presented with several components[9,10] (Figure 2).

Most externally lies a crucial **binding domain** which represents a monoclonal antibody fragment composed of a single light and heavy chain (**single chain variant fragment- scFv**) together with a linking peptide. This section determines the target of CAR-T cells, e.g. an extracellular domain of receptor on the malignant cell(most known and widely researched is the (CD) cluster of differentiation 19 surface receptor)[11]. Other targets may be soluble factors (such as TGF-β[12]). Another type of binding domain is a TCR like receptor binding domain which enables the CAR-T cell to recognize intracellular molecules via interface with MHC I[13, 14].Here, a balance is required to maintain affinity within an effective zone. Too little affinity will decrease the avidity of the CAR-T binding to its target which will lead to subpar results. On the other hand, too high affinity will cause the CAR-T cell to go through activation induced cell death (AICD) or cause an increase in the toxicity of treatment[15, 16]. Attached to the binding domain is a **hinge region,** aimed to stabilize and anchor the binding domain to the cell membrane while it is connected to the transmembrane domain. **Transmembrane domain (TM** connects the **intracellular domain** to the hinge region, and it also has a role in linking several factors in the CAR-T cell efficacy and longevity. Most known example is the CD3ζ which may increase dimerization and incorporation of CAR to resident T cells thus extending longetivity[17]**.** Some others are also tested, like CD8+ which has a greater tendency to release TNF-α and IFNɣ and reduced likelihood of activation induced cell death, while AICD[18] and CD28 transmembrane domains increase stability when connected to the intracellular domain**. Intracellular domain** comprises an effector mechanism of CAR, usually composed of CD3ζ, which contains several immunoreceptor based tyrosine activation motifs (ITAMs). When the single chain variable fragment (scFv) attaches to the appropriate target the signal is transduced through ITAMs congregation. This process requires a costimulatory molecule, which was mostly incorporated in the next generations of CAR-T cell receptors while some of them have acted as “armors” and were incorporated into later generations of CAR-T cells. A CAR-T receptor is usually named by its domains from exterior

to interior. It is important to point out that every CAR-T cell has domains listed above, however, the main differences among them are either the content specificity, costimulatory properties and further modifications.

3. CAR-T CELL GENERATIONS

The various generations and their components are outlined in Figure 2. and Table 1. and are described by the generations of production.

3.1. First generation

The first generation of CAR-T cells was relatively simplistic and non-independent, as it required infusion of IL-2 to promote T-cell survival but again it had relatively short longetivity.[2,82] It was composed of scFv, hinge region, TM domain and CD3ζ signaling domain which contained three ITAMs. These were not linked to any costimulatory molecule or any molecule enhancing survival, so the IL-2 infusion necessity and poor lifespan in vivo[19,20] were the main reasons of low efficacy in therapy[19,21].

3.2. Second generation

Second generation of CAR-T cells was designed to address shortcomings of the first generation. They were similar in most of their structure to the first generation, but the main difference was in the intracellular signaling domain, which contained more costimulatory molecules such as CD28 and, CD137 (1-4BB) along with the CD3ζ elements, allowing prolonged survival and expansion of CAR-T cell population without continuous external intervention. These costimulatory molecules were beneficial for the survival and stability of the CAR-T cell as some have increased the expansion of CAR-T population (CD28)[22, 23], while others (4-1BB) have exhibited increased tendency to promote memory cell formation and persistence of CAR-T cell population[24]. Overall, this generation has been more successful. as CAR-T cell therapy utilizing 4-1BB, has also shown efficacy in the treatment of hematologic malignancies[25,26] The first FDA approved CAR-T therapy (tisagenlecleucel) comes from this generation

3.3. Third generation

This generation utilized two distinct costimulatory domains (e.g. CD28/4-1BB/CD3ζ or CD28/OX-40/CD3ζ)- within its intracellular domain to promote T-cell survival and expansion These constructs have shown varying degrees of *in vitro* and *in vivo* levels of activation, proliferation and interleukin-2 (IL-2)

production[27,28]. However, early clinical trials have not shown significantly increased efficacy of third generation CAR-T cells versus second generation[29, 30]. More recent evidence shows a better adverse effect profile and increased persistence in vivo[31 32]

3.4. Fourth generation

The 4th generation has adopted a different approach to increase the longevity and functionality of CAR-T cells. Instead of adding a tandem of costimulatory domains, this generation of CAR-T cells has added armor proteins to T-cells. Simply, by genetic modifications CAR receptors were optimized, by instruction or constitutively, to secrete active cytokines (especially IL-12), or to express ligands (CD40L) that promote pro-survival microenvironment that is however more suitable for eliminating malignant cells[33]. Also, this modification has an additional effect of recruiting nearby immune cells to aid the CAR-T cell in its function. Dye to their tendency to form immune suppressing microenvironments Armored CAR-T cells are nowadays mainly utilized to treat solid tumors due to their tendency to form immune suppressing microenvironments. As armor proteins utilized in the 4th generation serve various functions in a cell their inclusion in CAR depends on the tumor microenvironment. Some major armor proteins are listed in Table 1.

Table 1. Armor proteins and their effect on CAR-T cell efficacy and survival.

Armor protein	Function	references
IL-12	This cytokine is crucial to T cell survival and proliferation, while also promoting CD4+ switch to Th1 subtype, promoting the anti-cellular function of CD8+ cells. These armored CAR-T cells, when activated release relatively small amounts of IL-12, avoiding the side effects related to systemic therapy with IL-12. There is currently no approved medical treatment with this subtype of CAR-T cell therapy, but clinical trials are underway for treatment of ovarian cancer. These armored CAR-T cells are known as T cells redirected for antigen- unrestricted cytokine- initiated killing (TRUCKS).	34–36
CD-40L	This ligand is expressed on dendritic cells, CD4+ T cells, B cells and macrophages. In T cell activation it is the costimulatory second signal which aids CD4+ cells to activate Tc (CD8+) cells. This has been shown to improve cytotoxic killing in vitro	37
4-1BB	This commonly used costimulatory molecule can also be part of the additional inducible effects. When attached with its ligand 4-1BBL, it promotes cellular survival and proliferation. This was further supported by both in vitro and in vivo results of armored 4-1BB CAR-T cells, which show better proliferation rates and survival compared with non-containing 4-1BB CAR-T cells	38

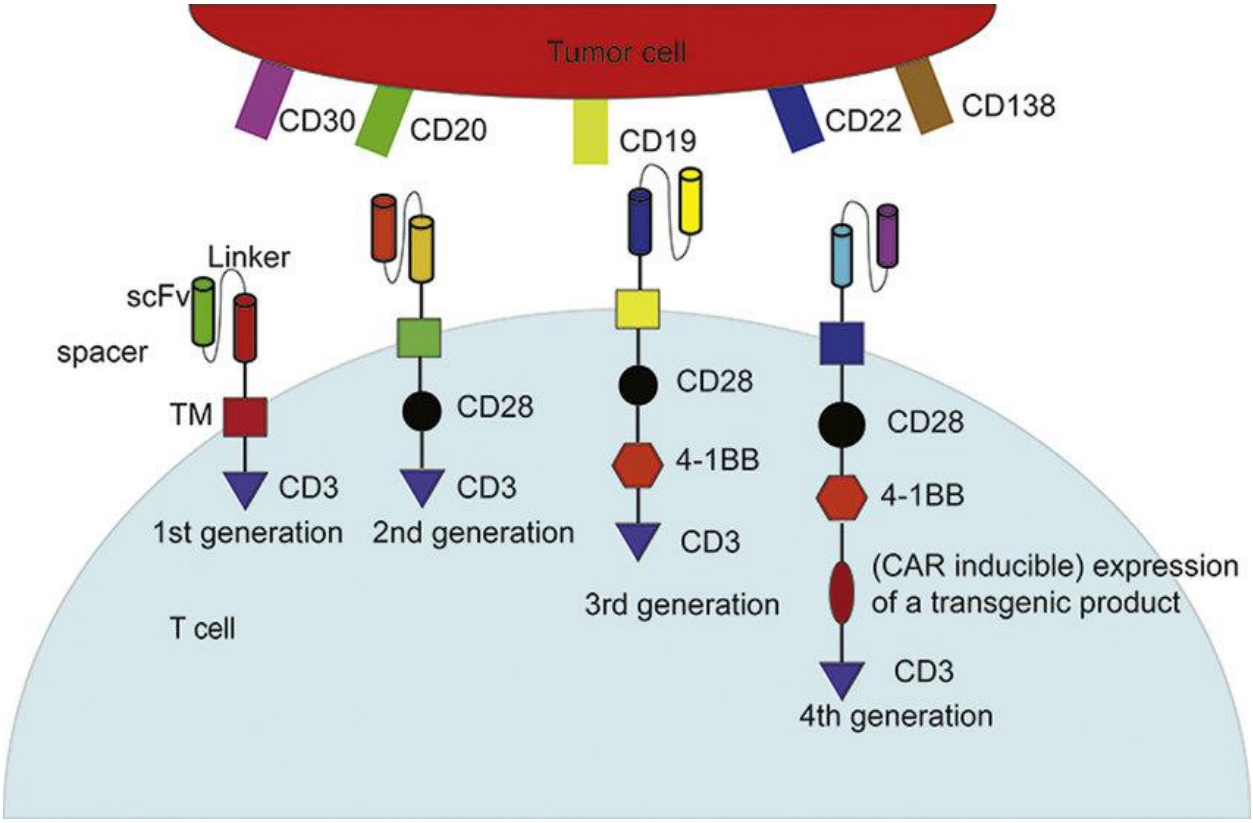

Figure 2: CAR-T cell domains throughout 4 generations and their targets[39].

3.5. Fifth generation [40]

The therapies using 5th generation CAR-T cells are currently still in development. This type has several potential novel mechanisms of action which are described below. (Table 2). It's principle of action is different from standard CAR-T cell therapy, allowing greater flexibility (Figure 3).

Table 2. The 3 main novel mechanisms of 5th generation CAR-T cellular therapy.

Type of 5th generation CAR	Function	clinical phase	references
Split, universal programmable (SUPRA) CAR model	The principle of operation is replacement of the scFv receptor of a standard CAR-T cell with a leucine zipper module, attached to the hinge, transmembrane and intracellular signaling domains. A leucine zipper containing scFv is then released with an attached leucine zipper domain as a form of monoclonal antibody. This allows physicians to modulate the activity of the SUPRA CAR-T cell and confer different targets with one CAR-T therapy.	Pre-clinical	[41]
Bispecific/dual signaling domains CAR-T cells	Bispecific CAR models-are CAR-T cells with two scFv domains attached together to the receptor, allowing more specific recognition but also reestablishing the costimulatory signal requirement. This allows for greater specificity of the treatment. Bispecific CAR-T cells are currently in phase 1 trial**.** A similar concept is utilized by employing two receptors with different signaling domains.	Phase I	[42,43]
Synthetic Notch receptor	Synthetic Notch receptor- a novel method of utilizing different response to the antigen-CAR binding via the notch signaling cascade. Here, an additional costimulatory molecule or an additional receptor is used to promote the release of various cytokines. During activation this mechanism ensures fine tuning and better specificity of release instead of simultaneous release of a bulk of cytokines- allowing a more controlled response with potentially less severe side effects during therapy.	Pre-clinical	[44,45]

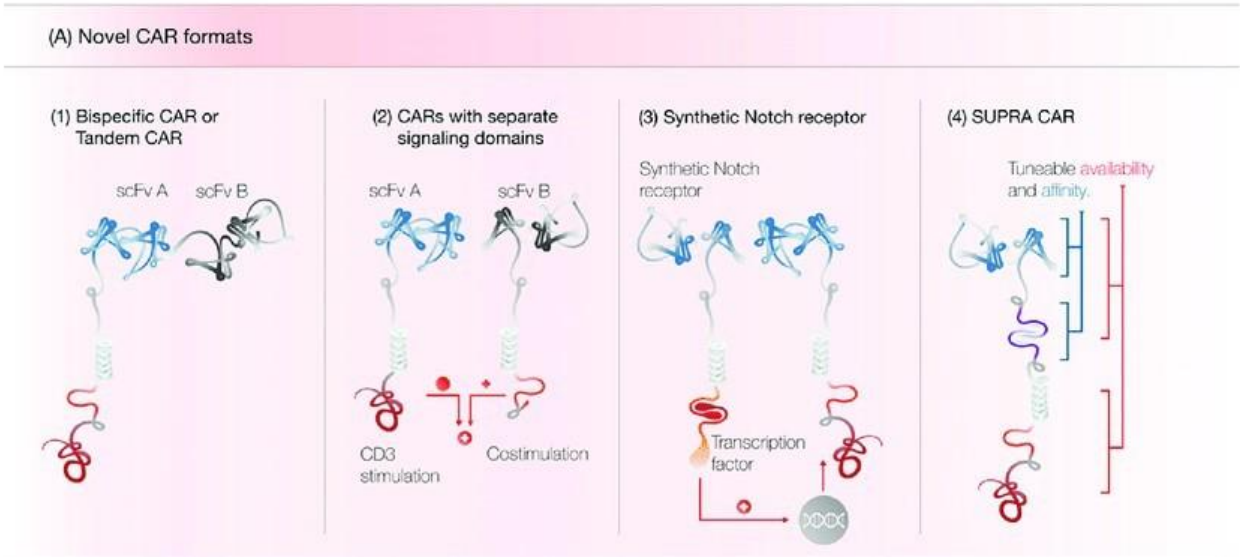

Figure 3. The 4 main 5th generation CAR-T cells in current research and their mechanism of action[46].

4. CAR THERAPY DEVELOPMENTAL TIMELINE

CAR therapy has first been developed in Japan in 1987[8], by a team lead by Yushikazu Kuwana, closely followed by Professor Zelig Eshar's and Professor Gideon Gross's contribution in 1989[47]. According to Professor Gideon Gross, these CAR-T cells were initially meant to study the mechanism of action of T cells in a controlled environment and as a therapeutic mean. These first generation CAR-T cells were not therapeutically useful since they poorly proliferated in vivo and required outside assistance in order to maintain survival[18,19] .This state had persisted until the development of second generation CAR-T cells in 2003, which has shown a capacity to eliminate leukemia cells in mouse model. In 2009 CAR-T cell were first used to treat relapsed/refractory leukaemia and in 2011 the first case of CAR-T cell treatment has been performed[26]. In 2012 a pivotal event occurred - a successful treatment of a 7-year-old patient-Emily Whitehead with non-treatment responsive ALL. Her subsequent complete remission of the disease has both revitalized and significantly increased the interest in CAR-T cell therapy[48]. As the year progressed more and more advancements were made in the field of CAR-T cell therapy, mainly in the structure and development of the CAR, such as generations of new CAR-T cells with more specific actions and lessened "on target off tumour toxicity"[49] or incorporation of cutting edge CRISPR system to the CAR-T cell procedure[50]. In 2017 another breakthrough occurred. Supported by the pivotal second phase trials ZUMA-1[51], JULIET[52] and ELIANA,[53] the FDA approved Tisagenlecleucel and axicabtagene ciloleucel for treatment of B-cell ALL and Diffuse large B cell leukaemia (DLBCL). Today, CAR-T

development is ongoing, and more and more techniques are discovered to aid in the efficacy, longevity and availability of CAR-T cell treatment as well as increasing the available repertoire of treatable malignancies with this therapy. Currently, CAR-T cell therapy is being explored as a treatment option for solid cancers, with new targets continuously being discovered and tested. As of the time of writing this thesis, according to clinicaltrials.gov, there are 1306 ongoing clinical trials using CAR-T cell therapy. Of them 730 are in phase 1, 358 in phase 2, 41 in phase 3 and 28 in phase 4, with an additional 277 reports available from early recruitment pre-initiation of phase 1 (Figure 4.). Currently, the main countries leading these experiments are the US, China, and the European Union.

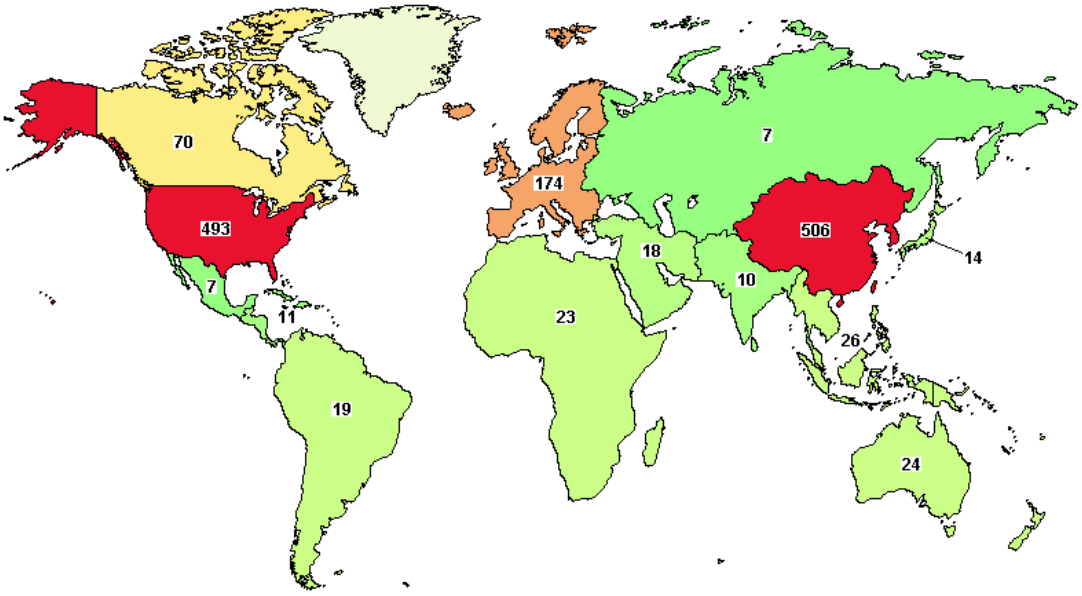

Figure 4. Map of clinical trials as of February 2021. Darker colors indicate greater number of studies. Source: clinicaltrials.gov

As of today, four CAR-T cell therapies have been approved by the FDA while three of those have also been approved by NICE (Table 3).

Table 3. The various FDA/NICE approved CAR-T cellular therapies.

Commercial name	Generic name	Company	Target	Indications	Approving agencies	References
Abcema	Idecabtagene vicleucel	Celgene Corporation	CD38	**Multiple myeloma-** refractory or relapsed only after 4 lines of therapy have failed in adult patients	FDA	[54]
Tecartus	brexucabtagene autoleucel	Kite Pharma, Inc. GILEAD	CD19	**Relapsed or refractory large B-cell lymphoma in adults, including diffuse and follicular lymphoma**, after 2 or more lines of therapy have failed, patients **up to 25 years of age with B-cell ALL , refractory or in second relapse**	FDA, NICE	[55,56]
Kymriah	tisangenlecleucel	Novartis	CD19	**Relapsed or refractory large B-cell lymphoma in adults, including diffuse and follicular lymphoma,** after 2 or more lines of therapy have failed, patients up **to 25 years of age with B-cell ALL , refractory or in second relapse.**	FDA, NICE	[52,53]
Yescarta	axicabtagene ciloleucel	Kite Pharma, Inc. GILEAD	CD19	adult patients with relapsed or refractory large B cell lymphoma after two or more lines of systemic chemotherapy have failed.	FDA, NICE	[57]

5. THE COMPLETE CAR PROCEDURE

CAR-T cell therapy is a classic example of bench to bedside medicine. The procedure uses almost exclusively autologous T-cells from the patient which are then modified and transplanted back to the patient. The purpose of the procedure is to supply T cells which exist as an independent population that could target cancer cells. CAR-T cell treatment is a multi-step procedure which requires specialized centres, multidisciplinary team, and close monitoring. The complete procedure is outlined in Figure 5.

The first step is **target identification,** meaning the identification of a cell population expressing the desired antigen and screening for the applicable candidates. Currently, CAR-T cell therapy is not the first line therapy and is normally utilized in more advanced tumors[58]. For CAR-T therapy that is currently approved, tumor or bone marrow aspirate is examined for applicable surface antigen (specifically CD19).

The second step is **baseline establishment,** where patient is checked for ferritin level, CBC, complete metabolic panel, lactate dehydrogenase (LDH), echocardiogram/multigated acquisition (MUGA)[59] [60] [61] and disease burden evaluation All analyses should be performed prior to initiation of therapy.

The **CAR-T cell production** includes several steps:

- Leukapheresis - This procedure involves extracting blood and solation of T cells from various subtypes, depending on the current need[62]. These filtered leukocytes are then either activated and proliferated or frozen in liquid nitrogen and sent to specialized centres. This procedure requires at least 500 WBC cells/microliter or 150 CD3+ cells/microliter in order to be succesful[63].
- CAR-T cell production - This is a complex process involving several steps and/or phases. First step is T cell selection, where viable T cells are selected based on their subtype and forced to proliferate. Several systems can achieve this purpose with differing results. The most commonly used population are CD3+ T cells[64,65], but evidence shows that other subtypes such as naive[66], central memory[67] and memory stem cells[68], might also be advantageous. This is followed by an activation and proliferation step to form CAR-T, which requires DNA manipulation in actively proliferating population of T cells. This can be achieved by several methods, all designed to consistently cause activation and proliferation of T cells, usually via artificial APC[69], antibody coated nanobeads, anti CD3 antibodies or Expamer technology[70]. Next, in the genetic modification phase, appropriate human gene-containing vectors are inserted, and the T cell acquires the

properties required for it to become a CAR-T cell. There are several vectors/mechanisms available today. The most utilized vector is σ-retrovirus vector and the first successful CAR-T therapy was formed utilizing it[11]. It was found to exhibit high gene expression and an established safety profile[71,72] . Most importantly, retrovirus vectors are more easily mass produced, enabling greater production of CAR-T cells[73]**.** However, they require an actively dividing cells to propagate the genetic modification. Lentivirus vectors, another choice, have better safety profile, especially with hematopoietic cell modification,[74] and lentivirus vectors can achieve genetic expression in non-dividing, non G0 phase cells. The main issues with lentiviral vectors are mass production and quality control. A different approach has also been introduced, i.e., transposon/transposase system - to transfer genetic material from the vector to the target. The currently utilized system, called "sleeping beauty", has shown promising results in reducing costs of production and adverse effect profile of CAR-T therapy[75]. After editing, the population of CAR-T cells after editing is then expanded in a bioreactor. There are several bioreactor types, with varying degrees of cost, transportation and storage methods and efficiency of expansion[76].

Quality control represents a crucial process in CAR-T cell manufacturing. The solution with cells is checked for sterility and lack of contaminants, but more importantly, the CAR-T cells are tested for their health status and function, specifically for cell population levels, morphology, antigen-target binding affinity, cytokine production and if applicable, armor protein release and response to activations signals.

Lymphodepletion and transport are done simultaneously. In order to achieve optimal CAR-T activation, expansion and persistence a lymphodepleting regiment must be performed prior to transplantation[77,78]. This lymphodepleting regiment is meant to decrease immunosuppression from surrounding lymphocytes, enable improved access to released cytokines[79], increase translocation of resident microbiota and promote IL-1 release[80] and enhance the ability of adoptive immune cells to traffic to the tumor site[81]. These lymphodepletion regiments are always accompanied by careful surveillance for opportunistic infections, with pneumocystis pneumonia prophylaxis as well as additional prophylaxis according to risk groups[58]. The treatment regimen utilized for lymphodepletion in most CAR-T cell treatment is a combination og cyclophosphamide (cy) and fludarabine (flu). Cyclophosphamide, a nitrogen mustard, has long been used in lymphodepleting regiments in allogenic hematopoietic cell transplant,[82] but addition of fludarabine, a purine analogue has been shown to reduce severity of adverse effects and improve CAR-T cell survival

compared to cyclophosphamide alone[83,84,85,86]. Lymphodepletion prior to solid tumor treatment is also done with cy/flu[87,88], but with higher doses.

CAR-T cells infusion and follow up are done after lymphodepletion, when autologous CAR-T cells are reinfused to the patient. The patient must remain in the hospital to allow for careful monitoring and surveillance for possible cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) development.

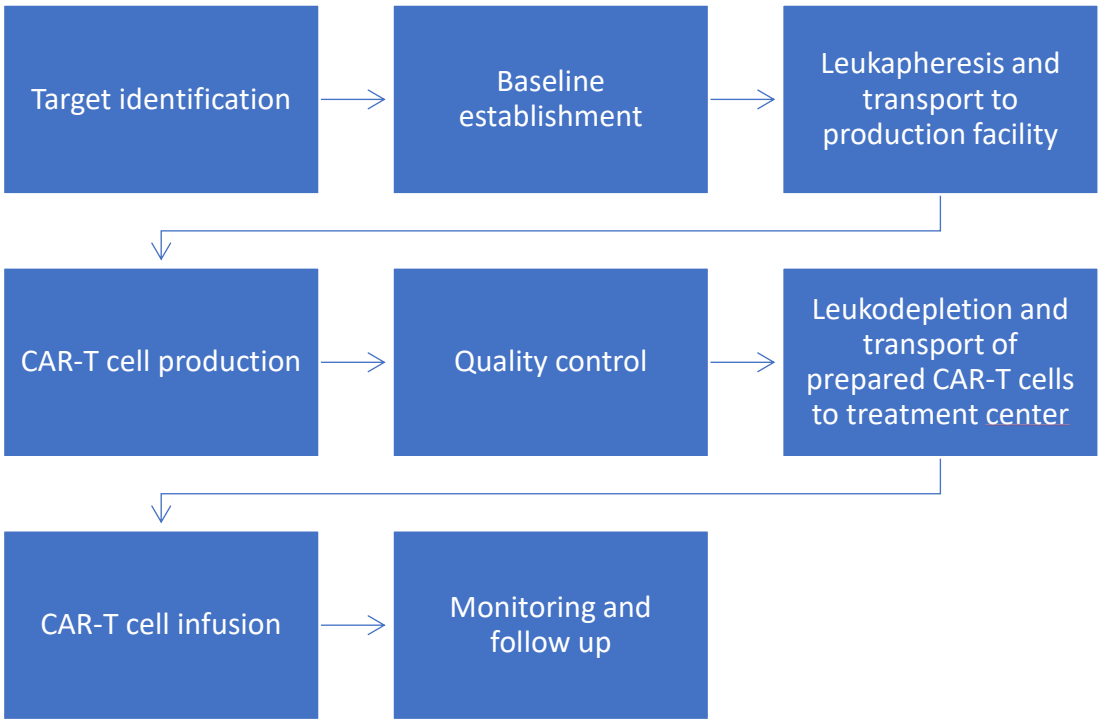

Figure 5. The complete CAR-T cell procedure. The production of CAR-T cells is performed in specialized facilities, follow up is in the hospital settings.

6. CAR THERAPY ADVERSE EFFECTS- MECHANISM, TREATMENTS AND PROGNOSIS

This section is focused on the main adverse effects documented in CAR-T therapy targeted against CD-19. These adverse effects are also well documented in CAR-T therapy for other targets with similar incidence[89 90 91].

6.1. Cytokine release syndrome

Cytokine release syndrome (CRS) is- a systemic inflammatory reaction caused by an acutely increased release of pro-inflammatory cytokines from WBC's present in the patient. These pro- inflammatory cytokines are responsible for the hallmark symptoms of fever, hypotension, hypoxemia, nausea, vomiting and in severe cases- a shock. CRS is the most common adverse effect, affecting 50%-93% of patients[58]. CRS is composed of two subclasses of signs and symptoms[92,93]. One is constitutional, expressed as fever with or without rigors, malaise, fatigue, myalgias, arthralgias, nausea, vomiting and headache. Other is non-constitutional, where symptoms and signs involve various organ systems (Table 4)

Table 4. The localized signs and symptoms of CRS by affected system.

Localization of dysfunction	Signs and symptoms	References
Skin	Macular rash, which may progress to desquamating, necrotizing rash	92,93
Gastrointestinal	Nausea, vomiting and diarrhoea	
Respiratory	Tachypnoea, Hypoxemia	
Cardiovascular	Tachycardia, widened pulse pressure (PP), hypotension, increased cardiac output (early) and potentially decreased cardiac output (late)	
Coagulation	Increased D-dimers, hypofibrinogemia with or without bleeding.	
Renal	Azotaemia, usually pre-renal (due to hypotension)	
Hepatic	Hyperbilirubinemia, increased liver enzymes in blood	
Neurologic	Headaches, confusion, delirium, seizures, mental status change, aphasia, hallucinations, tremor, dysmetria, altered gait	

6.1.1. Pathophysiology of CRS

CRS has a relatively poorly understood mechanism of activation. In CAR-T therapy, the chimeric effector cells are activated and in turn, via local cytokine release, activate bystander immune and non-immune cells (endothelial cells). The increased release of cytokines activates these immune cells without the proper activation and targeting cascade, leading to generalized inflammatory response. Key insight for the role of cytokines in CRS (then referred as cytokine storm) was achieved in a drug trial for TGN1412, a monoclonal anti CD28 antibody. Patients in that trial demonstrated markedly elevated levels of IL-2,6,10, TNF-α and IFN-ɣ[94]. In this acute inflammatory response, a special role has been discovered for IL-6. This interleukin has a pleotropic effect on various cells and successfully promotes differentiation of CD8+ T cells, plasma cells, Th-17, and thrombocyte production. Moreover, it enhances vascular permeability, VEGF production and angiogenesis while promoting collagen production leading to potential fibrosis. It also downregulates T-reg production, enhancing further the immune reponse[95]. IL-6 is released in acute inflammation and binds to a ligand, - IL-6R, forming a complex. This complex binds to GP-130, which dimerizes and propagates downstream intracellular signaling via the JAK/STAT pathway. Gp-130 is expressed in all cells, however, IL-6R only exists in hepatocytes and several types of WBC's. Via alternative splicing (in humans only) or metalloproteinases (in humans and in animal models)[96] [97] IL-6R is released in a soluble form (sIL-R). This soluble receptor can activate GP-130, which is then responsible for IL-6's inflammatory effects. When in high concentrations, sIL-6R/IL6 complex causes trans-signaling, where the increased concentration of the sIL-6R/IL-6 complex causes activation of immune or non-immune cells[98]. A known source of IL-6 in CRS are endothelial cells, whose dysfunction is a major part of CRS[99] and Immune effector cell associated neurotoxicity syndrome (ICANS)[100].

The contribution of other interleukins is also significant. IFN-ɣ is a well-known activator of immune cells, especially macrophages and is believed to contribute to secretion of high levels of pro-inflammatory interleukins, fever, chills, headaches, and fatigue[101]. TNF-α contributes to similar symptomology of IFN-ɣ, with the addition of watery diarrhea, vascular leakage, cardiomyopathy, lung injury and promotion of acute phase protein synthesis [101].

In some severe cases, CRS may progress to macrophage activation syndrome (MAS), which is similar in presentation and pathogenesis to hemophagocytic histiophagocytosis (HLH). This severe manifestation is often complicated by a lack of response to tocilizumab and its late onset[102]

6.1.2. Grading of CRS

There are various grading systems utilized to measure the severity of CRS in CAR therapy. The American society for transplantation and cellular therapy provides consensus guidelines which are adopted in the US and the UK[103] [104] The 3 main grading scales are the Lee scale, that utilizes common terminology criteria for adverse effects (CTCAE 4.0), the Penn grading scale and the CTCAE 4.0 and 5.0 scales. The consensus is formed from these main grading scales (Table 5.)

Table 5. The current consensus on CRS grading[103], adopted from: Lee at al.,2019 [103]

CRS parameter	Grade I	Grade II	Grade III	Grade IV
Fever*, not attributable to any other cause	Temperature ≥38.0C˚, with or without constitutional symptoms	Temperature ≥38.0C˚	Temperature ≥38.0C˚	Temperature ≥38.0C˚
	With			
Hypotension, not attributable to any other cause	None	Not requiring vasopressors	Requiring a vasopressor with or without Vasopressin	Requiring multiple Vasopressors (excluding vasopressin)
	And/or**			
Hypoxia	None	Requiring low flow nasal cannula*** or blow by	Requiring high flow nasal cannula, facemask, non breather mask or venturi mask	Requiring positive pressure (CPAP, BiPAP), or intubation nd mechanical ventilation

* If fever is treated by antipyretics or anti-cytokine therapy (Tocilizumab, Corticosteroids) then fever is no longer a required criteria for CRS grading and the grading will instead follow by hypotension and/or hypoxia

** CRS grade is determined by the most severe sign/symptom

*** Low flow nasal Cannula is defined by a flow of ≤6L/min, High flow is ≥6L/min.

6.1.3. Laboratory findings

Elevated levels of IL-1, IL-6, IL-10, IFN-ɣ, TNF-α, GM-CSF, CRP and low fibrinogen are common findings in active CRS.[92,103]

6.1.4. Risk factors [92]

The currently known risk factors for development of CRS are: High tumor burden (most recognized, strongest predictor)[105], the supposed mechanism could be a massive immune activation and the subsequent sequelae that follow. Lymphodepletion, especially when the regiment consists of fludarabine[105]. Concurrent infection which increases the risk of immune overactivation. High infusional dose and rate, the posited mechanism is similar to the high tumor burden etiology. Fractioned dosing regiment and some structural elements of CAR-T cells may impact the potential severity of CRS[106]

6.1.5. Prevention

Currently there are no known means to completely prevent CRS in CAR therapy. However, there have been several cohort studies and trials conducted that have attempted to prevent CRS by different mechanisms and actions[107] .These include timing of Tocilizumab (an anti IL6 monoclonal antibody) either during or before administration of CAR therapy to prevent development of CRS[108]**,** utilization of extracorporeal cytokine absorption as an adjunct to standard CAR therapy[109], and using autologous CAR with a built in suppresser of immune function (bivalent and synthetic notch receptor – table 2). Some evidence also exists that CD28 structural transmembrane (TM) elements within the CAR itself may affect cytokine release[106] when compared with other TM elements, namely CD28, and lastly, reduction of both the likelihood and severity of CRS during therapy can possibly be achieved by a strict dosing regimen, i.e by dose reduction per treatment.[110]

6.1.6. Treatment and outcomes of CRS

The current lines of therapy for CRS with outcomes are summarized in Table 6.

Table 6. The current lines of therapy for CRS with outcomes

Type of treatment	Line of treatment and indication	Mechanism of action	Common adverse effects	References
Tocilizumab	1st line. Administered to adults with grade 2 CRS and for children grade 3 CRS. Elderly with comorbidities decrease the threshold for administration of Tocilizumab	Inhibits IL-6 to prevent its binding to both the membrane bound and secreted IL-6R thus preventing both cis and trans signaling	Relatively safe, most common adverse effects are increased incidence of infections, slightly elevated liver enzymes, mild elevation of liver enzymes and infusion site reactions. Takes up to 7 days to be efficacious	[51,58,93,111–114]
Corticosteroids (CCS)	2nd line, administered to both adults and children who do not respond to first line.	Inhibits NF-kβ and lymphocyte maturation, stabilizes membranes, prevents neutrophil migration, attenuates inflammatory response.	Delayed wound healing, immunosuppression, altered mood, psychosis, hyperglycemia, hypertension, dyslipidemia, proximal muscle weakness, pancreatitis, osteoporosis, menstrual abnormalities, ocular dysfunction including glaucoma and cataracts, peptic ulcer. CCS do not attenuate the CAR-T response.	[90,114–116]
Anti-IL-1 monoclonal antibody (Anakinara) OR IL-6 monoclonal antibody (situlixumab)+high CCS	3rd line, given as treatment when the first two lines have failed, infection must be ruled out as a possible etiology.	Inhibits the inflammatory response by halting the cytokine cascade (IL-1 or IL-6 blockade)	Anakinara- injection site reactions and dyslipidemia are most common, Situximab (anti IL-6 mab) and corticosteroids possess the same adverse effects listed at first and second lines of therapy.	[58]

The current mainstay of treatment is Tolicizumab, a monoclonal antibody (mAb) against IL-6,. This monoclonal antibody prevents binding of IL-6 to both the membrane bound and secreted IL-6R, preventing both cis and trans signaling. The drug has good bioavailability and is relatively safe[111], although the response rate is not absolute (69% in the CTL-109 trial and 53% in the KTE-C19 trial)[112]. The response to Tocilizumab is not immediate and usually takes up to 7 days to take effect[93] . Tocilizumab is usually administered to adults with grade 2 CRS and to children with grade 3 CRS. In elderly people with comorbidities the threshold for administration of Tocilizumab is decreased[58]. The administration of Tocilizumab does not appear to negatively impact the efficacy of CAR-T thserapy[113, 51, 114]. Some patients, however, do not respond to Tocilizumab. Several medications are used in second and third line[90]. Corticosteroids (CCS), are well-known medications and work via various mechanisms: they stabilize membranes, prevent neutrophil migration to periphery, inhibit NF-kβ and lymphocyte maturation and have [114] various other activities. CCS are used as a second line therapy for patients who do not react to Tocilizumab. It is unclear whether CCS adversely affect the activity of CAR-T therapy because some recent studies indicate that CCS do not confer long term detrimental effects on CAR-T efficacy.[115] [116]. Third line treatment includes blockade of IL-1 (Anakinra) and IL-6 (Situlixumab) and administration of high doses of methylprednisolone may be administered. This third line is used if 2 rounds of Tocilizumab + CCS have failed to improve CRS[58]. In addition, any suspected CRS which is refractory to treatment carries a suspicion of an infection which must be ruled out.

CRS is by definition and acute condition. High grade CRS on its own does not leave any long-lasting damage and in fact other adverse effects linked to high grade CRS such as - cardiovascular events and cytopenia may cause prolonged morbidity.

6.2. Immune effector cell-associated neurotoxicity syndrome (ICANS)

ICANS is the second most common adverse effect affecting patients treated with CAR-T therapy, affecting 40-44% of children[117] [53] and 50% of adults[118] with 1-4BB domain. In other domains the incidence varies, ranging from lower incidence for adults with CLL (6-33%)[119] and relatively similar incidence albeit more severe appearance for CD-28 costimulatory CAR-T therapy (45% of affected had

severe ICANS). The presentation is often more severe with adults (up to 50%) than with children (13-24%). The adverse effect is characterized by several signs and symptoms[61 120] (Table 7). Additional potentially fatal adverse effects include[58 32,121]: cortical necrosis, acute cerebral hemorrhage during a resolving CRS episode, multifocal thrombotic angiopathy, subacute encephalomalacia[100]

Neurological area affected	Signs and symptoms	References
Cognitive function and attention	delirium, confusion and encephalopathy, this effect is the most common effect (66%) and is transient	61,120
Global	Altered state of consciousness- somnolence, difficulty to arouse, profound fatigue and rarely-coma, headaches- usually of the tension type	
Language and speech	difficulty in word findings, was usually coupled with delirium and changing state of consciousness	
Thalamic/global	Seizures- this adverse effect was more common in children and those who have already had a seizure disorder and in life threatening neurotoxicity	53,122
Pan encephalic	Acute Cerebral oedema a potentially fatal complication, this adverse effect is currently documented in anti-CD-19 CAR-T therapy alone and in different types of malignancies (NHL, CLL, ALL). This condition may develop several hours to days after initiation of treatment, often once CRS has begun to resolve.	121

Table 7. The most common signs and symptoms of ICANS, by affected area.

6.2.1. Pathophysiology of ICANS

The pathophysiological processes leading to the development if ICANS are still not fully understood. ICANS may appear with or without CRS and the mainstay of treatment for CRS (Tocilizumab) does not seem to be beneficial in ICANS therapy. This adverse effect has a monophasic appearance, appearing in a median of 4 days, peaking at day 7 and lasting for 5 days[120]. There are currently several elements recognized in the pathophysiological process of ICANS development. First is a robust cytokine release as laboratory tests have recognized elevated levels of pro-inflammatory cytokines, especially IL-6, IL-10, IL-15, Il-2, Il-1 receptor antagonist (RA) and CXCL-10[123]. The role of these cytokines is of yet unclear, but it is thought that they may contribute to endothelial damage and destruction of blood brain barrier (BBB) or to recruitment of bystander cells to attack normal cells (similarly to CRS). Considering the intensity of ICANS according to the type of CAR-T used, CD-28 costimulatory molecule appears to have a higher incidence of ICANS and severe ICANS in comparison to 1-4BB or CD-8 costimulatory molecule[124], but until now their role in the development of the condition is as of yet unclear. GM-CSF and bystander macrophage activation is another proposed mechanism for cytokine level elevation, and this one ICANS shares with CRS. Therapeutic blockage of GM-CSF has shown to decrease ICANS and CRS significantly in a xenograft model[125] yet there is currently no current evidence of a similar effect on humans. Next, a breakdown of BBB indicated by elevated cytokines and proteins is often prevalent in severe ICANS. There are several mechanisms suggested but best assertion comes from the endothelial activation[100] which leads to increased BBB permeability and progression of inflammation to the central nervous system.[123]

6.2.2. ICANS grading

Currently, there are two main systems for grading the severity of ICANS. CTCAE 5.0 and a more recent CARTOX grading system. Similarly, to CRS, the American society for transplantation and cellular therapy (ASTCT) formed a consensus grading system for ICANS[103] (Table 8), less robust than CTCAE 5.0, but being more focused towards the specific signs and symptoms in ICANS. It utilizes a sub scoring system to determine the level of encephalopathy, termed ICE score (Table 9)

Table 8. ASTCT consensus grading method for ICANS[103]

Neurotoxicity domain	Grade 1	Grade 2	Grade 3	Grade 4
ICE score*	7-9	3-6	0-2	0
Depressed level of consciousness**	Awaken spontaneously	Awaken to voice	Awaken only to tactile stimulus	Patient is either unarousable or requires repeat and vigorous stimuli to arouse, stuporous or comatose.
Seizure	N/A	N/A	Any focal or generalized seizure which resolves without intervention or evidence of nonconvulsive seizure on EEG which responds to intervention	Status epilepticus (generalized seizure lasting more than 5 minutes) or repetitive clinical or electrical seizures with no return to baseline in between.
Motor findings ***	N/A	N/A	N/A	Deep focal motor weakness (hemiparesis, paraparesis, etc.)
Elevated ICP/cerebral edema	N/A	N/A	Focal/local edema on neuroimaging****	Cerebral edema on imaging, decorticate or decerebrate positioning or cranial nerve VI palsy, or papilledema, or Cushing's triad (respiratory rate changes, hypertension, bradycardia)

* A patient with ICE 0 can be classified in ICANS 3 if caused due to global aphasia, if unarousable it is an automatic ICANS 4.

** Other causes of depressed consciousness must be ruled out 1st.

*** Tremors and myoclonus associated with CAR-T therapy may be assessed with CTCAE v5.0 but are not relevant to the consensus grading system

**** Brain hemorrhage is excluded from this grading system and can be graded according to CTCAE 5.0[126].

Table 9. The ICE score, part of the ASTCT consensus grading system for ICANS[103]

Type of test	Procedure 1	Procedure 2	Procedure 3	Procedure 4	Total
Orientation- 4 points	Orientation to year- 1 point	Orientation to month- 1 point	Orientation to city- 1 point	Orientation to hospital- 1 point	/4
Naming- 3 points	Naming object 1- 1 point	Naming object 2- 1 point	Naming object 3- 1 point	-	/3
Following commands- 1 point	Ability to follow simple commands (close your eyes and stick out tongue for example- 1 point	-	-	-	/1
Writing- 1 point	Ability to write a standard sentence- 1 point	-	-	-	/1
Attention- 1 point	Ability to count backwards from 100 by 10- 1 point	-	-	-	/1

*The ICE score has a range of 0-10, higher score is better. The score contributes to ICANS grading system.

6.2.3. Laboratory and imaging findings

Patients with ICANS have demonstrated increased cytokine release, especially of cytokines IFN-ɣ and IL-15[65,42]and macrophage activation. CSF findings demonstrated high levels of protein and white blood cells, consistent with BBB breakdown[127]. It should be mentioned that the presence of CAR-T cells in the CSF Can be found in patients both with ICANS and without it[128]. Increased levels of cytokines, especially TNF-α, IFN-ɣ and IL-6 in the CSF can be found with some cases showing higher cytokine levels in CSF than in peripheral blood. This raises the possibility that the these cytokines do not only enter the CNS from the blood, but are produced in the CNS[61]. Imaging results vary and are highly dependent on the subject in question and severity of ICANS. Imaging is normal in mild ICANS[129,122] .

The most common notable findings are T2 hyperintense symmetrical areas around the thalamus and deep grey matter structures, a pattern consistent with edema and possible micro hemorrhages[64,40]. In more severe cases of ICANS, cortical laminar necrosis or frank global cerebral edema could be recognized, heralding potentially devastating results[65,41].

6.2.4. Risk factors

Previous history of seizures, neurological events, and severe CRS (grade 3 and 4) may be possible elements which predispose an individual to develop more severe ICANS. In addition, higher disease burden (similarly to CRS), extramedullary disease and prominent rapid expansion of CAR-T cells may predispose a patient to develop ICANS[130,131]

6.2.5. Treatment and outcome of ICANS

Unlike CRS, the anti-IL-6 monoclonal antibody Tocilizumab has not been proven yet to be effective in reducing ICANS[53] and may even worsen it[64,40]. The current mainstay of the treatment is corticosteroids (dexamethasone) in two doses and a fast taper once the condition has resolved. There is an evidence that long term steroid treatment may not impact CAR-T therapy efficacy[132]. If the patient presents with seizures Levetiracetam has been proven effective in treatment[51]. However, there are no supporting evidence for the efficacy of prophylactic anti-seizure medication. There are still ongoing studies regarding the timing of administration of CCS and whether prophylaxis is possible. In 10% of patients who have been treated for longer than 3 months with CD-19 CAR-T neurological morbidity, including ischemic attacks, peripheral neuropathy and Alzheimer's dementia is displayed[133]

6.3. Cytopaenia

Cytopenia is a reduced level of circulating products of bone marrow including WBC, RBC and platelets and is the third most common adverse effect affecting patients who undertake CAR-T therapy. Cytopenias in general are expected due to the lymphodepletion regiment which is part of the preparation for IEC therapy. When prolonged, cytopenia predisposes the patient to opportunistic infections, anemia, and bleeding. The cytopenia may be partial (one or several cell lineages affected) or complete, in which case complete myelodysplastic syndrome must be ruled out. Cytopenia in CAR-T therapy is defined as persistent if it lasts more than 30 days after infusion of CAR-T cells. In several studies cytopenia has

occurred in approximately a third of patients[134,52,121] with greater incidence with administration of newer generations of CAR-T cells[54]. The symptoms are related to the type of cytopenia in question (Table 10).

Table 10. The most common forms of cytopenias by affected system and the common signs and symptoms per system.

Affected system	Signs and symptoms	References
Coagulation	Thrombocytopenia, increased bleeding times and hypocoagulability	134,52,121
Hematopoetic	WBC aplasia, which can be specific line up to pancytopenia. Increased incidence of infections are observed depending on the type of cytopenia. Anemia with either pure red cell aplasia can be found.	
Adaptive immune system	Hypogammaglobulinemia with pure B cell aplasia is observed.	

6.3.1. Pathophysiology of cytopenia

The general pathophysiology of cytopenia is well known. Decreased growth signals, bone marrow, invasion of non - productive cells, active destruction of bone marrow cells and nutritional deficiency may cause this condition. In CAR-T therapy cytopenia is less well understood. Immune system activation and introduction of CAR-T cells may tamper with proper growth signaling, thus decreasing the maturation of bone marrow cells. Some of the CAR-T cells are directed towards immature cells (CD-19 for example) and will actively destroy maturing cell populations. Another mechanism is the cytotoxic effect of CAR-T cells, which affects resident malignant cells and may also affect surrounding cells in the bone marrow, causing a decrease or halt in production of WBC's, RBCs, and thrombocytes. This adverse effect may have a biphasic pattern[135]. One proposed mechanism of late cytopenia is via an increase in SDF-1[136] (stromal derived factor 1), a chemokine which promotes B cell development and neutrophil development.

6.3.2. Grading of cytopenia

Cytopenia caused by CAR-T therapy is currently graded by the CTCAE 5.0 grading system[126,46] shown in table 11.

Table 11. CTCAE 5.0 grading system for cytopenia induced by IEC[126].

CTCAE term	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Anemia	Hemoglobin < 10.0 g/dL; 6.2 mmol/L; <LLN*-100 g/L	Hemoglobin < 8-10.0 g/dL; 6.2-4.9 mmol/L; <LLN- 80-100 g/L	Hemoglobin < 8.0 g/dL; <4.9 mmol/L; < 80 g/L, transfusion indicated	Life threatening consequences, urgent intervention required	Death
Bone marrow hypocellular	Mildly hypocellular or <= 25% reduction normal cellularity for age	Moderate hypocellular or >25% - <=50% reduction of normal cellularity for age	Severely hypocellular or >50% - <= 75% reduction of normal cellularity for age	Aplasia persistent for more than 2 weeks**	Death

* LLN= lower limit of normal

** Aplasia in CAR-T case is defined as aplasia 30 days post infusion due to the lymphodepletion regiment.

6.3.3. Laboratory findings

The laboratory findings depend on the deficiency, ranging from pure anemia to pancytopenia. Of note is CD4 and CD8 cells. CD4 cells have been shown to reconstitute later and in fewer numbers compared to CD-8 cells.[137]

6.3.4. Risk factors

The currently known risk factors for prolonged cytopenia are previous hematopoietic stem cell transplant (HSCT) within 1 year of pre-treatment, high disease burden and high grade CRS[86,62,57]

6.3.5. Prevention

Currently, due to the lymphodepletion regiment it is impossible to prevent cytopenia in CAR-T recipients.

6.3.6. Treatment and outcomes

Depending on the type and severity of cytopenia treatment may include[36,22, 103]: immunoglobulin (Ig) therapy, either intravenous or, if prolonged, subcutaneous (for hypogammaglobulinemia due to B-cell aplasia). Prolonged neutropenia may be treated with G-CSF (not GM-CSF) but only 14 days post-infusion and once CRS has resolved[85,61]. Anemia has been classically treated with transfusions and erythropoietin.

The long-term outcome is dependent on the accompanying risk factors and type of therapy. CD-19 CAR-T therapy and anti-leukemic CAR-T therapy are more cytotoxic and increase the risk of prolonged cytopenia. In contrast, therapy aimed at non haematological malignancies has not demonstrated any prolonged cytopenia[57,138].

6.4. Cardiotoxicity (Cardiovascular adverse effects – CAE)

Cardiotoxicity is a common adverse effect in CAR-T therapy as it affects around a quarter of the infused patients[,139,140] , children or adults[141, 60], who have received the therapy. Currently, there is no consensus regarding grading system of cardiotoxic adverse effect in CAR-T therapy. The various reported signs and symptoms shown in Table 12. have thus been adapted from CTCAE 5.0[126]

Table 12. The common signs and symptoms of cardiotoxic adverse events (CAE) due to CAR-T cellular therapy.

Affected cardiovascular system	Signs and symptoms	References
Contractile-cardiac	Decreased left ventricular ejection fraction (LVEF), new onset of heart failure or worsening of existing heart failure	139,140, 141, 60
Vascular	Hypotension	
Electrical/conduction-cardiac	Arrythmias, prolonged QT interval, sustained ventricular tachycardia (VF), wide and narrow complex tachycardias	
Myocardial cells	Increased cardiac enzymes- troponin, myocarditis	
pericardium	Pericarditis	

6.4.1. Pathophysiology of CAE

The mechanism behind the specific toxicity of CAR-T therapy to the heart is poorly understood. There is sufficient evidence, however for a positive correlation between CRS and the appearance of cardiovascular adverse effects[60,70, 142]. The cardiotoxicity may be exacerbated by previous treatment with anthracycline containing chemotherapy regimen (known cardiotoxic effects) and by previous cardiovascular conditions that reduce functional reserve and predispose patients to development of cardiotoxicity. High grade CRS may also incur disseminated intravascular coagulation (DIC) and consequent embolic strokes which may also affect the heart. In addition, tumor lysis syndrome and high tumor burden have also predisposed patients to develop cardiovascular adverse effect (CAE).

6.4.2. Grading of CAE

Unlike the other known adverse effects, there is no current consensus on a uniform grading system. CTCAE 5.0 is the method usually used.

6.4.3. Laboratory findings

When initiating the process of CAR-T therapy it is crucial to establish a cardiac function baseline. The following lab work should be taken as baseline and when suspecting CAE[58,22] . 1. Troponin levels, any elevation above baseline is considered pathological. 2. N terminal segment of pro-sBNP (NT-proBNP)- indicating possible heart failure or exacerbation of existing heart failure. 3. Echocardiography/ MRI to test LVEF, two dimensional speckle-tracking echocardiography derived strain to detect myocardial mechanic force changes[143]

6.4.4. Risk factors

Several recognized risk factors associated with CAE, such as previous history of heart disease and high grade CRS, can predispose to the development of CAE as well.[103,139,143]

6.4.5. Prevention

Timely intervention of high-grade CRS and possible early treatment with Tocilizumab may reduce the risk of developing CAE. Recognition and management of pre-existing cardiac conditions will help in providing the patient with additional functional reserve and also help in reducing the potential severity of CAE.

6.4.6. Treatment and outcome

The standard of care for CAE is the same as it is for any heart failure/cardiotoxicity, with the addition of cautious anti-coagulation therapy. Blood pressure normalization, rate and rhythm control and managing cardiac stress are the priority[144,145]. Clinical outcomes vary between patients however and the condition is usually acute and does not cause any substantial residual damage, although some rare mortality cases have been reported[146].

7. CHALLENGES IN CAR-T CELLULAR THERAPY

CAR-T therapy, although studied for a relatively long period of time is still in its infancy. Currently, there are only 4 approved second-generation CAR-T cell therapies and only one of those is for hematological malignancies. CAR-T therapy is one of the most studied subjects in hematology and is rapidly increasing (from around 120 studies in 2018 to over 1200 in 2021). CAR-T therapy, although promising and offering near limitless potential has to pass **some significant hurdles** in order to cement its position at the forefront of therapy for malignant diseases, hematological and solid alike.

7.1. Antigen escape and sensitivity

Antigen escape and sensitivity are listed first as they represent the most important feature of CAR-T cell. Namely even achieving complete remission in up to 94% of patients[52, 120,61] it had proven to be very efficient only in the short term. Up to 50% of patients relapse[124]. This relapse is partially due to antigen escape. It is based on the capability of malignant cells population to unergo a form of natural selection, in a way that malignant cells which do not express the antigen targeted by the CAR-T therapy survive and re-proliferate, inducing a more resilient relapse[119]. In the ZUMA-1 trial, 27.2% of patients in the phase 2 of the trial demonstrated CD-19 malignant cell populations[51] with similar results reported in other trials[147]. This antigen escape has several postulated mechanisms by which the malignant cell achieves this goal (Table 13).

Table 13. The various posited mechanisms of antigen escape and their pathophysiological mechanism.

Type of mechanism	Pathophysiological pathway	References
Acquired DNA mutations	Very common, as several studies have shown that frameshift mutations have affecting several exons coding for CD-19 and alter or truncate the CD-19 transcription, removing its expression.	[148,149]
Alternative RNA splicing	Mutations (specifically single nucleotide polymorphism- SNP) often change the target molecule, rendering the CAR-T cell obsolete. This has been demonstrated not only in CAR-T therapy but in immunotherapy in general. The mechanism apparently involves specific transcription factors, but further study is required to elucidate the exact mechanism	[149–151]
Epitope masking	A case demonstrated relapse of CD-19 B-ALL due to accidental introduction of the CAR genes into a B cell, rendering it a "decoy" cell which has masked the CD-19 epitope. This case study emphasizes the importance of proper standards of manufacturing and quality control	[152]
Decreased antigen density	Decrease in production or expression of an antigen may inhibit the action of CAR-T cell therapy. CAR-T cells require a larger density of expressed antigens to effectively operate, meaning that malignant cells with decreased expression of this antigen are able to evade the CAR-T cell and survive. A phase I trial for CD-22 CAR-T cell therapy has demonstrated a 70% clinical remission (CR) in treated patients with an 87% of CR patients demonstrating decreased antigen density	[153,154]

These mechanisms are some of the major factors behind the general lack of prevention of cytokine release in treated patients. The CAR-T cell is specific for the antigen in question and once that antigen is not present or does not meet the activation threshold the CAR-T cell will not function. Several methods have been suggested to combat this situation. One is a design of so-called bivalent CAR-T cell described in the 5th generation of CAR-T which offer more than one target for attachment which decreases the likelihood of antigen escape. A second possible option is a design of armored CAR-T cells. They are 3rd generation CAR-T cells which in addition to cellular based killing also release cytokines which promote an environment that enables immune activation, this approach enables a more efficient way to eliminate malignant cells and potentially reduce the likelihood of developing a population of resistant malignant cells. These CAR-T cells have shown a more tolerable adverse effect profile and an increase in CAR-T cell longetivity.[31] [32]

7.2. Improving persistence

One of the main postulated mechanisms of relapse and a primary hurdle to overcome it is the relatively low effective persistence of CAR-T cells. There have been two main reasons cited for this low persistence. **One is a lack of survival signals**.as CAR-T cells, due to their design are able to activate themelves against tumor cells without utilizing the standard pathway of immune activation. The drawback to this form of activation is an incomplete inflammatory pathway, leading to low or non-existent formation of memory cells. Once the CAR-T cell has "treated" it's target it does not receive any survival signals and proceeds to anergy. The second **reason, a T cell exhaustion** was first described in patients with lymphocytic choriomeningitis virus (LCMV)[155]. It represents the loss of effector functions of a T cell and even frank cell death due to persistent antigen stimulation. Usually, an increase in inhibitory and apoptotic receptors on T cell surface is observed[156],and issue is compounded also by the tumor microenvironment which suppresses immune function and promotes apoptosis[157]. Another main cause for this exhaustion lies in the structural element of the CAR-T cell. Here, several studies have shown that CD28 costimulatory domain is more sensitive than 4-1BB domain to T cell exhaustion, causing the CD28 subtype to have a significantly shorter lifespan[158, 38] , from a median of 30 days in CD28 domain to a median of 168 days with the 4-1BB domain[122].

The role of CAR-T cell persistence in disease relapse is of yet not completely clear[124] as several studies have demonstrated similar rates and duration of relapses in both 4-1BB and CD28 domains[159,,133] . Nonetheless several improvements in structural and costimulatory domains in newer generations have been offered to improve CAR-T cell persistence. (One improvement comes from a novel method which includes administering artificial T antigen presenting cells (T-APC) which will periodically activate CAR-T cells, providing them with the necessary stimulation to continue expansion and persistence[160]. This method is currently being tested in a pilot study (NCT03186118) and is expected to be completed in 2033. T-APC cells can potentially be administered as an off-shelf solution as they require much less preparation than other therapies. Utilizing different subtypes of T cells as origin of CAR-T cells is also one of the proposed solutions, such as T stem cells that have greater potential in developing to memory cells, thus improving persistence[161]. Another option is the usage of immune checkpoint inhibitors. As previously stated, increased expression of immune checkpoint receptors and apoptotic receptors is the hallmark of T cell exhaustion. It was shown that usage of approved immune checkpoint inhibitors improves CAR-T cell survival[162, 163], especially in the hostile tumor microenvironment of solid cancers, with promising preliminary results[164].

7.3. Commercialization

Currently, CAR-T cell therapy belongs to the area of personalized medicine as is strictly limited to autologous T cells However, this method, while accurate and with a very low chance of rejection makes the CAR-T cell therapy less commercially viable. There are several potential methods/solutions based on allogenic T-cell infusion to enable this therapy to be more accessible, less costly, and ultimately more available. The main hurdle which must be over crossed is reducing or eliminating graft versus host disease (GVHD) which is the main limiting factor of propagating allogenic CAR-T cells. All developed methods attempt to allow safe usage of allogenic CAR-T cells[165]. Establishing a source of CAR-T cells: Building a “bank” of CAR-T cells which are readily replicable. Several sources have been suggested and shown in Table 14.

Table 14. The various sources, physiology and allogenic source potential of T cells.

T cell source	Physiology of T cell population	Allogenic potential	References
Peripheral blood mononuclear cells (PBMC)	Mature or naïve T cells from peripheral blood	Low allogenic potential due to variable TCR, and HLA haplotypes, making them more likely to initiate and maintain GsVHD,	[165]
Umbilical cord blood (UCB) T cells	These subsets of T cells have a less active nuclear factor of activated T cells (NFAT and thus exhibiting a different, less sensitive self-antigen response.	Greater allogenic potential than PMBC and less likely to initiate GVHD,	[166,167,168]
Induced pluripotent stem cells (iPSC)	PBMCs can be "reprogrammed" to become pluripotent stem cells, potentially serving as a reservoir of stem cells which can be further programmed and matured to be utilized as CAR-T cells. Theoretically, a "bank" of various common HLA haplotypes could be made utilizing those iPSC and may serve as a source of manufacturing for a substantial, possibly indefinite amount of time	Very high allogenic potential, potentially can establish a "bank" of HLssA subtypes.	[165]
Non αβ T cells (NK cells)	NK cells serve as an interface point between the adaptive and innate immune system. NK cells are potent anti-tumor and anti-viral cells and operate via a complex interaction via various inhibitory and activating signals. Their dysfunctionality was observed in certain solid malignancies while they have shown significant antitumor activity[169].	High allogenic potential due to very low self -reactivity. Almost unable to initiate GVHD. The main hurdles which must be overcome is maintaining their persistence paucity in the bloodstream	[169]

7.4. Solid tumor therapy

The next big step in CAR-T cell therapy is the application of CAR-T cells in solid cancers. Globally solid cancers, comprise the majority of both new cancer cases and cancer deaths[170] and thus treating them effectively could lengthen and improve millions of lives. Solid tumors however, pose a challenge to cellular therapy (Figure 6). Firstly, a lack of access to the tumor may be a problem as solid, unlike hematological malignancies, have an environment is relatively poorly perfused. This feature serves a dual purpose where firstly it is more difficult for immune cells to arrive on site and exert their influence, and secondly, the tumor microenvironment is immunosuppressive, containing many cytokines and soluble receptors which cannot be washed out by blood flow, thus imparting the malignant cells with immune evasion[171]. Lack of tumor antigen can be another problem. Currently, no single prominent antigen that can be utilized as a target for CAR-T on most solid tumors has been discovered thus far. This is compounded by the fact that some antigens are shared between normal cells and tumor cells, increasing the risk of on target off tumor toxicity[172, 173.]. Lastly, the microenvironment of solid tumors is definitely immunosuppressive with chemokines (CXCL5[174], CXCL12[175]) expressed on tumor cells which suppress lymphocyte migration. Moreover, solid tumor cells secrete TGF-β which acts by altering the resident lymphocytes to promote an environment which is unfavorable for T cell survival and proliferation. Moreover, PD-1 expression promotes anergy to lymphocytes interfacing with the tumor cell, adding an additional layer of protection from the immune system.

These are all issues that must be overcome to effectively fight against solid cancers. They might seem insurmountable currently, but some trials are ongoing to optimize CAR-T cell therapy against solid cancers. As of 2021., there have been 300 ongoing studies in early phases of trials with relatively promising results.[177]

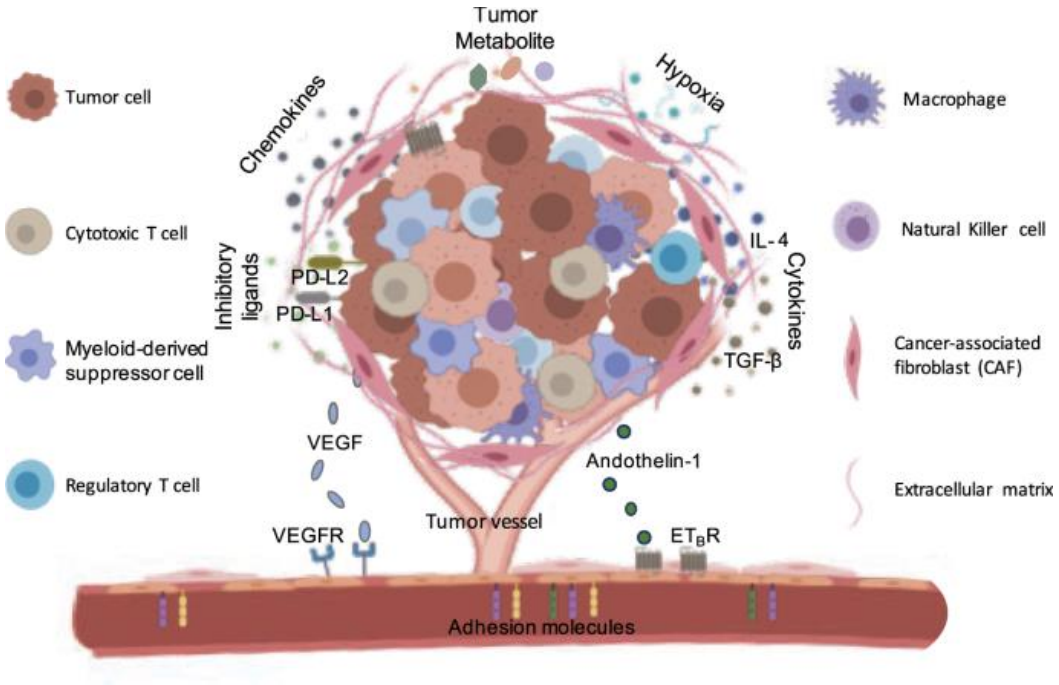

Figure 6. The solid tumor microenvironment[176]

8. DISCUSSION

CAR-T therapy has shown to be a relatively successful treatment, achieving up to 94% remission in patients with malignancies that are currently approved for this therapy and, more importantly, complete remission in about half of thes patients[178 ,179]. As of today, 4 CAR-T cell therapies have been approved by the FDA and 3 of those have also been approved by NICE. Those include: ABCEMA (idecabtagene vicleucel), an anti CD38 CAR-T cell, indicated for multiple myeloma, refractory or relapsed after 4 lines of therapy have failed in adult patients not approved by NICE; TECARTUS (brexucabtagene autoleucel), an anti-CD19 CAR-T cell, indicated for mantle cell lymphoma, either refractory or relapsed in adult patients; KYMRIAH (tisangenlecleucel), an anti CD19 CAR-T cell, indicated for adult patients with relapsed or refractory large B-cell lymphoma, including diffuse and follicular lymphoma, after 2 or more lines of therapy have failed in patients up to 25 years of age with B-cell ALL, refractory or in second relapse, and YESCARTA (axicabtagene ciloleucel), an anti CD19 CAR-T cell, indicated for adult patients with relapsed or refractory large B cell lymphoma after two or more lines of systemic chemotherapy have failed. As we can clearly see from the list of approved CAR-T cell therapies, it is utilized late in hematological malignancies. Many factors contribute to the fact that CAR-T cell therapy is often placed last in the lines of therapy. The preparation process is lengthy and expensive, often requiring an external commercial laboratories to process and produce the specific CAR-T from patient derived T cells, taking upwards of a month and costing hundreds of thousands of dollars[180]. Compared to the standard regimes the cost is five to six times higher compared to the first line treatments[181].

The undeniable effectiveness of CAR-T therapy is limited by the continued presence of the pre-determined antigen. Several mechanisms of escape exist, including formation of antigen negative tumor cells, alternative splicing, antigen masking and decreased expression, all leading to relapse Without a viable visible antigen the CAR-T cells cannot target the tumor cells and they undergo anergy. Many mechanisms have been posited to alleviate this issue, with the 5th generation Bivalent CAR-T cells and SUPRA CAR-T showing promising results.

Toxicity in terms of pre-treatment and side-effects is another major issue because CAR-T cells require lymphodepletion[77, 78] which places the patient in an immunosuppressive state. Also, CAR-T therapy itself is highly toxic with CRS and neurotoxicity being a common occurrence. CRS affects 57%-93%[121,51] of patients, mainly depending on the treatment type, burden of disease and age of patient. This CRS can

range from mild constitutional symptoms to massive cytokine storms which may threaten the patients' lives. Thankfully CRS can be managed with several lines of therapy, the first of which is the anti-IL-6 Mab tocilizumab. with a 69% response rate[112] Tocilizumab is not absolutely efficient but several other lines of therapy exist, including corticosteroids and anti-IL-1 MAb. CRS has not been shown to directly cause significant morbidity and is an acute condition. The second most common adverse effect is neurotoxicity, a less understood effect with an incidence in patients of 40-54%[61, 120] . This effect is one of the causes of death due to cerebral edema in some cases of CAR-T cells treatment. An additional toxicity which affects CAR-T treatment is on target off-tumor toxicity, which may cause serious morbidity and possibly mortality. Here, the proper hospitalization management and training of emergency medicine personnel in early recognition of alarm symptoms could reduce morbidity and mortality. New generations of CARs with better specificity and flexibility aim to increase the treatment efficacy. Among them, SynthNotch CAR receptors with their newly designed features, seem to have a much finer tuning of T-cell activation, creating a safer and more accurate "magic bullet" [44].CAR-T therapy is currently limited to haematological malignancies which make a minority of cancers[170]. Solid cancers, however, pose a different set of challenges. Hostile microenvironment, persistent hypoxia, extensive recruitment of immunosuppressive cells, limited access to the tumor itself and a lack of unique antigens have shown to be a major hindrance in the development of CAR-T therapy geared towards solid cancers. As of today, only a third of CAR-T trials are geared towards solid cancer, and until now no CAR-T therapy has been FDA approved for them. All these factors limit the current effectiveness of CAR-T in solid tumors, but promising usage of armored CAR-T cells, manipulation of pre-treatment regiments and novel injection methods all attempt to increase the effectiveness of CAR in solid cancer treatment

Although the current results are promising, one must keep in mind that most, if not all CAR-T cellular therapy studies have a relatively low group size (n) and thus have low statistical power. Even meta-analysis relies on studies with 30-100 subjects[182]. There are various reasons for this issue, but mainly the current indications for treatment and the cost of treatment have confined the availability of CAR-T treatment to a select few. In the future with increased efforts in adoptive cellular therapy and hopefully, the development of true allogenic CAR-T cellular therapy a broader and more substantial statistical base can be acquired.

To end, our immune system is the single most effective anti-cancer medication we have. Every day various intracellular and extracellular surveillance tools we naturally possess remove pre-cancerous cells and help

to maintain our cellular population healthy and normalised. When a malignant transformation does develop, one of the most essential steps in its development is immune evasion. With CAR-T cellular therapy we can harness this powerful tool and remove tumors in a highly specific and targeted way, creating a true “smart bullet”. Unfortunately, current CAR-T therapy does suffer from high toxicity and various other issues which prevent it from being truly at the forefront of both haematology and oncology, some of which may be caused by its current indications and usages. However, the future for CAR-T cells is bright, with many new and exciting technologies on the horizon, from CAR-T cells which create their own immunogenic environment via cytokine releases to a fine-tuned CAR-T cell which responds only towards the tumor with a variety of different mechanisms and even CAR-T cells which have a receptor base which can switch depending on the situation. All these ground-breaking technologies which are currently being developed show us the potential that this therapy can achieve. In the future, I believe that CAR-T therapy will be available as an “off the shelf” therapy for many malignancies and in the far future may even be offered as an immune booster or prophylaxis to high-risk patients. The first monumental success in 2012. has opened the way for an exciting and novel field of cellular therapy, utilizing cutting edge technologies from various fields to achieve what was once in the realm of science fiction: a personalized, targeted therapy towards diseases which were once considered incurable.

In conclusion, CAR-T therapy is a very promising line of therapy, and with utilizing advanced technologies and personalised approach it represents a remarkable achievement in cancer therapy. However this treatment is not without its issues, from a rigorous pre-treatment regiment, high costs, significant toxicity, and an inconsistent lasting remission that all keep this therapy on the side-lines. We must remember that the current approved therapy is three generations behind the newest CAR-T cells currently in phase I trials. Due to many trials, it is obvious that CAR-T cellular therapy will continue to expand, possibly encompassing most if not all malignancies and provide both a treatment option and a hope to previously refractory or incurable cancers.

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11. CV

Yaniv Izhaki Kotchinsky was born on 13.05.1991 in Tel Aviv, Israel. He was born to Gila and Isaac.

In 2009 he has finished high school and enlisted to the Israeli defense force. He has served there for 4.5 years as an officer.

In 2014 Yaniv has started Medical school in the Zagreb international medical program. He has achieved the Dean’s awards on his first year.

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