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CAR-T cell: an epitome for the cure of hematologic malignancies

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ABSTRACT: There is an increasing reliance on modern cancer therapies on immunotherapeutic approaches such as immune checkpoint inhibitors and adoptive cell therapy (ACT), which includes tumor-infiltrating lymphocytes (TILs), T cell receptor (TCR)-modified T cells, and chimeric antigen receptor (CAR). CAR-T cell therapy provides a unique approach to redirect T cells against distinct tumor antigens. It has generated widespread interest in oncology following several clinical successes in patients suffering from chemorefractory B cell malignancies. Since CAR-T cell therapy is a novel treatment, it does not have a clearly defined protocol. However, a rough protocol for CAR-T cell production is outlined in this article. The manufacturing of clinical-grade CAR-T cells under Current Good Manufacturing Practices (cGMP) is a very critical step in CAR-T cell production. However, this step has also become a bioprocessing bottleneck that needs to be surmounted for CAR-T cell therapy to reach a global patient population. CAR-T cells have a wide-ranging application in treatment of cancer. The first trials on B-ALL patients were conducted at MSKCC with conditioning chemotherapy of cyclophosphamide only. In case of CML patients, CAR-T cells that target the IL-1RAP protein have demonstrated the ability to selectively target the quiescent CML stem cells in various preclinical studies. Apart from CML, CAR-T cells can also be used to treat Acute Myeloid Leukemia (AML). For example, CD7 targeting CAR-T cells have shown effective cytotoxic effect against AML.

Keywords: Chimeric T cell; ALL; NHL; CML; AML.

1. INTRODUCTION

CAR-T cell therapy is a type of immunotherapy in which the patient's T cells are changed in the laboratory so that they bind to cancer cells and kill them. William Coley (1862-1936), a New York Surgeon is credited to be the father of Immunotherapy because he was the first person to seriously investigate the association between infection and cancer remission. Coley observed that apparently one of his patients was cured of cancer after two attacks of erysipelas, that is caused by an acute infection with bacteria *Streptococcus pyogenes* [1]. He became interested in the connection between immunity and oncology thereafter. In 1909 the Nobel laureate, Paul Ehrlich suggested that cancers may occur at an "overwhelming frequency" if it were not for the immune system. Unfortunately, Coley's success could not be reliably reproduced, as result immunotherapy was not widely accepted by the scientific and clinical communities. The idea of using the

immune system of the host to treat cancer is not a recent idea. The elimination of malignant cells during its initial transformation, is a process known as immune surveillance. Scientists have known about this phenomenon for decades and therefore had the idea that the immune system can be exploited to fight against cancer. Immunotherapy according to the National Cancer Institute (NCI) can be defined as a type of therapy that makes use of substances to stimulate or suppress the immune system to assist the body in fighting cancer, infection and other diseases. While some types of immunotherapies only target certain cells of the immune system, others affect the immune system in a more generalized manner. According to the Cancer Research Institute (CRI), there are five types of immunotherapy which include the use of Adoptive cell therapy, Cancer Vaccines, Immunomodulators, Oncolytic virus therapy and Targeted antibodies. Adoptive cell therapy (ACT) is a rapidly emerging immunotherapeutic approach. There are several types of ACT but the one that has been most promising in clinical development is CAR-T cell therapy. CAR-T cell therapy belongs to a subclass of medicinal products known as Advanced Therapy Medicinal Products (ATMP). These are medicines for human use based on cell therapy, gene therapy and tissue engineering. In CAR-T cell therapy the patient's T cells are equipped with a synthetic receptor known as CAR (Chimeric Antigen Receptor) [2]. In 2017, the US Food and Drug Administration (FDA) approved two CAR-T cell therapies, one to treat children with acute lymphoblastic leukemia and the other to treat adults with advanced lymphomas. The first CAR-T cells were developed by Yoshikazu Kuwana et al. in 1987 which was followed by Gideon Gross and Zelig Eshhar at the Weizmann Institute, Israel in 1989 [3]. Now CAR-T cells are different from naturally occurring T cells or engineered T cells because while these cells recognize their cognate antigens in the context of specific major histocompatibility complexes (MHC), the CAR-T cells are MHC independent [4]. Dr. Renier J. Brentjens from Memorial Sloan Kettering Cancer Center in New York, once said that giving CAR-T cells to patients is like "giving patients a living drug". When human cells undergo malignant transformation, Tumor-associated antigens (TAA) appear. The identification of TAA has been the focus of intense research so that TAA can serve as a target for immunotherapy of malignant diseases. CAR-T cells are defined as recombinant fusion or chimeric proteins combined with an antibody-derived targeting fragment and a signalling domain that can activate T cells [5]. The chimeric antigen receptors (CAR) are engineered receptors and can graft any specificity onto the immune effector cell (T cell). CARs consist of three domains: ectodomain (extracellular antigen recognition domain), a transmembrane domain and an endodomain (cytoplasmic signalling domain). Since the development of CARs in 1989, there have been four generations of CAR-T cells based on the structure of the endodomain [6]. Adoptive cell therapy aims at directing T cells towards tumor lesions but due to the limited number of T cell receptor chains (TCR) and the high frequency of loss of MHC presented antigen by cancer cells new strategies were adopted. It was shown that an antibody-derived binding domain in the extracellular domain and a TCR-derived signalling domain in the intracellular domain is capable of recognizing specific antigens on target cells as well as activating engineered T cells upon engagement. CARs are recombinant transmembrane molecules [3]. One of the most famous case of CAR-T cell therapy benefiting a patient was the story of Emily Whitehead who was suffering from recurrent ALL. She has now reached 8 years of cancer free survival. Her story is a testament to the ability of CAR-T cell therapy to positively influence the lives of many cancer patients [7]. Initially, CAR-T cell therapies focused on Acute Lymphoblastic Leukemia (ALL). More than 80% of children diagnosed with ALL will receive treatment that includes intensive chemotherapy. However, there are cancer patients who will eventually relapse even after chemotherapy or stem cell transplant. In those cases, the options of treatment available are very disappointing. Relapsed ALL is the leading cause of death from childhood cancer. Several trials of CAR-T cells were done

on young adults and children with ALL. In one of these trials, we see autologous T cells transduced with CD19-directed Chimeric Antigen receptor with the help of a lentiviral vector. These CAR-T cells are known as CTL019 or tisagenlecleucel. It is the first CAR-T approved by the US Food and Drug Administration (FDA). It is then infused in patients with relapsed or refractory ALL. CAR-T cell therapy against CD19 was found effective in the treatment of relapsed or refractory ALL. Even in patients whose stem cell transplantation had failed, CTL019 was found effective and was associated with high remission rates. Durable remissions were noted in that trial [8]. Kite Pharmaceuticals funded a study in which a phase 2 trial was conducted where 111 patients were enrolled with diffuse large B-cell lymphoma, primary mediastinal B-cell lymphoma and transformed follicular lymphoma which had become refractory. Those who received that therapy had high levels of durable response with an acceptable safety profile [9]. This trial and other earlier trials led to the FDA's decision to approve Kite's CAR-T cell product, axicabtagene ciloleucel (Yescarta^{TM}) for patients with lymphoma. If CAR-T cells are to be in clinical use then a safe, reliable and reproducible process for CAR-T cell production following Good Manufacturing Practices (GMP) needs to be created. Many GMP facilities have developed and refined protocols for CAR-T cell production. Now T cells need to be genetically targeted to an antigen viral vector transduction using gamma-retroviral or lentiviral vectors has emerged as one of the most efficient method [10]. The production of CAR-T cell begins with the collection of T cells from patients using a laboratory procedure called leukapheresis. These isolated T cells are then activated using antibodies specific for CD3 or CD28. The activated and proliferating T cells make it susceptible to viral transduction. Upon transduction the cells are expanded until the required CAR-T cell dose needed for therapy is achieved. Here we discuss in depth the use of CAR-T cells in cancer immunotherapy against Non-Hodgkin Lymphoma (NHL) and B Cell Acute Lymphoblastic Leukemia (B-ALL) and Chronic Myeloid Leukemia (CML). Early trials were conducted on NHL patients at places like Memorial Sloan Kettering Cancer Center (MSKCC), Fred Hutchinson Cancer Research Center (FHCRC), Baylor College of Medicine (BCM), etc. The patients were infused with 1st or 2nd generation CAR-T cells. The results from these trials suggested that to get optimal results, 2nd generation CAR design and pre-infusion conditioning chemotherapy was required [11]. The next phase of trials conducted at NCI and University of Pensylvania (UPENN) gave the first clear evidence of clinical activity against indolent B-cell malignancies [12,13]. In these trials there was use of significant host conditioning chemotherapy consisting of cyclophosphamide and fludarabine or bendamustine, respectively. While both the NCI and UPENN trials were expanded, a difference in efficacy was seen between the UPENN and NCI groups in terms of Complete Remission (CR) rates. The difference was attributed to the more aggressive conditioning chemotherapy given to NCI patients. But higher CR rates associated with increased conditioning chemotherapy came with worsened toxicity. The subtypes of NHL that were treated included Chronic Lymphocytic Leukemia (CLL), Diffuse large B cell Lymphoma (DLBCL) and primary mediastinal B cell Lymphoma, etc. [14,15]. In 2015 FHCRC investigators reported that in patients treated with cyclophosphamide and fludarabine showed increased persistence and expansion of CAR-T cells as well as an improved CR rate compared to patients treated with cyclophosphamide only. These suggest that conditioning chemotherapy provides an anti-lymphoma benefit [16]. The early success seen in CD19 CAR-T cell therapies for NHL suggested the possibility of using CAR therapy for B cell malignancies. The first trials on B-ALL patients were conducted at MSKCC with conditioning chemotherapy of cyclophosphamide only. There were concerns that because B-ALL patients have pancytopenia due to aggressive lymphodepletion, a sufficient number of T cells could not be collected and modified with CAR. In the end, the CR rate after CD19 CAR therapy was 88%. Moreover, high rates of molecular CR were observed,

raising the hopes for durable remissions [17]. In the case of CML patients, CAR-T cells that target the IL-1RAP protein, have shown the ability to selectively target the quiescent CML stem cells in various preclinical studies. Thus, it has the potential to be an effective cure for CML [18]. Other than CML, CAR-T cells can also be used to treat Acute Myeloid Leukemia (AML). Various AML-specific antigens are being targeted in preclinical and clinical trials to develop a viable strategy to treat AML using CAR-T cells. For example, anti-CD123 CAR-T cells have shown encouraging results in the treatment of Myelodysplastic syndrome (MDS) and CD7 targeting CAR-T cells have shown effective cytotoxic effect against AML [19]. With the improvement of the field of immunotherapy, the focus of cancer treatment has moved on from solely focusing on the disease site to focusing on the specific biologic characteristics of the tumor and how it interacts with the intrinsic immunologic ability or "cancer immune set-point" of the patient to fight against the disease. Unlike other therapies, our immune system has two advantages, the capability to remember and the ability to detect and eliminate different tumor variants as they emerge. Although CAR-T cell therapy is still in its clinical infancy, with both clinical and preclinical advances occurring rapidly CAR-T cell therapy has the potential to treat hematologic malignancies, solid tumors, autoimmune diseases, and inflammatory skin conditions.

2. MANUFACTURE OF CAR-T CELLS

All the major parts of CAR-T cell manufacturing are relatively standardized. There are six major steps in the production process which includes apheresis collection, T cell activation, gene modification, activation and ex-vivo expansion, quality assessment and formulation, delivery and administration in consecutive order (Figure 1). One important point to be noted is that Cancer patients often undergo chemotherapy or radiation therapy, and thus lymphodepletion before administration of the final CAR-T product. However, evidence points to the fact that CAR-modified immune cells from patients who have received radiation and chemotherapy perform much worse in cancer eradication when compared to patients who have never undergone radiation or chemotherapy. So, when CAR-T therapy will gradually become a first line therapy a much wider range of application along with higher efficacy for CAR-T therapy will be seen. Improved results from cell immunotherapies due to better starting materials will result in an increased probability of success.

3. PROTOCOL ON MANUFACTURING OF CAR-T CELLS

3.1. Apheresis collection

Peripheral blood mononuclear cells (PBMCs) are collected generally from peripheral blood. The collection of T cells can vary significantly depending on the patient, disease and collection factors. In patients who have advanced malignancy and an extensive history of treatment, it might be difficult to collect enough T cells for the CAR-T cell manufacturing process. A ficoll density gradient centrifugation and automated cell washers are employed to isolate T cells [20,21]. Instruments such as COBE2991, Fresenius Kabi LOVO and Haemonetics Cell Saver 5+ can help to separate red blood cells and platelets. CliniMACS Plus and Prodigy systems can isolate subsets of T cells such as CD4+, CD8+, CD25+ or CD62L+ T cells. These instruments are important because the Magnetic bead-based systems, like the CliniMACS system, with its anti-CD3+, anti-CD4+, or anti-CD8+ microbeads can select or deplete specific T cell types within the PBMCs allowing T cell expansion and administration of the final cell product that has a defined CD4:CD8 ratio [20]. In clinical trials, the use of CAR-T cells expanded from the CD3+ population is widespread.

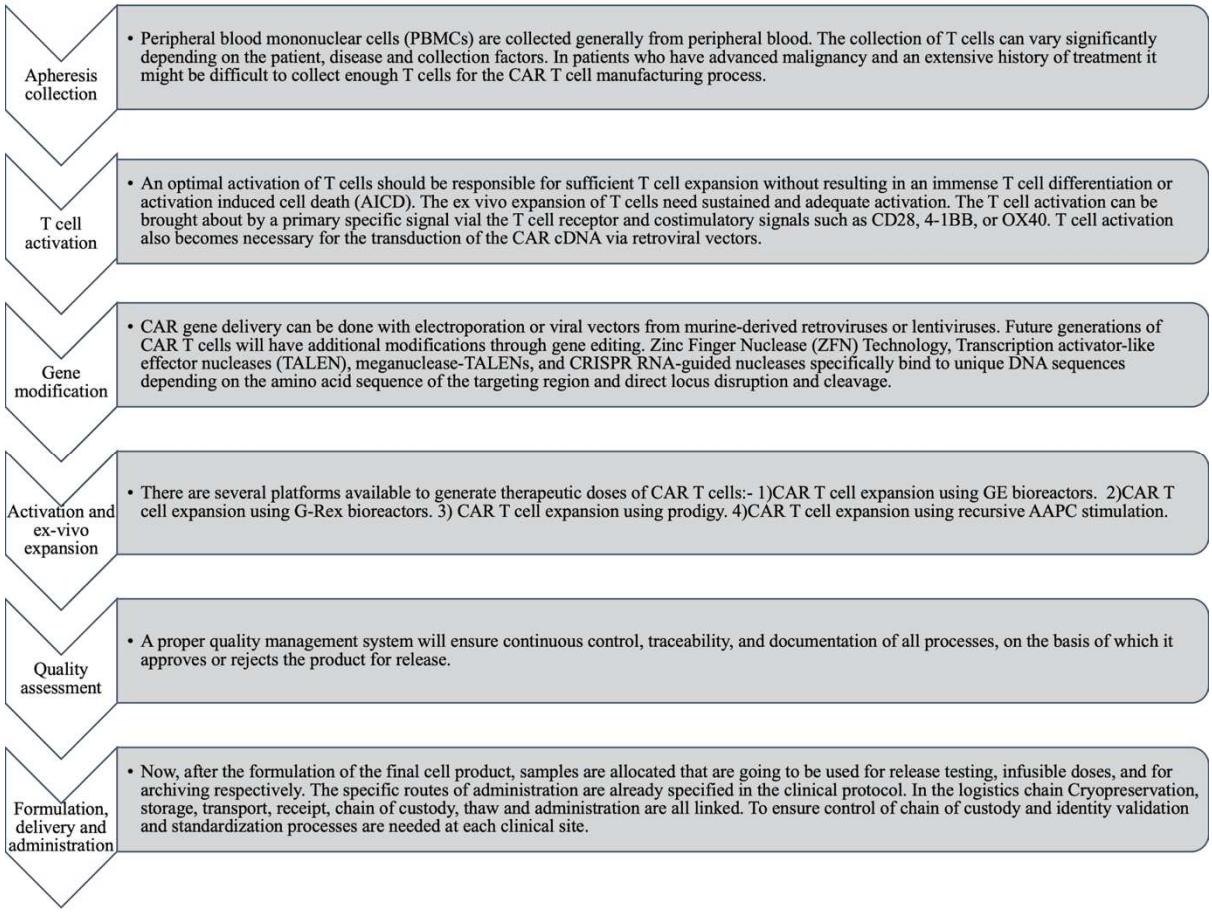

Figure 1. Steps in the manufacturing of Chimeric Antigen T Receptor cells.

T cell subsets can have functional advantages according to studies, so the selection, transduction and expansion process of these subsets have been developed on a clinical scale. The CAR-T cell production from defined T cell subsets appears to be beneficial. However, it is hard to find a T cell subset with an optimal therapeutic effect and minimum toxicity levels that can survive a robust and easy-to reproduce manufacturing process [22].

3.2. T cell activation

An indispensable part of CAR-T cell production is T cell activation. Optimal activation of T cells should be responsible for sufficient T cell expansion without resulting in an immense T cell differentiation or activation-induced cell death (AICD). The ex vivo expansion of T cells needs uniform and continuous activation. T cell receptor and costimulatory signals such as CD28, 4-1BB, or OX40 are responsible for conducting the signal that activates T cell. Which is necessary for the transduction of the CAR cDNA using retroviral vectors.

3.2.1. Cell-based T cell activation

Antigen-presenting cells (APCs), such as dendritic cells (DCs), are responsible for mediating physiological T cell activation. DCs are difficult to be used in the laboratory and clinical setting and DC potency varies from patient to patient. Therefore, DCs do not have much practical use in CAR-T cell therapy. As a result, simplified activation strategies are used to avoid the use of APCs as endogenous activators for ex

vivo T cell activation [20]. Artificial antigen-presenting cells (APCs) are another cell-based T cell activation approaches. However, the expansion and selection of GMP-grade HLA-matched aAPC lines are quite complex and need the dedication of more resources [23].

3.2.2. Beads-based T cell activation

Off-the-shelf clinical-grade T cell activation reagents like the Invitrogen CTS Dynabeads CD3/28, the Miltenyi MACS GMP TransAct CD3/28 beads, etcetera, have made the ex vivo T cell activation procedure simple.

3.2.3. Antibody coated magnetic beads

These are beads like Dynabeads CD3/28 which are uniform super-paramagnetic beads covalently coupled to CD3 and CD28 antibodies. This reagent allows for the selection and activation of T cells in a single step when used along with the Dynal ClinExVivo MPC magnet. Dynabeads are a clinical-grade reagent used for selecting and activating CD3+ T cells in early clinical trials. Apart from Dynabeads, Miltenyi ExpAct Treg beads are also used, which are paramagnetic beads conjugated to CD3-biotin, CD28 and anti-biotin monoclonal antibodies. Both Regulatory T cells and normal T cells can be expanded different beads to T cell ratios of ExpAct Treg beads [22]. It is mandatory to remove the magnetic beads at the end of the manufacturing process [24].

3.2.4. Antibody coated nanobeads

Antibody coated nanobeads like Miltenyi MACS GMP TransAct CD3/28 beads are polymeric nano matrix conjugated to CD3/CD28 monoclonal antibodies. It is biodegradable and does not need removal before formulation. However, before activating T cells, upstream T cell purification must be performed [22,25].

3.2.5. Expamer technology

A recent development among T cell activation reagents is the Expamer developed by Juno Therapeutics. It has a unique core Streptamer technology that can isolate viral-specific lymphocytes. Reports confirm that Expamers are soluble and can be easily bound and removed from the cell surface. These properties allow Expamers to instantaneously start and stop a T cell activation signal. It is an efficient T cell stimulation reagent, inducing T cell receptor (TCR) signalling and activating T cells to support retroviral transduction and expansion [26-28].

3.2.6. Activation with anti-CD3 antibodies

When anti-CD3 monoclonal antibodies disturb the CD3 complex on the T cell surface in the presence of IL-2, T cells are activated. Production of autologous and allogenic CD19 CAR-T cells by activating and expanding patient PBMCs with anti-CD3 monoclonal antibody OKT3 can be done using this method [15,29].

3.3. Gene modification

CAR gene delivery can be done with electroporation or viral vectors from murine-derived retroviruses or lentiviruses [30]. Future generations of CAR-T cells will have additional modifications through gene editing. Zinc Finger Nuclease (ZFN) Technology, Transcription activator-like effector nucleases (TALEN), meganuclease-TALENs, and CRISPR RNA-guided nucleases specifically bind to unique DNA sequences depending on the amino acid sequence of the targeting region and direct locus disruption and cleavage [31].

3.3.1. Viral transduction

Viral vector systems are gamma-retroviral vector and lentiviral vector; both belonging to a family of retroviruses and are commonly used as gene delivery systems that can achieve high transduction efficiency rates. Retroviruses are used because they allow for a long-term gene expression by integrating the viral DNA into the host DNA. No genotoxic effects resulting from gene transfer into differentiated cells, including T cells are known. Only a few cases are known arising from patients treated with genetically modified T cells where virus-mediated transformation has been witnessed. In one case, where a CLL patient was treated with CD19-specific CAR-T cells, a lentiviral vector mediated insertion of the CAR transgene was observed which in turn resulted in the disruption of the methylcytosine dioxygenase TET2 gene [31]. In a different case, the CAR gene was unintentionally introduced into a single leukemic B cell in the course of the production process, which lead to insertional mutagenesis resulting in tumor escape in a patient. The patient relapsed after treatment with CD19-specific CAR-T cells with a CD19 negative leukemia [32]. No report on accidental insertion for gamma-retroviral vectors has been found. Thus, gamma-retroviruses are often regarded as a safe vector system for clinical ACT. Despite the few cases of virus-mediated transformation, the viral gene delivery system primarily used is lentiviral transduction. It is used in the production of *Kymriah*® (Tisagenlecleucel) which is used for the treatment of ALL or Diffuse Large B-cell Lymphoma (DLBCL) [33,34]. Retroviral transduction was part of the ZUMA-1 trial with Axicabtagene Ciloleucel for treating r/r large B cell lymphoma [35,36]. However, a major bottleneck in the production of retroviral vectors is the intensive and often expensive process of vector production. Appropriate cleanroom facilities and the need to perform vector release testing for the retrovirally or lentivirally transduced cells are usually an expensive affair even for big pharma companies.

3.3.2. Plasmid-based gene delivery

An alternative to viral transduction-based CAR gene delivery is the "Sleeping Beauty" transposon/transposase system [37]. Compared to electroporation of naked DNA, transposon system is a much more efficient method of gene transfer. It is also economically beneficial relative to other methods, as no GMP-grade virus generation is necessary. However, it is a labor-intensive process. The transposon system works using two DNA plasmids, one containing the transposon encoding the CAR transgene and another plasmid expressing the transposase that is necessary for excision and insertion of the transgene [10,20,38].

3.3.3. Genome editing

Genome engineering tools like CRISPR/Cas9 technology allow for the specific genomic disruption of multiple gene loci. Combined with adeno-associated virus (AAV) vector repair matrix, the CRISPR/Cas9 system can be utilized to integrate the CAR encoding DNA into the T cell receptor α constant (TRAC) locus. This leads to uniform expression of CAR, an improvement in the potency of T cells, and an inhibition of T cell differentiation along with exhaustion [39]. Lentiviral transduction along with CRISPR/Cas9-mediated genome editing is used to produce PD-1 deficient CD19-specific CAR-T cells, which has better anti-tumor and therapeutic efficacy [40]. Upon analysis, the applied gene transfer systems currently focus on transduction and clinical efficacy, safety and costs. The optimal gene transfer system has not yet been defined and further investigation is necessary.

3.4. Activation and ex vivo expansion

There are several platforms available to generate therapeutic doses of CAR-T cells.

3.4.1. CAR-T cell expansion by GE bioreactors

The GE WAVE bioreactor system is used to expand T cells. It is intended to be used as development and manufacturing equipment for the expansion of cells. A single-use, gamma-irradiated bag, known as Cellbag™ is used for cultivation. The cellbag bioreactor is situated on a rocking base that will maintain bag inflation which rocks the cell bag for quick transfer and mixing. Automatic feeding and waste removal are done by perfusion functionality of the WAVE bioreactor. This platform is widely used to expand cells for supporting phase ½ clinical trials [24,41].

3.4.2. CAR-T cell expansion by G-Rex bioreactors

It is a new platform, where the cell culture flask has a gas-permeable membrane at the base. The membrane makes sure that cells can grow to a high density without any compromise in its gaseous exchange. The advantages of this platform are Low seeding density, one-time upfront feeding regimen, growing cells in an incubator, and the reduction in volume at the time of harvest. However, cell expansion will be affected if cells are disturbed when they are in culture. So, cell samples cannot be taken in-process [42].

3.4.3. CAR-T cell expansion by prodigy

The CliniMACS prodigy system is a new technology that can prove to be a viable avenue for expanding CAR-T cells. The Prodigy system is an amalgamation of a cell washer, cell cultivation device and the CliniMACS magnetic cell separation system. Reports reveal that NK cells can be successfully separated from leukapheresis products. It is then expanded to reach a dose of clinical relevance using Prodigy. Prodigy also allows for lentiviral transduction of T cells with CARs [43,44].

3.4.4. CAR-T cell expansion using recursive AAPC stimulation

Lentivirally modified aAPCs such as K562 cells can direct stable expression as well as secretion of an array of co-stimulatory molecules and cytokines. The combination of various genes and their expression on the aAPC is necessary for the activation of human T cells. aAPCs can be tailored to meet the requirements for optimal propagation of T cell subsets [22]. K562 is a myelogenous human leukemic cell line that does not express HLA class II, HLA class I A or HLA class I B alleles and can be modified genetically to express a wide variety of co-stimulatory molecules such as CD40, CD40L, CD70, CD80, CD83, CD86, CD137l, ICOSL, GITRL, and CD134L to facilitate T cell expansion.

3.5. Quality assessment

CAR-T cell products' quality can vary from donor-to-donor. The quality of CAR-T cells should be under careful monitoring and should be integrated into the manufacturing process. The quality of CAR-T cell products depends on the quality of the raw materials and reagents used in the process. Thus, a proper quality management system based on different principles of quality control will ensure continuous control, traceability, and documentation of all processes, on the basis of which it approves or rejects the product for release (Figure 2). Accrediting organizations for cell therapy such as Foundation for the Accreditation of Cellular Therapy (FACT) or The Joint Accreditation Committee of ISCT-EBMT (JACIE) have standards that follow regulations outlined in Title 21 CFR, Parts 210 and 211 as governed by CBER of the FDA (Figure 3).

The FDA Investigational New Drug Application (INDA) based on assays and specifications prescribes a level of purity, identity, sterility, safety and potency for the product. To ensure conformation to FDA regulations, release testing is conducted. Testing for immunophenotyping, Gram stain, endotoxin, bacterial and fungal testing, and mycoplasma testing are generally conducted [45].

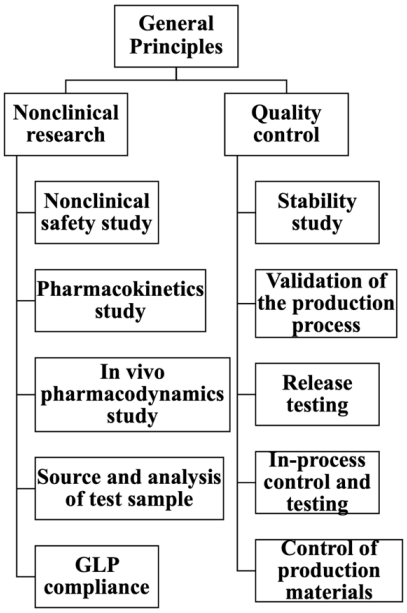

Figure 2. General Principles for the quality control of CAR T cell therapies.

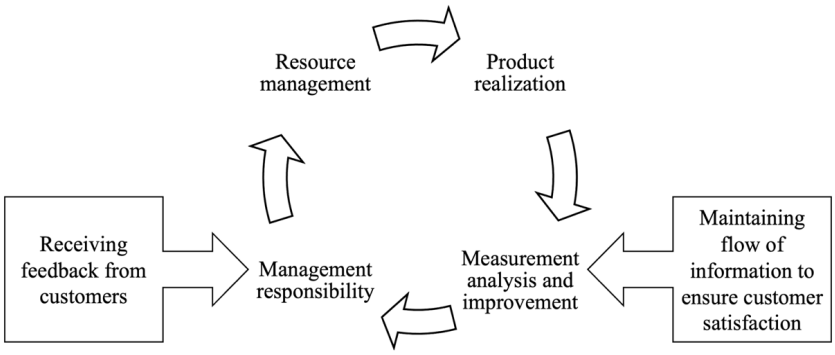

Figure 3. Quality management system according to FACT/JACIE guidelines.

3.5.1. Quality assessment of manufacturing facilities

GMP facilities with ISO5 cell processing cleanrooms is necessary for manufacturing CAR-T cells [46]. The facilities must have the proper equipment like analytical equipment; manufacturing process equipment; facilities systems, and environmental monitoring equipment.

3.5.2. Quality assessment of ancillary components

The clinical manufacturing process of CAR-T cells requires raw materials and components that are approved for human use (Figure 4). Established acceptance criteria should be met by the certificate of analysis provided by qualified vendors and has to be reviewed for each lot. The quality control laboratory must routinely test the raw materials to guarantee product integrity. Backup vendors should also be established to reduce the risk of supply chain interruptions. Complex biological materials like viral vectors must have separate release testing procedures and regulations that deal with them [10].

3.5.3. Quality assessment of manufacturing process

CAR-T cell therapies can be successful if we can establish a steady, robust and easy to reproduce manufacturing platform. Each of the constituents of the platform must pass individual testing. The qualification process helps to address unanticipated challenges during the evaluation and qualification of each component. Thorough documentation and analysis of the entire process are essential to execute a successful CAR-T cell manufacturing operation. [47].

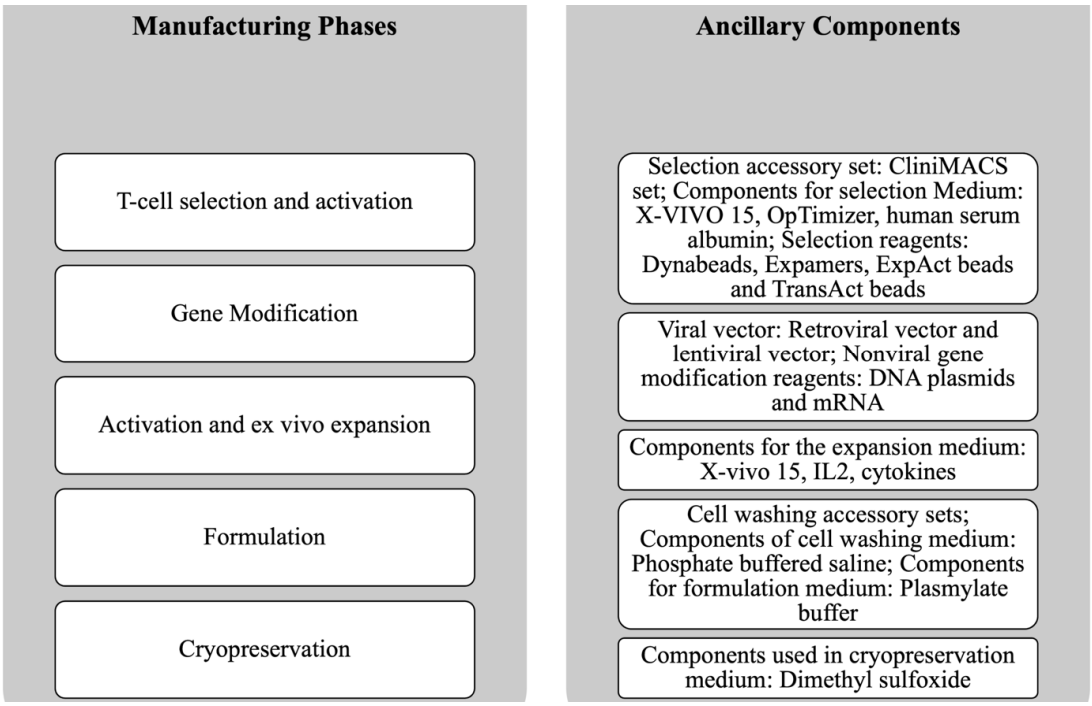

Figure 4. Manufacturing phases of CAR T cell manufacturing and various ancillary components required in those phases.

3.6. Formulation, delivery and administration

The manufacturing process of CAR-T cells is labour intensive. It is a complex process. Moreover, resolving issues like quality control and single lot release adds to the manufacturing cost. Academic centres still have the most experience in CAR-T manufacturing. Partnerships with academic centres are therefore important. Now, after the formulation of the final cell product, samples are allocated that are going to be used for release testing, infusible doses, and for archiving respectively (Figure 5). The specific routes of administration are already specified in the clinical protocol. In the logistics chain Cryopreservation, storage, transport, receipt, chain of custody, thaw and administration are all linked. To ensure control of the chain of custody, identity validation and standardization processes are needed at each clinical site. Cryopreservation is an important part of the process as it is required to transport the final product from the manufacturing site to the clinical centres as well as it is needed for mandatory quality-control tests. Even though the functionality of the CAR-T cells was reduced directly after thawing, their viability, gene transfer efficacy and recovery are not affected up to 90 days. An incubation at 37-degree Celsius overnight can lead to a complete restoration of the functionality of these CAR-T cells completely negating the effects of the harsh freeze-thaw process [48].

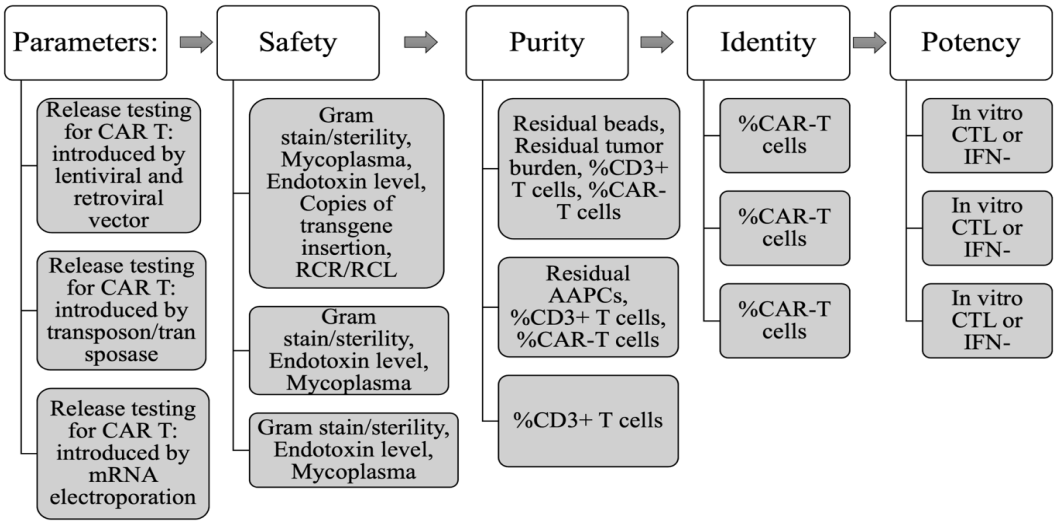

Figure 5. A summary of CAR T cell release tests.

4. ROLE OF CAR-T CELLS IN TREATMENT OF HEMATOLOGIC MALIGNANCIES

The interest in CAR-T cell therapy has risen steadily since 1993, when the first-generation CARs were developed by Zelig Eshhar of the Weizmann Institute in Israel. In 2014, the FDA even granted the designation of 'Breakthrough' therapy to CD19-directed CAR-T cell therapy. Finally, in 2017 CAR-T cell therapy crossed the regulatory finish line when the FDA approved Kymriah (tisagenlecleucel) for the treatment of relapsed, refractory acute lymphoblastic leukemia (ALL) in children and young adults. It was the first gene therapy available in the United States. Thus, it makes sense that over the years a lot of clinical trials on CAR-T cells have taken place. By 2016, there were 220 clinical trials on CAR-T cells that were documented. 188 of these trials were ongoing and nine were long-term follow-up studies. It was more than 20 years ago that the first CAR-T cell trials were initiated and its patients had advanced epithelial ovarian cancer or metastatic renal cell carcinoma and the trials were targeting the folate receptor and carbonic anhydrase IX (CAIX) respectively. The subsequent two registered clinical trials with published results reportedly had single patients with neuroblastoma or follicular lymphoma reaching complete response. Consequently, the breakthrough in CAR-T cell therapy was however achieved over the following years when CD19-specific CAR-T cells were used to target B-cell malignancies. In these trials, unlike previous trials, complete or partial response was reported not by just single individuals. In some trials, majority of the patients reported complete or partial response. This resulted in an exponential growth in the number of CAR-T cell trials conducted [49]. According to the International Clinical Trials Registry Platform (ICTRP) of the World Health Organization (WHO), in 2020 alone, there were 265 new CAR-T cell clinical trials that were registered. In a review article by Hartmann et al. the status of various clinical trials on CAR-T cell therapy registered at clinicaltrials.gov as of 2017 was illustrated along with the phases that different clinical trials [49] (Figures 6 and 7). It can be said that CAR-T cells have shown promising results in hematological cancers.

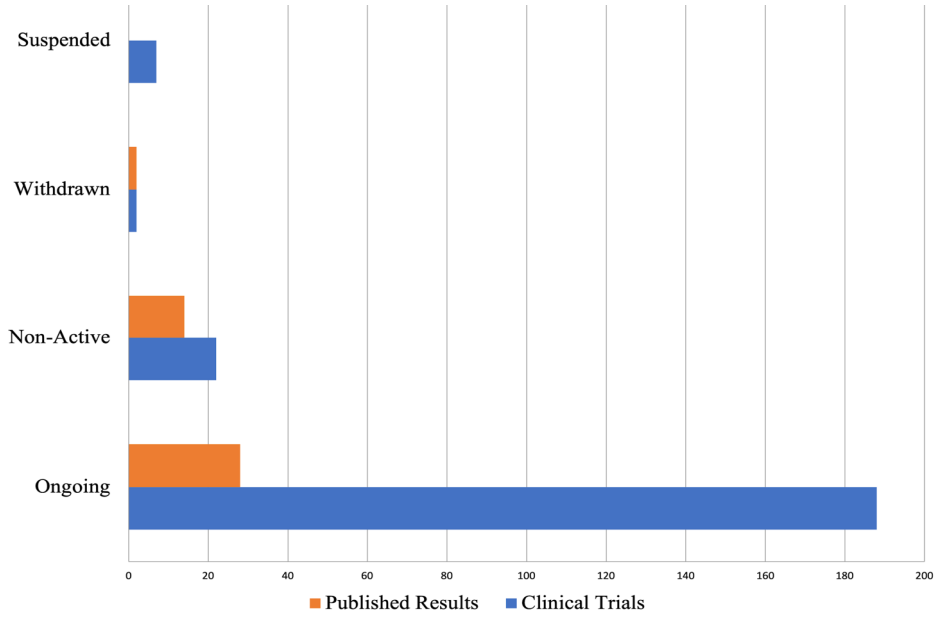

Figure 6. Status of published CAR T cell gene therapy trials registered at clinicaltrials.gov. The total number of clinical trials (red bars) are compared to the number of published clinical trials (blue bars) in various categories. In the 'suspended' category there were no clinical trials with published results [49].

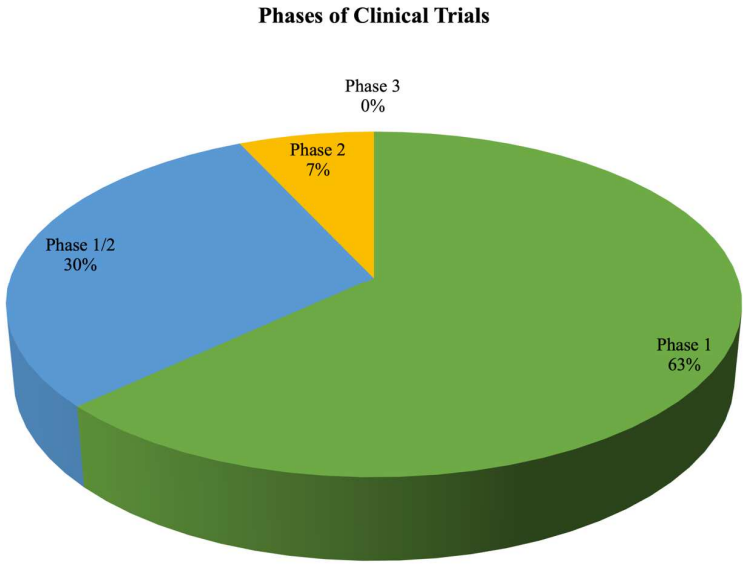

Figure 7. Phases of CAR T cell gene therapy trials. Long-term follow-up studies are not being included here. For 9 trials their phase classification could not be determined. There were no trials in phase 3 [49].

4.1. ALL

Chimeric Antigen Receptors (CAR) can be defined as engineered molecules which are the result of combination of an antigen recognition domain with one or more intracellular T cell signalling domains. Through gene transfer techniques like lentiviral and retroviral transduction, CARs are integrated into T cells redirecting their specificity to target specific tumor antigens using an MHC independent mechanism. In the treatment of ALL the most important CAR is the one that target the CD19 molecule, which is a biomarker for B lymphocyte development, and shows a higher expression in B-ALL [50]. An important factor that determines the functionality of the CAR-T cells are the components of its intracellular signalling domain.

First generation CARs were incorporated only with the CD3ζ intracellular domain. They were able to engage and respond to antigen but they had limited in vivo expansion and persistence, resulting in limited efficacy. Second generation CARs with co-stimulatory domains such as CD28 or CD137 showed better outcomes in patients. These second-generation CAR-T cells that target CD19 have been responsible for complete remission (CR) rates of 67-93% in pediatric patients as well as adult patients suffering from relapsed and refractory (r/r) acute lymphoblastic leukemia (ALL) [51]. It is important to note that prior to infusion of anti-CD19 CAR-T cells, patients usually receive chemotherapy to induce lymphodepletion and enhance CAR-T cell expansion and persistence in vivo [52]. Chemotherapy may also benefit the patient through leukemic cytoreduction, which improves the efficacy and decrease the toxicity of CAR-Ts. Treatment of ALL, especially fatal, relapsed/refractory (r/r) B-ALL is well suited to CAR-T cell therapy. CAR-Ts are successful because of their immunologic characteristics. These characteristics include the specificity of a targeted antibody, ability to expand in vivo resulting in an amplified anti-tumor response and the potential of CAR-T cells to persist for a longer term. The in vivo activation and expansion of anti-CD19 CAR-Ts is responsible for high remission rates, it also leaves no minimal residue disease (MRD) but unfortunately correlates with neurologic activity and cytokine release syndrome (CRS). Durability of the remissions with the anti-CD19 directed CAR-Ts are variable across different studies with efforts to minimize both CD19 positive and CD19 negative relapses. Between 2013 and 2015 three academic groups had published five reports describing the clinical trials and outcomes of CD19 targeted CAR-T cells for adults and children with relapsed/refractory B-ALL (Table 1). The first report came from Memorial Sloan Kettering Cancer Center (MSKCC). It was a phase I trial using a CAR 19-28z T cells with a dose of 3×10^6 CAR-T cells/kg [17,53]. Cyclophosphamide was used for conditioning chemotherapy before infusion with CAR-T cells. After the therapy the CR rate was 88%. These were deemed to be high quality remissions because there was no detection of disease when high-sensitive molecular assays like deep-sequencing or real-time PCR were used. Molecular Complete Response (CR) rate was reported at 75%. Such results certainly raised the hopes for durable remissions but certain patients did suffer from toxicities. However, such patients could still receive an Allogenic Stem Cell Transplantation (allo-SCT) [18]. CAR-T cells did expand very rapidly but they also contracted similarly, as a result, after 2-3 months very low levels of CAR-T cells could be detected. Another study was done in University of Pennsylvania (UPENN). The conditioning chemotherapy was done using fludarabine and cyclophosphamide, and it was done a week before the adoptive transfer of 19BBz CAR-T cells. The dose administered ranged from 0.8×10^6-17.4×10^6 CAR-T cells/kg. The CR rate was reported to be 90%, while the molecular CR rate was at 73% [9]. In this UPENN study the CAR-T cells were engineered to contain anti-CD19 attached to TCRZeta and 4-1BB signalling domains. The T cells' capability of long-term persistence directly affected the clinical outcomes of the patients participating in the study. Nearly 68% of the pediatric patients expressed persistence of T cells for 6 months, while in some patients these T cells lasted for nearly 2 years [9]. Some patients however did relapse due to the loss of CAR-T cells, loss of CAR-T cell function and also CD19-negative clonal escapes. A similar study was conducted by National Cancer Institute (NCI). The patients were treated with fludarabine and cyclophosphamide before infusion of CAR-T cells. These phase I trials evaluated 19-28z CAR-T cells in children and young adults (1-30 years old). The B-ALL patients in these trials had a CR rate of 70% and the molecular CR rate was 60%. The interpretation of these results deemed CAR-T cell therapy completely safe, feasible and responsible for anti-leukaemic activity in children and young adults with B-precursor ALL [54].

Table 1. A list of published clinical trials on CD 19 CAR-T cell therapy for treatment of relapsed/refractory B ALL.

Site	n	Median age	Post-Allo SCT	CAR-T dose per kg	CR (%)	CRm (%)	EFS	Notes
MSKCC	16	50	25%	3×10^6	88	75	NA	All patients are adults, 1 patient received less than study dose
UPENN[a]	25[b] 5[#]	11[b] 47[#]	60%	0.8×10^6 – 21×10^6	90	73	67% at 6 months	25 Pediatric patients 5 adult patients
NCI	21	NA	38%	1×10^6 – 3×10^6	67	57	51.6% at 9.7 months	All pediatric/young adult patients (1-30 years of age). Less than study dose - received by 2 patients

CAR: Chimeric Antigen Receptor; allo-SCT: allogenic stem cell transplant; CRm: molecular CR; EFS: Event free survival; MSKCC: Memorial Sloan Kettering Cancer Center; UPENN: University of Pennsylvania; NCI: National Cancer Institute; NA: Not available; [a] One patient with CD19+ T-ALL; [b] Pediatric Cohort; [#] Adult Cohort.

4.2. NHL

B-cell-Non-Hodgkin Lymphoma (NHLs) are generally sensitive to chemoimmunotherapy, but fatalities still occur among patients due to relapsed/refractory (R/R) disease. Rituximab (anti-CD20 monoclonal antibody) was one of the last major breakthroughs that showed promise in the treatment of the most common subtypes; such as diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma [55]. Results of high dose chemotherapy and autologous stem-cell transplantation (auto-HSCT) use, however, have been inadequate. In contrast, use of autologous, genetically modified T cells (CAR-T cells) targeting CD19 antigen has been responsible for noticeably high response rates (RR) and has the potential to lead to long-term disease-free survival. The Food and Drug Administration (FDA) has approved axicabtagene clioleucel (axi-cel) [for DLBCL/primary mediastinal large B-cell lymphoma (PMBCL)] and tisagenlecleucel (tisa-cel) (DLBCL) after two or more lines of systemic therapy [9,34]. The European Medicines Agency (EMA) approved both products in 2018. FDA approved another product lisocabtagene maraleucel in 2021 [56]. Each of these CAR-T products are unique and its clinical outcomes and toxicity profiles depends on various factors like construct, co-signalling domain (4-1BB vs CD28), viral vector used for transduction (lentiviral vs retroviral), intensity of lymphodepletion, composition of CAR-T product, CAR-T cell dose, and disease subtype. CD19 CAR-T cell therapy commonly has an increased response rate and deliver an enduring response against R/R DLBCL, mantle cell lymphoma, follicular lymphoma. DLBCL is the most common type of aggressive NHL and accounts for nearly 30-40% of all cases [57]. CAR-T cell therapy consisting of CD19 directed CAR-T cells has been proven to be effective against B-cell malignancies. These autologous T cells are genetically modified using retroviral or lentiviral vector containing DNA encoding a CAR. The CAR contains a CD19-recognition domain, a costimulatory domain, either CD28 or 4-1BB, and a CD3z intracellular signalling domain. The early studies were conducted in institutions like the NCI which conducted the first study. It demonstrated the clinical activity of CAR-T cells in DLBCL using the CD3ζ-CD28 CAR-T construct (this was licensed later as axi-cel by Kite Pharmaceuticals). The lymphodepletion regimen used a combination of cyclophosphamide (60 mg/kg) followed by a dose of 25 mg/m^2 of fludarabine applied daily for 5 days [16]. Out of the seven patients evaluated under this trial, five patients had CR, while two patients had a partial response. The duration of response (DOR) had ranged from somewhere between 38 to 56 months in patients with ongoing responses, as reported in a long-term follow up report [58]. In another report from the NCI, there were 22 aggressive B-cell lymphoma patients. The chemotherapy conditioning administered was

of a lower dose. After the treatment, while there was a lymphodepleting action, the associated hematologic and nonhematologic toxicity was reportedly less. The patients with DLBCL had an overall response rate (ORR) and CR rate of 68% and 47% respectively, while, the median duration of remission was 12.5 months and the 12-month progression-free survival (PFS) was at 63.3% [59]. In 2015, at the American Society of Hematology (ASH) meeting, important updates on NHL were presented regarding trials conducted at the Fred Hutchinson Cancer Research Center (FHCRC), where CAR-T cells using a 4-1BB costimulatory domain was developed. In mouse models, CAR-T cells manufactured using purified CD4+ or CD8+ central memory (TCM) or naïve (TN) T cells in a 1:1 CD4:CD8 ratio were more effective in the elimination of CD19+ tumour cells when compared with CAR-T cells manufactured from effector memory T cells (TEM). Based on this data from mouse models, in the FHCRC phase I clinical trial, the CAR-T cells infused had a defined subset, i.e., a fixed 1:1 ratio of CD8+ T central memory cells to CD4+ T cells. The trial result was positive with ORR and CR rates of 63% and 33%, respectively. For patients with DLBCL and TFL, the ORR and CR rates were 67% and 38%, respectively. JUNO Therapeutics has licensed the CAR construct used in this trial for development as JCAR017 [17]. In the University of Pennsylvania (UPENN), another 4-1BB CAR-T (CTL019) construct was developed. In the trial, 38 patients were included. The chemotherapy included many regimens that were left to the physician's discretion. The median dose of CTL019 was at 5.79×10^6 CAR-T cells/kg. The DLBCL patients had ORR and CR rates of 50% and 43% respectively. At the last follow up PFS was at 43% while the median duration of remission was 28.6 months. Some patients had no significant differences in their outcomes after treatment [60,61]. Due to the successes of the early single-center studies, multicenter studies that included several academic institutions accompanied with various pharmaceutical companies were designed. Three phase I/II multicenter clinical trials: ZUMA-1, JULIET and TRANSCEND-NHL-001 tests different types of anti-CD19 CAR-T cell constructs [62]. In the ZUMA-1 trial, the Axi-cel used utilizes a CD28 costimulatory domain. Both the tisa-cel and liso-cel used in JULIET and TRANSCEND-NHL-001 respectively uses a 4-1BB costimulatory domain [63]. The CARs that used CD28 apparently had more rapid in-vivo expansion and higher peak area under the curve levels but could not persist long-term and might have suffered from rapid metabolic exhaustion. The patients who were enrolled in these trials were adults, had DLBCL, high grade B-cell lymphoma, transformed follicular lymphoma (tFL), had R/R disease and some had undergone two prior lines of systemic therapy. The ZUMA-1 trials had patients with PMBCL, while TRANSCEND-NHL-001 had patients with follicular-lymphoma grade-3B and other transformed indolent lymphomas. Both JULIET and TRANSCEND-NHL-001 allowed bridging therapy, however, such therapy was not permitted in ZUMA-1 [63]. The ZUMA-1 clinical trial was the first multicenter trial to evaluate the viability of CAR-T cell therapy against refractory DLBCL. The clinical trial had a phase I and phase II portion. It used the NCI CD3ζ/ CD28 CAR construct (KTE-C19, now known as axi-cel). The lymphodepleting regimen involved cyclophosphamide (500 mg/m^2) and fludarabine (a dose of 30 mg/m^2 applied for 3 days). Axi-cel infusion followed the chemotherapy regimen at a dose of $1\text{-}2\times10^6$ CAR-T cells/kg. The objective response was 71% with four out of seven patients achieving CR (57%) within a month. Three patients had ongoing CR at 12 months. Reversible grade 3 neurotoxicity and CRS was reported among this cohort [35]. However, one fatality occurred due to intracranial bleeding which was considered unrelated to axi-cel. The patient had experienced a grade 4 CRS and grade 4-encephalopathy. Before chemotherapy and CAR-T cell infusion the patient had a high inflammatory state [36]. In the phase II portion of the trial, necessary changes in the safety evaluation were made and baseline C-reactive protein (CRP) assessment was included. The delaying of CAR-T cell infusion in patients with fevers until appropriate work-up was also

completed. The phase II portion of the ZUMA-1 trials and the phase I portion had similar eligibility criteria with two cohorts, cohort 1 for DLBCL and cohort 2 for PMBCL and TFL [64]. 119 patients were enrolled, 70% of them were refractory and 21% had relapsed within 12 months of auto-HCT. For different reasons, ten patients were unable to receive axi-cel. Out of 119 patients, 7 patients received an axi-cel infusion in phase I, while the others received it at the end. The manufacturing success of CAR-T cells was 99%, with the median time for delivery being 17 days. Compared to the historical cohort of SCHOLAR-1, it had an eightfold higher CR rate. As of August 11, 2018, 101 patients were assessable for activity in phase II and were followed up for a median of 27.1 months. 84 patients had an objective response (83%) while 59 patients had a complete response (59%). The median overall survival was not reached, and the median PFS was 5.9 months while the median Duration of Response was 11.1 months. Out of the 108 patients assessed in both phase I and II of the trial 52 (48%) had a grade 3 or worse serious adverse events (SAE). Two treatment-related deaths had been reported (due to haemophagocytic lymphohistiocytosis and cardiac arrest). The 2 years follow up data from Zuma-1 indicate that axicabtagene clioleucel can result in durable responses and overall survival of greater than 2 years [37]. The JULIET trials used the CTL019, anti-CD19 CAR using a 4-1BB costimulatory domain. It is a phase II multicenter global study in patients with refractory DLBCL. CAR-T cells were produced centrally. In contrast to ZUMA-1 trials, cryopreserved apheresis products were used and bridging chemotherapy was allowed according to clinical discretion for patients with rapidly progressive disease. Two regimens of lymphodepleting chemotherapy were applied and it consisted of fludarabine 25 mg/m^2 and cyclophosphamide 250 mg/m^2 for 3 days or bendamustine 90 mg/m^2 for 2 days. The patients who were eligible were ≥18 years with r/r DLBCL, had received ≥2 lines of therapy, and were ineligible for or had failed Autologous stem cell transplant (ASCT). A total of 167 patients were enrolled and 115 patients were infused with a single dose of tisagenlecleucel [65]. CTL019 could not be administered to fifty patients due to failure to manufacture CAR-T cells or change in disease/patient status. The median dose administered of tisangenlecleucel was 3.0×10^8 CAR-positive viable T cells (range: $0.1\text{-}6.0\times10^8$). Among the patients infused, 90% and 93% received bridging therapy and lymphodepleting chemotherapy, respectively. 51% had refractory disease with at least three lines of therapy, while 49% had received prior auto-HCT. The median time from infusion to data cut-off was reported at 19.3 months. All the 99 patients who were part of the main cohort were evaluable, and their ORRs and CRs were 54% and 40%, respectively. These patients also had a PR of 13%. The ORR was consistent across all prognostic subgroups (post-auto-HCT, and double/triple-hit lymphoma). The relapse-free survival for 12- and 18-month was at 64%. The 12 and 18 month OS probability in all patients were at 48% and the 18 month OS probability in all patients was at 43%. The median Duration of response (DoR) was not reached, while the median OS for all patients was 11.1 months and CR patients never reached it. Like ZUMA-1, 54% of patients who had originally achieved PR finally reached CR. CTL019 also did not cause any death, however, three patients died within 30 days of infusion due to disease progression [35]. In the multicenter trial known as TRANSCEND-001, a 4-1BB CAR-T cell construct was used. The construct used a defined 1:1 CD4:CD8 T cell ratio and was developed at FHCCR. The CAR-T cell product used is known as Lisocabtagene maraleucel (JCAR017). The patients were divided into two group, the FULL and CORE cohorts. Patients with R/R DLBCL, TFL, FL grade 3b, MCL, RT, DLBCL, arising from MZL, and PMBCL were included in the FULL cohort. Patients with R/R DLBCL and TFL were included in the CORE cohort [66]. The conditioning regimen used fludarabine (30 mg/m^2 dose) and cyclophosphamide (300 mg/m^2 dose) daily for 3 days, followed by infusion of CAR-T cells. A single flat dose of JCAR017 was applied at one of two dose levels, either at DL-1S at 5×10^7 cells or at DL-2S at 1×10^8 cells. A small number of

patients received a double dose of JCAR017 at 5×10^7. The second dose was administered 14 days after the administration of the first dose. 344 patients had undergone leukapheresis, of whom 269 patients received at least one dose of lisocel between Jan 11, 2016, and July 5, 2019. Median follow-up for OS for all the 344 patients who had undergone leukapheresis was 18.8 months. Out of the 256 patients that could be evaluated, only 186 patients achieved OR (73%, 95% CI 66.8-78.0) and 136 patients had a CR (53%, 46.8-59.4). Nine patients had a dose-limiting toxicity, one of these patients died of diffuse alveolar damage following a dose of 5×10^7 CAR-T cells. Patients did suffer from grade 3 or worse adverse events like neutropenia (60%, 161 patients), anaemia (37%, 101 patients), thrombocytopenia (27%, 72 patients), cytokine release syndrome (42%, 113 patients) and neurological events (30%, 80 patients). Although under evaluation, use of liso-cel or JCAR017 might lead to high ORR among patients with R/R large B-cell lymphoma. The TRANSCEND-NHL-001 study is estimated to be completed by December, 2022 [67,68]. The outcomes of CD19 CAR-T therapy trials for CLL and different varieties of NHL according to the chemotherapy regimen are detailed in Table 2 [51].

Table 2. A summary of CD19 CAR therapy clinical trials for CLL and other NHL before 2012: outcomes depend upon conditioning chemotherapy.

Site	Number of Patients	Disease	Conditioning Regimen	Gene Transfer	Cell Dose/kg	Response Rates
MSKCC	3	CLL	None	Gamma Retrovirus	$1.2–3.0 \times 10^7$	CR 0 % PR 0 %
MSKCC	4*	CLL	CY	Gamma Retrovirus	$0.4–1.0 \times 10^7$	CR 0 % PR 25 %
NCI	7	CLL	FLU/CY	Gamma Retrovirus	$0.3–4.0 \times 10^6$	CR 43 % PR 43 %
UPENN	14	CLL	FLU/CY, PC, Benda	Lentivirus	$0.14–11 \times 10^8$ **	CR 29 % PR 28 %
NCI	14	Other NHL	FLU/CY	Gamma Retrovirus	$0.3–5.0 \times 10^6$	CR 36 % PR 36 %

Outcomes of CD19 CAR therapy trials for CLL and other types of NHL according to the conditioning chemotherapy regimen. CY: Cyclophosphamide. FLY/CY: Fludarabine cyclophosphamide. PC: Pentostatin cyclophosphamide. Benda: Bendamustine. PR: Partial Remission.

*: - A 5th patient could not be evaluated and therefore not included in the trial.

**: - Total CAR-T cell dose is given as dosage in per unit kg was not available.

4.3. AML

Patients with myeloid malignancies such as acute myeloid leukemia (AML) have to deal with a major problem which is the risk of relapse after conventional chemotherapy. Relapsed disease is also the major cause of death after diagnosis with AML. At the moment, allogenic hematopoietic stem cell transplant (allo-HSCT) is the only potentially curative treatment option available. Allo-HSCT can eliminate residual leukemia cells through its graft-vs-leukemia effects. Unfortunately, despite its history of success, relapse after allo-HSCT can still happen and is a major challenge and is associated with poor prognosis. CD19 directed CAR-T cell therapy has shown remarkable success in certain types of B-cell malignancies. However, what limits the use of CAR-T cells in myeloid malignancies is the lack of a dispensable antigen [69]. Most myeloid antigens are often co-expressed on normal hematopoietic stem/progenitor cells (HSPCs), which if depleted will lead to unacceptable myeloablation. Among the clinical trials conducted on AML, the first clinical trial that reported demonstrable biological activity of CAR-T cells in AML was published in 2013 by Ritchie et al., where a

second generation CD28-ζ CAR was directed against the Lewis Y antigen. Even with limited efficacy, this was an important study as it demonstrated CAR-T cell biological activity in AML patients in the absence of overt hematopoietic toxicity [69]. Currently there are more than twenty CAR-T cell clinical trials which are recruiting patients suffering from AML (Table 3). However, in the absence of mature clinical data, we have to rely upon case reports and pilot studies reporting the use of CAR-T cells in AML treatment. Most of the target antigens are CLL-1, CD33, or CD123, with CD33 and CD123 being appealing targets as both are extensively expressed in AML blasts. CLL-1 which is also known as CLEC12A is also a viable target for CAR-T cells due to its high expression in AML and reported absence in healthy HSPCs as well as its rare expression in non-hematological cells [70,71]. A dual-specific CD33-CLL-1 CAR-T cell was created, supported by pre-clinical data on specificity and anti-tumor potency of these cells. A human trial using these dual-specific CAR-T cells was conducted. A preliminary report by Liu et al. explained that two patients with refractory/relapsed (R/R) AML were treated with the dual CAR-T cells following chemotherapy preconditioning with fludarabine and cyclophosphamide. Both the patients had a minimal residual disease $(MRD)^{-ve}$ complete remission with pancytopenia within 3 weeks of CAR-T cell infusion. Later on they underwent anti-thymocyte globulin (ATG)-based HSCT with subsequent hematopoietic recovery [71,72].

A clinical trial of CD123-specific CAR-T cells was conducted, at the University of Pennsylvania where CAR-T cells was manufactured via mRNA electroporation. Patients who had R/R AML received lymphodepleting chemotherapy prior to administration of CAR-T cells. Although no anti-tumor responses were observed some level of CAR-T cell bioactivity was detected, which was demonstrated by the cytokine release syndrome (CRS) and/or fever experienced by all treated patients. No overt vascular, hematologic or neurologic toxicity was reported despite expression of the target antigen on healthy hematopoietic tissues [73]. Due to this favorable safety profile, clinical trials were conducted that targeted the CD123 antigen. A study conducted by the City of Hope Medical Center (clinicaltrials.gov identifier: 02159495) reportedly used second-generation CD28-ζ CAR-T cells targeting CD123 manufactured by lentiviral transduction. After preconditioning chemotherapy six patients who were suffering from refractory AML were administered either 50 or 200 million CAR-T cells. One of two patients who received the 50 million dose achieved a transient morphologic leukemia-free state. Blast reduction went from 77.9% to 0.9% upon receiving infusion of CAR-T cells. Two of the four patients who got the higher dose experienced complete remission and went on to HSCT. Grade 1 or 2 CRS was reported in most patients, but no dose-limiting toxicity like cytopenia was observed at the time the report was made [74]. From these trials it can be said that CAR-T therapy can prove to be potent immunotherapy treatment. However, it has to be kept in mind that there are a lot of barriers that can limit the full therapeutic potential of CAR-T cells. For example, AML antigens are usually also shared by normal HSPCs or their progeny, there are no leukemia-specific cell-surface antigens that can be good targets for CAR-T cells. Manufacturing of CAR-T cells also becomes challenging as T cell expansion is inhibited by AML blasts or T cell damaging chemotherapy, and finally AML is a heterogenous and complex disease that can evade the immune system by utilizing various immunosuppressive mechanisms. To overcome these obstacles potential avenues to reduce toxicity to healthy tissues while simultaneously utilizing the full therapeutic potential of CAR-T cells has to be exploited. Rationally designed clinical trials that are informed by a complete understanding of the AML-effector cell-microenvironment axis have to be implemented in order to create a successful immunotherapy regimen [69].

Table 3. Clinical trials on CAR-T cell therapy in myeloid malignancies that are currently recruiting. Information is collected from www.clinicaltrials.gov

Disease	Interventions	Identifier ID	Phase	Location
AML	Biological: anti-ILT3 CAR-T	NCT04803929	Early Phase 1	Zhejiang Provincial People's Hospital, Hangzhou, Zhejiang, China
	Biological: CD123/CLL1 CAR-T Cells	NCT03631576	Phase 2 Phase 3	Fujian Medical University Union Hospital, Fuzhou, Fujian, China
	Biological: CAR-T CD19	NCT04257175	Phase 2 Phase 3	Chaim Sheba Medical Center, Ramat Gan, Israel
	Biological: CLL-1, CD33 and/or CD123-specific CAR gene-engineered T cells	NCT04010877	Phase 1 Phase 2	Shenzhen Geno-immune Medical Institute, Shenzhen, Guangdong, China
	Biological: CLL-1. CAR-T cells	NCT04219163	Phase 1	Texas Children's Hospital, Houston, Texas, United States
	Biological: Third-generation anti-CD123 CAR-T cells	NCT04014881	Phase 1	Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China
	Biological: CART therapy in Acute myeloid leukemia (AML)	NCT03473457	Not Applicable	Southern Medical University Zhujiang Hospital, Guangdong, Guangdong, China
	Biological: CAR-γδT	NCT04796441	Not Applicable	Hebei yanda Ludaopei Hospital, Yanda, Hebei, China
	Drug: MLM-CAR44.1 T cells, cyclophosphamide and fludarabine from -5 to -3	NCT04097301	Phase 1 Phase 2	IRCCS San Raffaele, Milan, Italy, IRCCS Ospedale Pediatrico Bambino Gesù, Roma, Italy
	Biological: Chimeric antigen receptor T cell	NCT04835519	Phase 1	Beijing Boren Hospital, Beijing, Beijing, China
	Biological: anti-CLL1 CART	NCT04884984	Phase 1 Phase 2	The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, China
	Biological: CD123 CAR-T cells	NCT04272125	Phase 1 Phase 2	Chongqing University Cancer Hospital, Chongqing, Chongqing, China
	Other: Chimeric Antigen Receptor T cells (CAR-T)	NCT04692948	Not Applicable	Anhui Provincial Hospital, Hefei, Anhui, China
	Other: Sample collection	NCT04169022	Not Applicable	CHU Besançon, Besançon, France
	Biological: CD123 CAR-T cells	NCT04265963	Phase 1 Phase 2	920th Hospital of Joint Logistics Support Force, Kunming, Yunnan, China
	Biological: CD33CART	NCT03971799	Phase 1 Phase 2	California, Maryland, Pennsylvania, USA
	Biological: CART-38	NCT04351022	Phase 1 Phase 2	The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, China
	Drug: Cyclophosphamide Drug: Fludarabine Biological: KITE-222	NCT04789408	Phase 1	Washington University School of Medicine, Saint Louis, Missouri, United States The University of Texas MD Anderson Cancer Center, Houston, Texas, United States
	Biological: UCART123v1.2	NCT03190278	Phase 1	USA
	Biological: Humanized CD7 CAR-T cells	NCT04762485	Phase 1 Phase 2	The First Affiliated Hospital of Soochow University, Suzhou, China
	Drug: CD123-CAR-T Drug: Cyclophosphamide Drug: Fludarabine Drug: Mesna Drug: Rituximab	NCT04318678	Phase 1	St. Jude Children's Hospital, Memphis, Tennessee, United States
	Biological: CART-19	NCT03896854	Phase 1	The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, China

	Drug: cyclophosphamide Biological: Autologous CD123CAR-CD28-CD3zeta-EGFRt-expressing T Lymphocytes Other: laboratory biomarker analysis Biological: Allogeneic CD123CAR-CD28-CD3zeta-EGFRt-expressing T-lymphocytes Drug: Fludarabine Phosphate	NCT02159495	Phase 1	City of Hope Medical Center, Duarte, California, United States
	Biological: CD7-specific CAR gene-engineered T cells	NCT04033302	Phase 1 Phase 2	Shenzhen Geno-immune Medical Institute, Shenzhen, Guangdong, China
AML/ MDS	Biological: Chimeric Antigen Receptor T cells Biological: peptide specific dendritic cell	NCT03291444	Phase 1	Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong, China
	Biological: CYAD-02 Drug: ENDOXAN Drug: Fludara	NCT04167696	Phase 1	USA and Beligium
AML /MDS /MPN	Biological: CLL1-CD33 cCAR-T cells	NCT03795779	Early Phase 1	The General Hospital of Western Theater Command, Chengdu, China
	Biological: CD123-CD33 cCAR-T cells	NCT04156256	Early Phase 1	Chengdu Military General Hospital, Peking University Shenzhen Hospital, China
CML	Other: biological samples	*NCT02842320	Not Applicable	Hôpital Nord Franche-Comté, Centre Hospitalier Régional Universitaire de Besançon, CHU de Dijon, CHI de Haute-Saône, France

Acronyms used: AML: Acute Myeloid Leukemia; MDS: Myelodysplastic Syndrome; MPN: Myeloproliferative neoplasms; CML: Chronic Myeloid Leukemia; CAR; Chimeric Antigen Receptor.

*The trial is active but is currently not recruiting.

4.4. CML

Myeloid leukemia represents a mixed group of cancers of blood and bone marrow. It arises from the clonal expansion of hematopoietic myeloid lineage cells. Chronic Myeloid Leukemia is an indolent disease that requires lifelong treatment with a risk of progression to fatal blast crises. Unlike most cancers, the cause of CML can be traced to the BCR-ABL1 oncogene. This arises due to a genetic abnormality, the Philadelphia chromosome, a shortened chromosome 22 which is a result of the reciprocal translocation of the long arms of chromosome 9 and chromosome 22. This results in the formation of BCR-ABL fusion gene. The BCR-ABL gene also gets translated in the p210 BCR-ABL1 oncoprotein which is present in almost all CML patients [75]. However, CML treatment has reached a point where patients can have a normal predicted life expectancy. From radiotherapy and cytoreduction using traditional chemotherapy to targeted therapies with tyrosine kinase inhibitors (TKIs) and allogenic stem cell transplantation (allo-SCT) associated with IFNα, there are a myriad of treatments available to patients. However, discontinuation rates for TKI can be substantial, in part because of intolerance and toxicity, potential risk in pregnancy, and medico-economic reasons [76]. TKI discontinuation studies points to the fact that TKIs may cure the disease in up to half of patients with CML. But, current TKIs are a suppressive instead of a curative therapy. This will lead to a continuous long-term administration which might result in unforeseen adverse effects [77]. However, detection of the BCR-ABL1 breakpoint fusion gene by long-range or reverse PCR, reveals that a quiescent primitive CML stem cell compartment persists after TKI treatment by remaining insensitive which then risks

becoming a source of relapse in the future. CML has certain characteristics like: BCR-ABL fusion region peptides that elicit CML-specific T cell responses, the potential for autologous dendritic cell vaccination, the role of natural killer (NK) cells, the anti–BCR-ABL efficacy of T-helper or cytotoxic T lymphocytes, the restoration of immune control associated with programmed cell death-1 (PD-1) inhibition in molecular (RM3.0) or deep-response CML patients, immune surveillance evasion after downregulation of MHC-II expression by CML cells, and the well-known graft versus-leukemia effect of allo-SCT. These characteristics point to CML as an immune-sensitive disease [78]. CML, therefore, can be a candidate for immunotherapies. CAR-T cell which have shown exceptional success against B cell malignancies come up as a viable option. Several antigens that are selectively expressed on CML cells are known. Interleukin-1 receptor-associated protein (IL-1RAP) is one example of such antigen. CAR-T cells that target IL-1RAP in quiescent CML stem cells can lead to permanent cure, because CML is an immune system-sensitive disease. The IL-1RAP protein which have been mentioned earlier as a surface biomarker, is a co-receptor of the IL-1 and IL-33 receptor, and also is a very attractive candidate to target CML or AML hematopoietic stem cells (HSCs). The IL-1RAP targeting by antibodies demonstrated a potent antibody-dependent cellular cytotoxicity (ADCC) against HSCs in vitro and also in a xenograft murine model of CD34+/CD38- AML or CML HSCs [79,80]. Therefore IL-1RAP is a viable cell surface tumor-associated antigen to target with immunotherapy approaches. It is a suitable target because it is upregulated in all CMLs but not in normal HSCs, unlike other CML HSC markers which are expressed in only a portion of BCR-ABL1 cells. This allows for the discrimination between normal HSCs and CML cells. Warda et.al in his preclinical studies demonstrated that the IL-1RAP CAR-T cells had a 1:1 Effector to target cell (E:T) ratio. Less than half of the monocytes were targeted while more than 90% of the leukemia cells were destroyed. The destruction of the monocytes was speculated to have a deleterious effect on CRS and toxicity because there has been reports that held the IL-1 secreted from monocytes and macrophages responsible for CRS and neurotoxicity after infusion of CAR-T cells [81]. The absence of A3C3 immunostaining in healthy TMA also supported the argument that IL-1RAP targeted CAR-T cells might be associated with limited side effects. These preclinical studies in effect proves that the IL-1RAP targeted CAR-T cells are able to selectively target quiescent CML stem cells and provide a potential cure for CML which otherwise can be considered to be a lifelong pathology. There are also multiple case reports that demonstrated the ability of CAR-T cells to treat CML. In one such study conducted by Zhang, et al., a 56-year-old man was diagnosed with chronic phase Philadelphia positive (Ph+) CML. The flow cytometry analysis showed that the percentage of lymphoblasts in the bone marrow was 55.09%. CD34, CD38, HLA-DR, CD10, CD19 and CD22 antigens were present. The BCR-ABL/ABL ratio was 101.01% without mutations in BCR-ABL kinase domain. Dasatinib was initially given at a dose of 70 mg daily, and changed into 50 mg daily for maintenance [82]. In March 2018, after the bone marrow examination it showed that there were 88% of lymphoblasts in the bone marrow and the BCR-ABL/ABL ratio was 112.65%. Meanwhile the T315I mutation was detected. Subsequently, Dasatinib was immediately discontinued, and combined chemotherapy was administered until authorization for CD19-targeted CAR-T cells clinical trial (ChiCTR–OCC–15007008) was obtained. In July of 2018, the patient was administered autologous CAR-T cells at the dose of 5×10^6/kg after conditioning chemotherapy (Fludarabine 30 mg/m^2/day at day 1-3 consecutively and Cyclophosphamide 750 mg/m^2/day at day 2, 3). A grade 3 of Cytokine Release Syndrome (CRS) which is associated with CAR-T cells expansion was detected and managed by Tocilizumab combined with methylprednisolone and supportive treatment. No significant neurotoxicity was observed. The flow cytometry which detects phenotype of lymphoblasts showed minimal residual disease (MRD) less than that of 0.01% [83]. T315I mutation also disappeared in bone

marrow 2 weeks after infusion of CAR-T cells. BCR-ABL transcripts, however, were still noticeable with a ratio of 26% which further increased to 46% after four weeks without T315I mutation and lymphoblasts progression. Since, Ph+ leukemic clones without T315I were still present, the patient was treated once more with a daily 70 mg dose of dasatinib. After three months there were no additional mutation along with T315I mutation detectable and the patient's BCR-ABL1/ABL ratio dropped to 015%. The patient was still in remission fourteen months after CAR-T cell infusion [82]. Therefore, it can be said that CAR-T cell therapy has the potential to offer a permanent solution for AML However, these case reports are not enough to provide a framework for a long-term solution. Researchers have not even begun to explore the budding landscape of CML CAR-T therapy. Further research is required to comment on the clinical feasibility of CAR-T therapy for CML treatment.

5. CONCLUSION

CAR-T cell therapy is one of the most promising adoptive cell therapies (ACT). CAR-T cell therapy has positioned itself as an alternative therapy in many cases where none existed before. It has seen immense success in the treatment of hematologic malignancies, along with progress in the fields of antigen targeting, combined application of immune cells and synthetic small molecule drugs and intracellular signal domains. It also combines two distinct characteristics, i.e., the specificity of gene therapy and the wide-ranging response associated with cellular therapy. However, engineered T cells that can treat solid tumors still remain an insurmountable challenge. Widespread adoption of clinical-grade CAR-T cell therapy also remains a challenge and can only become possible when there is a proper way to reproduce the high-quality manufacturing needed for this technology [84]. The process employed for manufacturing tisagenlecleucel is a perfect example of how to scale out, streamline and optimize a complex manufacturing process in order to ensure a steady supply of high-quality product to the patient population. The manufacturing process was optimized by focusing on key areas of enhancement, without compromising the product integrity or potency. Another one of the biggest challenges to the widespread marketability of CAR-T cell therapy is its high Cost of Goods (CoG) [85]. The reason for high CoG is a bioprocessing bottleneck due to the current instrumentation and methods being expensive, time-consuming and difficult to scale, as well as high cost of viral vectors. To reduce the CoG, a complete reengineering of the biomanufacturing process, integrating a complicated, multistep process into a closed, modular, benchtop system and effective biomanufacturing near patients are needed. Reducing the high CoG, improving precision and persistence of ACTs, reducing the processing time, improving the starting materials of immunotherapies and development of scalable systems are some obstacles that needs to be surmounted for immunotherapies like CAR-T to reach broader patient populations.

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