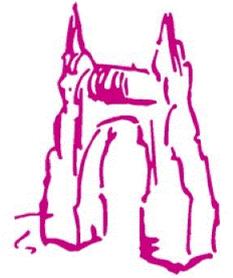




UNIVERSITAT DE
BARCELONA



FACULTAT DE FARMÀCIA I
CIÈNCIES DE L'ALIMENTACIÓ

Final Degree Project

Chimeric Antigen Receptor T-cell Therapy: Bioengineered immunocellular approach to Acute Lymphoblastic Leukaemia

Universitat de Barcelona

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Main area: Immunology

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Pharmacology

Physiology and Pathophysiology

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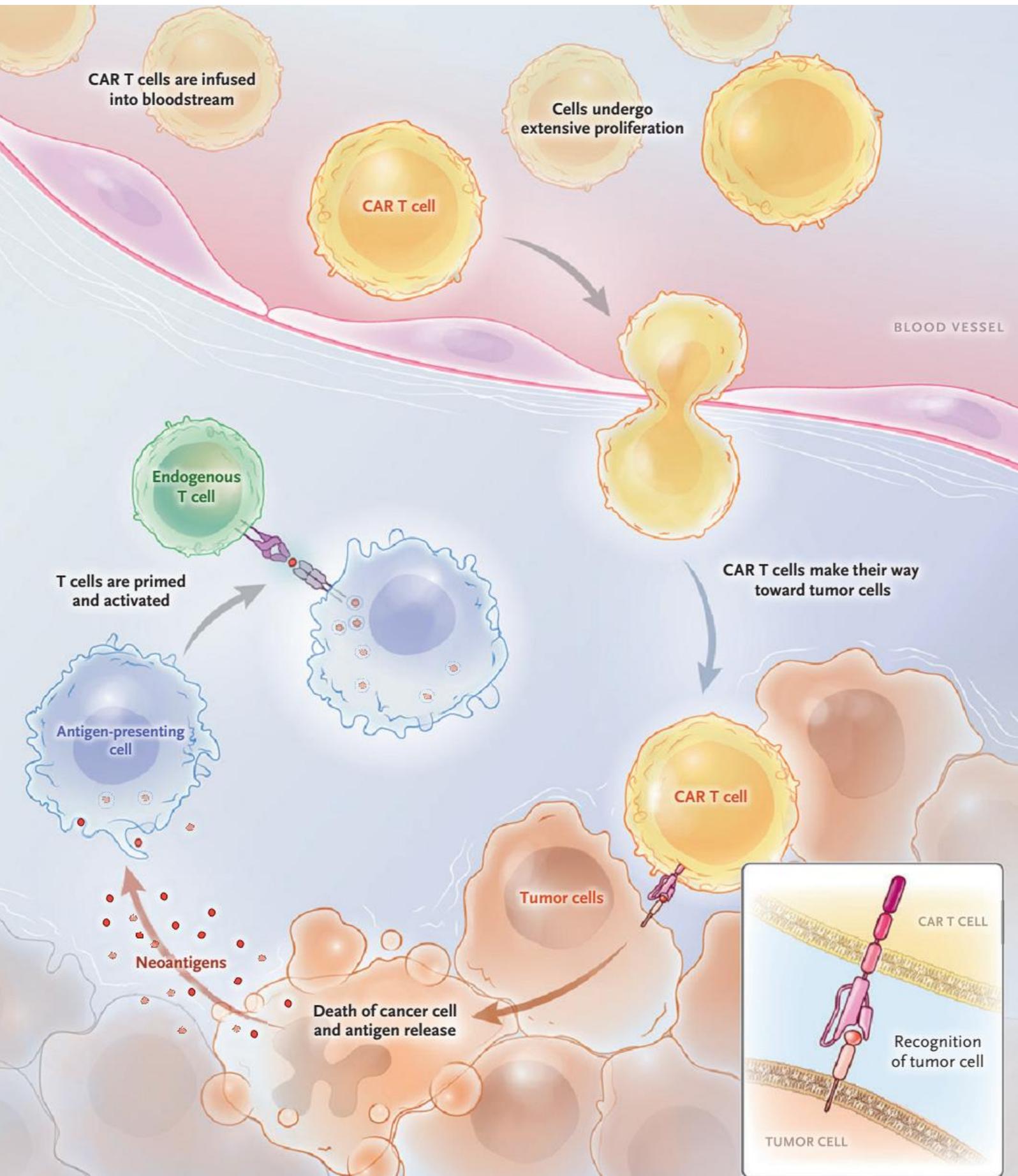


Figure from June CH, Sadelain M. Chimeric Antigen Receptor Therapy. *N Engl J Med.* 2018; 379: 64-73.

“The optimal agent would combine high paratropism with low organotropism,
Paul Erlich”

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Abbreviations

ACT: Adoptive Cell Therapy

AEMPS: Spanish Agency of Medicines and Medical Devices

ALL: Acute Lymphoblastic Leukaemia

allo-SCT: allogeneic hematopoietic Stem Cell Transplantation

APC: Antigen-Presenting Cell

BFM: Berlin-Frankfurt-Münster

CART: Chimeric Antigen Receptor T-cell Therapy

CNS: Central Nervous System

CR: Complete Remission

CRS: Cytokine Release Syndrome

CSF: Cerebrospinal Fluid

EMA: European Medicines Agency

ESMO: European Society for Medical Oncology

FAB: French-American-British

FDA: Food and Drug administration

GVHD: Graft–Versus–Host Disease

HCVAD: hyperfractionated cyclophosphamide, vincristine, anthracycline and dexamethasone

ICU: Intensive Care Unit

IVIG: Intravenous Immunoglobulin

MHC: Major Histocompatibility Complex

MRD: Minimal residual disease

NCI: National Cancer Institute

OS: Overall Survival

R/R: Relapse or Refractory disease

scFv: single chain variable fragment

SEFH: Spanish Hospital Pharmacy Society

TAA: Tumour Associated Antigens

TCR: T-Cell Receptor

TKI: Tyrosine Kinase Inhibitors

TRUCKS: T-cells redirected for universal cytokine killing

WBC: White Blood Cell

WHO: World Health Organisation

1. Abstract

Acute Lymphoblastic Leukaemia (ALL) is one of the most prevalent cancers in children and an almost fatal disease for adults. There is an urgent need to develop new drugs because of the lack of good treatments for these patients, especially those with relapsed or refractory clinical disease. Chimeric Antigen Receptor T-cells (CARTs) is a potent cellular cancer therapy consisting of autologous patient's T lymphocytes, reprogrammed through gene editing to express a surface receptor against a particular tumour antigen. Autologous T cells transduced with anti-CD19 receptors may become a breakthrough for the treatment of B-cell ALL, since they bypass the need for antigen presentation usually affected by tumour immunosuppressive microenvironment. Different CARs have been designed, during the last years, and several institutions have tested its efficacy in clinical studies. Roughly, treated patients presented high remission rates with long-term durations, thus becoming a relevant alternative for those otherwise untreatable patients and granting commercial authorisation by the leading two regulatory agencies: Food and Drug Administration and European Medicines Agency in 2017 and 2018, respectively. This intersection between adoptive cell therapy, bioengineering tools and immunotherapy may have applications beyond cancer such as in autoimmunity and infectious diseases. The primary goal of this review is to present the current evidence of the efficacy of CART treatment and the main problems related to its toxicity and manufacturing, as well as its therapeutic value for ALL.

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La Leucèmia Limfoblàstica Aguda (LLA) és el tipus de càncer més prevalent en nens i una malaltia pràcticament fatal en adults. L'absència de noves teràpies pel tractament de pacients amb malaltia refractària o en recidiva posa de manifest la necessitat urgent de nous agents amb una eficàcia rellevant. Les cèl·lules T amb receptor d'antigen quimèric són una teràpia immunocel·lular potent que consisteix en la reprogramació dels limfòcits T del pacient a través de l'edició gènica per expressar un receptor a la seva superfície que reconegui un antígen tumoral concret. Les cèl·lules T autòlogues transduïdes amb receptors anti-CD19 poden arribar a suposar un gran avenç pel tractament de la LLA de cèl·lules B, ja que és capaç d'evitar la necessitat de presentació d'antigen que acostuma a estar inhibida pel microambient immunosupressor del tumor. Al llarg dels anys, diferents dissenys i generacions han sigut estudiats de diferents institucions. Pràcticament tots ells presenten taxes de remissió elevades a llarga durada, representant una alternativa rellevant per aquells pacients sense més possibilitats terapèutiques i permetent també l'autorització de comerç per part de les dues agències regulatòries principals: L'Administració d'Aliments i Medicaments dels EEUU al 2017 i l'Agència Europea del Medicament al 2018. Aquesta intersecció entre la teràpia adoptiva de cèl·lules, les eines de bioenginyeria i la immunoteràpia podrien tenir aplicacions més enllà del càncer en autoimmunitat i malalties infeccioses. L'exposició de les evidències actuals i les principals característiques pel que fa a toxicitat, eficàcia i fabricació, així com el seu posicionament concret per la LLA, són qüestions tractades en aquesta revisió bibliogràfica.

2. Integration of different fields

The present bibliographic review integrates different science fields. The main one is immunology, since the treatment herein explained benefits from adaptive immune system as a mechanism of action to strive against leukaemia. It is closely related to the proposed secondary areas of application: Molecular Biology, Pharmacology and Physiology and Pathophysiology. Molecular Biology allows understanding of the manufacturing mechanism, as it is a bioengineered autologous cell from the patient. Pharmacology helps with comprehension of the toxicity, pharmacodynamics and pharmacokinetics tested in clinical trials. Finally, Physiology and Pathophysiology enables a better knowledge of the targeted disease: acute lymphoblastic leukaemia. All of them can be considered essential for an integrated view of the topic discussed in this review.

3. Introduction: Acute Lymphoblastic Leukaemia

3.1 General Characteristics: Epidemiology, Incidence Rates and Aetiology

Acute Lymphoblastic Leukaemia (ALL) is a hematologic type of cancer caused by a malignant transformation of the lymphocytic progenitors, and its main feature is the presence of immature lymphocytes, denominated lymphoblasts, and their accumulation in the bone marrow and peripheral blood (i.e., extramedullary sites). ALL represents 72% of all leukaemia types and is considered an aggressive cancer. The disease follows a bimodal distribution, which means there are two peaks of age for presentation. The first peak appears around five-aged children, and ALL is the most frequently diagnosed cancer during childhood, representing 25% among all. Notwithstanding the high rates of incidence in the paediatric population, 80% of total ALL cases, the survival rates have risen from 60% to 90% in the past few decades. Even though, possible long-term toxicities associated with aggressive chemotherapeutic regimens concerns because it may decrease life quality in those cured patients.

In contrast, it represents a challenging disease for adults, owing to frequent relapses and poor long-term survival. The second peak of age at diagnosis is around 50s and represents 20% of ALL and 0.2% of all cancers. Despite low incidence, it represents a fatal disease with survival rates of 30% at six months. In summary, it is a devastating disease for adults and has an extremely high incidence in children (1,2).

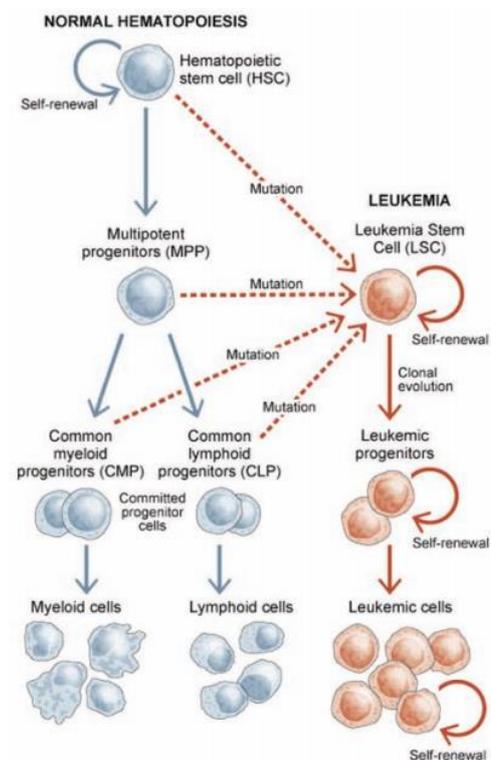


Figure 1. Leukaemia cells transformation from haematopoietic stem cell (3).

Although the pathogenesis is known to involve aberrant proliferation of lymphoid cells, the aetiology remains unidentified. Several risk factors such as genetic syndromes, age, viral infections and exposure to radiation have been observed, but none of them seems to be a sufficient causal agent (4).

3.2 Clinical Manifestations

Despite the lack of specific and distinctive symptomatology, patients with B-cell ALL can develop a combination of so-called '*B symptoms*' that include fever, unexplained weight loss and night sweats. Moreover, infection, easy bruising or bleeding, dyspnoea and fatigue are signs attributed to low functional blood cells count. Occasionally, joint pain appears as the first symptom in children and can be dangerously misinterpreted as a normal process in growing ages unless accompanied by other symptomatology. Splenomegaly and hepatomegaly are present in roughly 20% of patients who have infiltration in these organs as an extramedullary debut. The occurrence of central nervous system (CNS) involvement as the first manifestation with neuropathies and meningeal infiltration happen in approximately 5 to 8% patients (2).

3.3 Diagnosis and Classification

The presence of $\geq 20\%$ lymphoblasts in the bone marrow or peripheral blood is indicative for ALL diagnosis. Further assessment including morphology, immunophenotyping, cytogenetic and molecular studies is valuable for classification and risk-stratification (4). The historical categorisation of subtypes was established according to the French-American-British (FAB) morphological criteria based on cell size and characterisation. This system did not correlate with treatment and prognosis implications, resulting in the World Health Organization (WHO) determining in 2008 a classification, in which immunophenotypic and cytogenetic characteristics of blasts presenting prognosis values were combined (5).

- Immunophenotype allows distinguishing between precursors from B- or T-cell lineage. The 75% of ALL cases correspond to B-cell ALL and the remaining to T-cell malignancies.
- Cytogenetic analysis permits to characterise the individual mutations that provide molecular features contributing to malignant transformation. The prognosis depends on this subset classification. Meaningful genetic determinants are presented in *Table 1*.

Type of ALL	Cytogenetic abnormality	Protein	Genes involved	Comments
T cell	NA	NA	<i>RAS/PTEN</i> , <i>FBW7</i> , <i>PICALM-MLLT10</i> , <i>NUP214-ABL1</i> fusion, <i>MLL</i> , <i>SET-NUP214</i> fusion, <i>PTPN2</i> , <i>NOTCH1</i> , <i>BCL11B</i> , <i>JAK1-PIF6</i> , <i>IL7R</i> , <i>EML-ABL1</i>	Poor outcomes with <i>RAS/PTEN</i> and <i>JAK1</i> Favorable outcomes with <i>FBW7</i> (~20%) and/or <i>NOTCH1</i> (>60%)
B cell	Near-hypodiploid	NA	<i>FLT3</i> , <i>NFI</i> , <i>KRAS</i> , and <i>NRAS</i>	Occurs in 70% of cases
	Ph-like	NA	<i>NUP214-ABL1</i> , in-frame fusions of <i>EBF1-PDGFRB</i> , <i>IKZF1</i> deletions, <i>BCR-JAK2</i> or <i>STRN3-JAK2</i> , rearrangements/mutations in <i>CRLF2</i> , cryptic <i>IGH-EPOR</i> rearrangements <i>IGH-CRLF2</i>	Occurs in 15% of cases. Utilization of TKIs and/or <i>JAK2</i> inhibitors and mTOR is possible
	Hyperdiploid	NTSC2	<i>TP53</i> mutations, <i>CREBBP</i> , <i>NTSC2</i> mutations	Occurs in 6% of cases
	Positive <i>BCR/ABL</i> (Ph ⁺)	Ikaros	<i>IKZF1</i>	Outcome is poor. Occurs in approximately 80% of Ph ⁺ cases
	Low-hypodiploid	NA	<i>TP53</i> disruptions, <i>IKZF2</i> , <i>CDKN2A/B</i> locus deletion	Occurs in 91% of cases

Table 1. Frequent genetic determinants in ALL (4).

3.4 Risk-Stratification and Prognosis

Risk-stratification considering all the characteristics of the patient at diagnosis is crucial to lead them in a suitable treatment strategy that could achieve the best possible remission rate. Apart from that, a precise description of surface markers could reveal potential treatment targets. Moreover, physicians accomplish an accurate idea of prognosis (6). Age is an intrinsic unfavourable risk factor due to comorbidities and less tolerability for chemotherapy translated into decreased long-term survival numbers as age increase, being 90% in children, 60% in adolescents and young adults and only 40-50% in adults. Although most adults treated achieve high Complete Remission (CR) rates between 80 and 90%, many of them experience relapses and 5-overall survival is dramatically low (2).

3.5 Treatment Overview

During the last decades, paediatric patients have experienced an increase in cure rates thanks to dose intensification chemotherapy. However, such a strategy stratified to adults has not achieved the same success, mainly due to appeared toxicity and even death, arriving just to 30-40% long-term remission rates with high-frequency relapse. This fact shows the necessity for targeted agents less intense than chemotherapy that do not impair health structures and allows long-term survival without durable toxicities. The goal setting an approach is to optimise treatment regimens according to characteristics at diagnosis of each patient (5). Therapy aims to establish remission, defined by the presence of all the following criteria (2):

- No more than 5% normocellular blasts in bone marrow
- No signs or symptoms of the disease and neither CNS leukaemia or other extramedullary infiltration
- All laboratory finding within normal limits:
 - White Blood Cell (WBC) count (4.5 to 11 x10⁹/L)
 - Haematocrit (35-50%) and haemoglobin level (12 to 17.5 g/dL)
 - Platelet count (150 to 170 billion/L)

3.5.1 Frontline Treatment

Treatment regimen at first diagnosed ALL is analogous in children and adults, differing just in intensification, lower in elderly. It consists of three consecutive phases of induction, consolidation and long-term maintenance chemotherapy all along with CNS prophylaxis. Overall treatment can last at least 1 year and an additional 2 for maintenance (4,6). Purpose of treatment is to:

- Abolish disease
- Recover normal haematopoiesis
- Prophylaxis of sanctuary sites (i.e., areas where leukaemia cells are protected from systemic chemotherapy): CNS and testis mainly
- Prevent the survival of resistant blasts that can develop a relapse

Phases of treatment are described below (4):

1. Induction therapy: eradication of leukemic cells and introduction to complete remission

Berlin-Frankfurt-Münster (BFM) protocol regimen of therapy includes 8 cycles, each one alternating 2 different parts known as “A” and “B”. Part A of the cycle consists of hyperfractionated cyclophosphamide, vincristine, doxorubicin and dexamethasone (HCVAD). Dexamethasone is slightly preferred among prednisone owing to higher concentrations in cerebrospinal fluid (CSF) although greater toxicity. Part B includes high-dose methotrexate and cytarabine. Patients with Philadelphia chromosome disease take Tyrosine kinase inhibitors (TKI) as well. CNS prophylaxis considers intrathecal chemotherapy administered twice each cycle at least during 4 cycles. The number of cycles increases in the case of a high-risk patient. When CNS leukaemia is present at diagnosis, cranial radiation is added. Additionally, hematopoietic growth factors are included after a completed cycle to quicken bone marrow restoration and to enable the continuation with dose intensification.

Whether achieved complete remission after first induction, patients undergo consolidation chemotherapy. For patients with high-risk classification and available donor, allogeneic hematopoietic stem cell transplant (allo-SCT) is considered.

2. Consolidation therapy: elimination of residual malignant blasts persisting after induction therapy

Used drugs are similar to those in the induction phase but differing in dose or associated with specific targeting drugs for particular subsets. For example, in the case of paediatric, adolescents and young adults L-asparaginase is added in to improve outcomes. However, for adults, it is too toxic and achieves more unfortunate results. A more extended phase of maintenance usually follows it.

3. Maintenance therapy: avoid relapse and achieve long-term remission

Long-term treatment period consisting of daily 6-mercaptopurine, weekly methotrexate, monthly vincristine and pulses of prednisone or dexamethasone can last from 2 to 3 years. Elimination of this phase tends to produce adverse outcomes.

4. Central nervous system prophylaxis

Although CNS leukaemia, diagnosed when >5 WBC/uL in the CSF, present at diagnosis time is uncommon ($<10\%$) it can become 75% after a year without prophylaxis. When CNS leukaemia appears, the prognosis is lower, especially when relapse in adults. Overall survival (OS) hardly arrives at 6 months, and cure is restricted to allo-SCT. Methotrexate and cytarabine are given intrathecally in high-doses and decrease the possibility of CNS relapse by 4%. Radiation is a more aggressive option and can result in cognitive damage and neurologic adverse effects, in which the development of brain tumour is included.

3.5.2 Stem Cell Transplantation and Minimal Residual Disease

Recently, minimal residual disease (MRD) status postulates as a prognostic factor for patients who achieve CR after consolidation therapy. Having MRD⁻ means that the probability of identifying a blast by flow cytometry is $\leq 1 \times 10^{-3}$. When patients present MRD⁺, they are reassigned to the high-risk group and thereby are considered for transplantation. In the case of MRD⁻, the prognosis for patients is significantly favourable, and they usually benefit from maintenance chemotherapy. The disparity in 5-year OS is notable, considering 75% vs 33% for MRD⁻ and MRD⁺, respectively. MRD⁺ usually occurs when chemorefractory disease, resulting in high numbers of relapse whether no alternative approach is tried: 90% of the MRD⁻ subgroup relapses within 4 to 5 months. In summary, MRD is the only prognostic factor announcing success once treatment has started, setting the goal for every new therapy to accomplish, CART therapy as well (5,6).

3.5.3 Salvage Treatment for Relapsed and Refractory ALL (R/R ALL)

Regardless of the high amount of initial CR, 40 to 50% of adults suffer a relapse or refractory disease (r/r) ALL. In those situations, allo-SCT percentage of cure is limited to 30% and only 10% of patients indeed receiving it (4). Ongoing strategies capable of attaining second complete remission (CR2) after relapse with great OS are missing, but pioneer compounds in the exciting and compelling field of immunotherapy can reshape treatment. All advances done in the last decades are focused on the molecular understanding of the disease that allows a refinement regarding prognosis factors and targets for novel therapies (5).

3.5.4 Targeted Novel Therapies: monoclonal antibodies and CAR T cells

3.5.4.1 Monoclonal antibodies: *Blinatumomab* and *Inotuzumab Ozogamicin*

Blinatumomab is a bispecific monoclonal antibody that combines CD19 and CD3. It enables the redirection of immune T cells to the CD19 antigen expressed in leukemic cells, resulting in its lysis. It was approved by Food and Drug Administration (FDA) in December 2014 for r/r ALL. It is administered as an infusion for 28 days every 6 weeks due to shown long-term CR with MRD⁻ disease in 60% patients (4).

Inotuzumab Ozogamicin is an immunoconjugate directed to CD22 antigen and linked to calicheamicin, the compound responsible for double-strand DNA break. It has shown a median overall survival of 7.4 months (5).

3.5.4.2 Chimeric Antigen Receptor (CAR) T-Cell therapy

CAR therapy uses bioengineered T lymphocytes expressing a receptor led to specific antigens of leukemic cells. Such a mechanism of action is fascinating and may represent a real breakpoint for r/r treatment and even have first-line regimen consideration in the future (4). This work not only focuses on such an approach but also on its clinical implications.

4. Objectives

The main aim of this project is to perform an exhaustive bibliographic research about one recently developed immunotherapy referred to as Chimeric Antigen Receptor bioengineered T cells, applied to the treatment of refractory or relapsed Acute Lymphoblastic Leukaemia, which holds poor outcomes with conventional therapeutic regimens. The bibliographic research mainly consists of how therapeutic establishment is included within standard regimens and the advantages that offer, as well as the different issues involved. Secondary goals complementary to the main one allowing all concepts integration are:

- Contextualise through epidemiology and statistical numbers the necessity for innovative approaches in refractory and relapsed B-cell ALL for both, paediatric and adult patients: **What is the context for the emergence of a new therapeutic approach?**
- Key points checked when a new therapy is under development: efficacy and toxicity through studies and draw main conclusions. **Which are the mainstays to comprehend about a recently developed therapy?**
- Study the feasibility for a recently approved therapy and its inclusion in treatment: **What do chimeric antigen receptor T cells need to be considered worthwhile in hospital practice?**

5. Materials and Methods

Compiled information used to elaborate this bibliographic inquiry has been based on research in articles or reviews published in Pubmed and Scopus databases. Materials were filtered by citation number, journal impact factor and year of publishing, considering imperative those reported as much recently as possible to date. Keywords for strategic research were: *“Acute Lymphoblastic Leukaemia” [Mesh] AND (“Chimeric Antigen Receptor” [All Fields] OR “CART therapy” OR “anti-CD19 T cells”)* combining them with or without Boolean operators.

Furthermore, web pages of different regulatory organisations such as the Food and Drug Administration (FDA), the European Medicines Agency (EMA) and particularly, the Spanish Agency of Medicines and Medical Devices (AEMPS) were consulted to widen the information about legal and cost-production concerns. Additionally, the National Cancer Institute (NCI), as well as the European Society for Medical Oncology (ESMO) were examined for CAR T cells consensus opinions. Some specific doubts of meaning were resolved and sought in the dictionary of medical terms to obtain a more accurate definition.

6. Results

6.1 Chimeric Antigen Receptor T-cell Therapy

6.1.1 Background: How did they come up?

Considering the high occurrence of acute lymphoblastic leukaemia new cases in children and its severity in adults, numbers and percentages reveal the necessity of new therapies that improve the response to treatments and the overall survival; especially in those patients undergoing refractory and relapsed B-cell ALL, where standard regimens has already been tried and failed (2).

The introduction of cancer immunotherapy marked a milestone in patients' response against tumour cells. Thereby, biological drugs such as antibodies, cytokines or cellular therapies included in this young field are now possible stratagems to fight cancer. Adoptive cell transfer therapies (ACT), considering autologous tumour infiltrating lymphocytes in patients with metastatic melanoma or allogeneic donor lymphocyte infusion in patients with relapsed leukaemia were initiated in the 1980s. Additionally, the development of genetic engineering techniques was the complementary breakpoint essential to make possible the launching of Chimeric Antigen Receptor T-cells as a state-of-the-art conceptual therapy originally conceived by Zelig Eshhar and colleagues in 1989 (7). All in all, immune-oncology has reshaped the field of ALL treatment and given new perspectives herein exposed.

Chimeric Antigen Receptor T-cell Therapy (CART) involve the expression in patients' T lymphocytes membrane of a genetically engineered receptor capable of redirecting and enhancing its effectivity by recognising tumour antigens in a major histocompatibility complex (MHC)-independent manner, oppositely to the physiological T-cell receptor (TCR). CARs combine the capacity of antigen recognition of antibodies and the antitumor activity of T-cells. The basic structure of the genetically modified receptor thus expresses extracellularly an antigen-binding domain of immunoglobulin nature linked by a transmembrane domain to the intracellular T-cell receptor signalling moieties that activate the T cell (8).

6.1.2 Mechanism of Action: How do they act?

Physiological T lymphocytes are a potent antigen-specific cellular effector of adaptive immunity that eliminates viral and tumour cells. However, in most cancers, tumour cells create an immunosuppressive microenvironment in their site that allows them escaping from the host's immunity by avoiding major histocompatibility complex or downregulating the HLA expression. In contrast, redirecting the manufactured cells expressing a transgenic chimeric antigen receptor that recognise the tumour cells without antigen presentation, it is possible to overcome tumour escape (8). The interaction between the effector cell and tumour cell is different through the T-cell receptor and CAR receptor, as *Figure 2* illustrates.

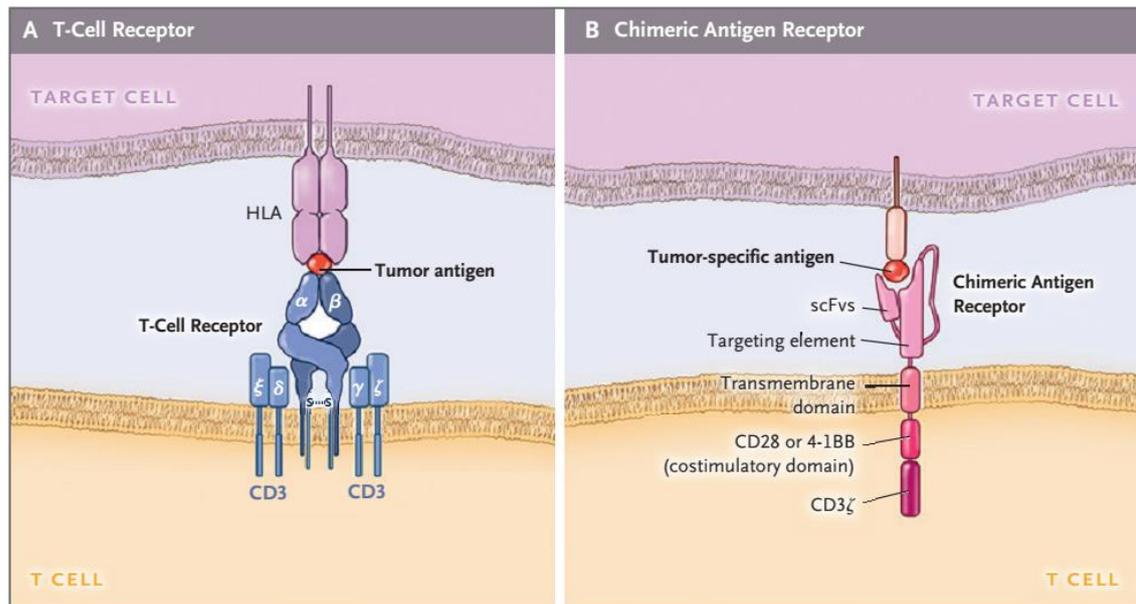


Figure 2. Structure comparison: TCR (A) vs CAR (B) interaction with the target cell. TCR requires HLA presentation, while CAR needs just the antigen to be expressed on the surface to recognise it (7).

Once CART infusion, cells travel to the cancer site and recognise its antigen, triggering conformational changes in the receptor that allows the cell activation and a potent cytotoxic response, which finally destroy the tumour cell. The killed cell exposes a significant number of antigens that can be processed by antigen-presenting cells (APC), such as dendritic cells. APC activate more effectors from both, innate (natural killers and myeloid cells) and adaptive (T- or B- lymphocytes) immune responses by cross-priming. Besides that, the mentioned array of following actions is crucial for CART effectiveness: activation, expansion, recruitment and foremost, persistence (7).

6.1.3 CAR Construct and Structure: How do they look like?

Since CAR-engineered cells launching, different generations have been developed to enhance efficacy, specificity and persistence of the T-cells. Nevertheless, the extracellular domain of murine origin maintains as a single-chain variable fragment (scFv) throughout all different generations. The endodomain that handles cellular response has been modified across generations to achieve the desired properties (9).

The initial proposed structure, termed first-generation CARs, was composed by the extracellular scFv of monoclonal antibodies linked by a spacer to a transmembrane domain connected with CD3 ζ , the intracellular signalling moiety derived from endogenous T-cell receptors. Although efficacy showed in preclinical trials, lack of enough proliferation during the clinical application and thus, no significant antitumor effects *in vivo* led to the evolution of new molecules. Second-generation, also referred to as dual-signalling CARs, incorporates a costimulatory domain linked to the CD3 ζ for sustained and enhanced T-cell responses. It is still to determine whether CD28 or 4-1BB (CD137) is preferable. They seem to contribute adding different properties to the T-cell. CD28 appears to improve the

cytotoxic potential rapidly but promotes cells withdrawn from the blood. Conversely to observed with 4-1BB, which has slower kinetics but ameliorates the persistence (10). Third generation or multiple-signalling CARs include the two costimulatory domains within their gene construct. Any study regarding the comparison between generations has been conducted; all information is based on preclinical data. Further aspects of this review focus on second-generation CARs, since it is the one that gained FDA approval and the used in available clinical data that set the basis for therapy (9). The structural differences between the three generations are depicted in *Figure 3*.

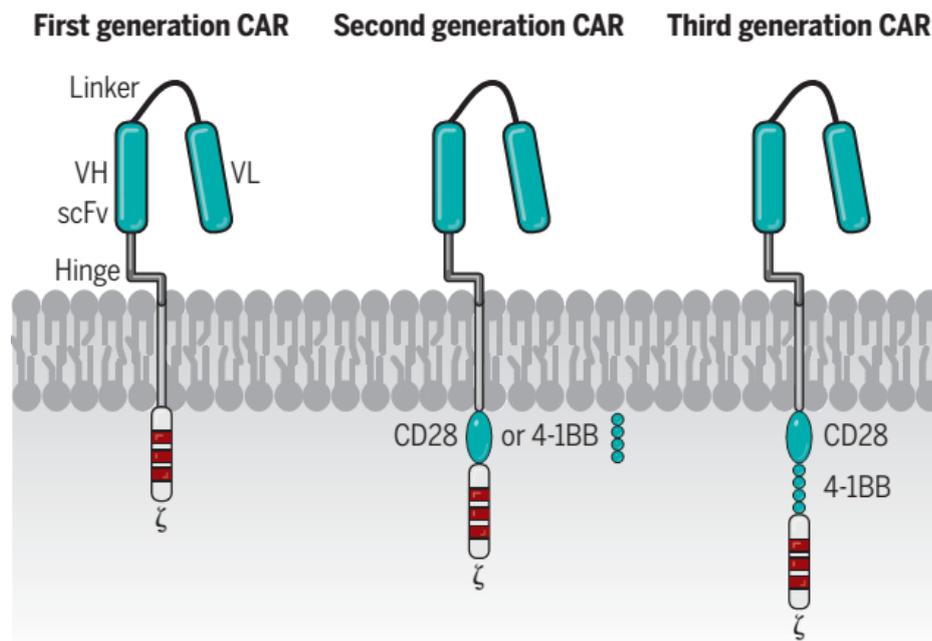


Figure 3. First three generations of chimeric antigen receptors showing their structural differences in composition.

VH, variable heavy; VL, variable light chain; scFV, Single-chain fragment variable (9).

Although considered as persistent living drugs as a positive feature because of their tumour control capability, CART unrestrained activation could lead to severe side effects and toxicity. Therefore, investigators seek the balance between active profile and safe performance by finding suitable CAR construction.

Fourth-generation CAR T-cells redirected for universal cytokine killing (TRUCKs) are now under development. A cytokine expression cassette is inserted in the vector encoding for CAR construct allowing the deposition of pro-inflammatory cytokines in the targeted tumour site, enhancing the attraction of immune cells and enabling its response toward those cancer cells inaccessible before. TRUCKs focus more on solid tumours because their locus usually is more restricted than in haematological malignancies as we discuss herein (11). More information about TRUCKs and its future implication is exposed in *6.6 Challenges and Perspectives* section.

6.1.4 CD19 Antigen: the nearly ideal target

Chimeric antigen receptors can recognise a large number of tumour associated antigens (TAA) from virtually all characters, not only from protein nature but also carbohydrates or gangliosides. The extracellular portion of the receptor that mimics antibodies' properties is the part that achieves antigen recognition. Choosing the targeted antigen against which T cells would be directed to apply the therapy in ALL patients was one of the most challenging points during the development of the therapy (12). Ideally, a targeted antigen would contain the following requirements:

- Tumour specificity, assuring a directional therapy and preventing side effects.
- Ubiquitously and broad expression, allowing the development of a useful approach for all the cases of the considered disease.
- Not expressed on healthy cells, decreasing toxicity.
- Membrane molecule, preventing steric problematic entering the cell.

Unfortunately, such a perfect antigen is hard to find, and almost all the feasible candidates are also self-expressed on normal tissues. In those cases, an attractive alternative antigen could be the one whose expression belongs to a particular cell lineage, with a replaceable function or with some feature that distinguishes it from normal cells. Of note, whether the target molecule is the same in cancerous and only a lineage of healthy cells, but it involves possible off-tumour side effects in vital organs, is directly precluded. Although it could seem trivial, a proper election is of utmost importance for a fruitful yield. Precisely for that reason, CAR therapy applied to solid tumours delay compared to haematological cancer. Even when managed to choose a good candidate, whether just minimally expression in normal tissues, it still carries inherent off-tumour side effects in healthy cells as the main drawback. As favourable, off-tumour toxicities only occur when active therapy. Possible off-tumour side effects should be considered in advance and be easily controllable (13).

All considered, CD19 became the chosen antigen for B-cell malignancies. Likewise, it is the most exhaustive studied one until today. CD19 expression is broad during all phases of B-cell development and thus, can be found in higher concentration relative to other potential targets of B-malignancies, such as CD20 or CD22. Furthermore, foreseeable side effects considering healthy B-cell depletion results in manageable toxicity and enable the engineered receptors to bypass antibody response against the murine components of the receptor (14). By contrast, the main hindrance is that CD19-targeted T-cell is only effective against B-ALL, which represents only one of the two possible phenotypes of this leukaemia. In the case of T-cell ALL blasts, none potential antigen has been found yet, since the leukemic blasts share all the antigens with normal T-cells and T-cell aplasia is not as manageable as B-cell (13).

6.1.5 T-cell Engineering and Manufacturing: A scale up production

Once a patient undergoes CAR T cell therapy, it starts all the manufacturing process to obtain autologous CAR-expressing T-cells. When initially therapy set in motion, the manufacturing of CART cells occurred in almost all cases in the same institution that later on treated the patient. As therapy has evolved and the number of clinical centres offering it has increased, the necessity of scaling up manufacturing processes has dramatically increased. The primary goal for an effective procedure is to ensure traceability and minimise all the possible variability that was previously in reduced scale impossible to fulfil. The manufacturing method, which can last from 10-days to 3-weeks, is following enumerated (15):

- 1. Leukapheresis and T cell isolation:** removal of patient's immune cells from blood to harvest the leukocytes while the remaining components return into the circulation. Counterflow centrifugal elutriation separates blood cells by cell size and density. Generally, no separation depending on T cell subsets occurs, and the cell population following the whole process is in the ratio present in the patient's peripheral blood as is explained according to this manufacturing model based on tisagenlecleucel preparation, the FDA approved CART (15). Nevertheless, lately, some defined ratios such as 1:1 CD4+: CD8+ postulate to result in better potential. Selection of subsets lies in the selection of specific antibody-bead conjugates that can achieve such ratios. Heterogeneous composition of T lymphocytes population can manifest some differences in pharmacodynamic profiles (16). *Figure 4* shows the leukapheresis and isolation phases.

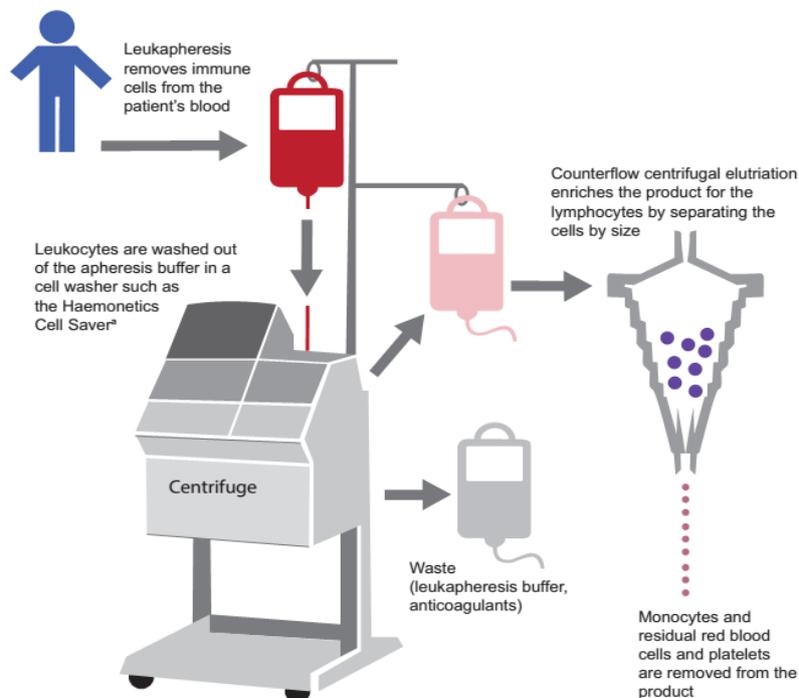


Figure 4. Leukapheresis from the patient, leukocytes wash out and counterflow centrifugal elutriation enriching the product from lymphocytes (15).

2. **T cells activation:** expansion and activation *ex vivo* are crucial for effective product achievement. There are different approaches such as beads coated with anti-CD3/anti-CD28 monoclonal antibodies or the used in tisagenlecleucel manufacturing, which is artificial antigen-presenting-cells (15).
3. **Transduction:** T cells incubated with the vector encoding the receptor in a cell culture medium. The CAR construction can be introduced into the primary T-cell by different approaches. Mainly, two strategies can be distinguished: non-viral based RNA-methods and viral-mediated transduction. In the first alternative, the CAR construct integrates into the cell genome, leading to transient gene expression. It appears to be translated into less on-target off-tumour side effects of the therapy since it is a short-term expression and healthy cells are less attacked. Additionally, they present no risk of mutation insertion. Overall, it is considered a safer option; but, it is a temporary approach that needs several repeated infusions to control the disease. On the other side, viral transduction lasts longer due to the integration within the genome, but because of the integration, it carries a risk of mutagenesis near an oncogene and consequent development of another malignancy. Lentiviral vectors are preferred among other viral strategies such as gammaretroviruses because frequently they integrate away from cell promoters preventing any dysregulation. All those differences have clinical consequences such as times of infusion (8). *Table 2* gathers the abovementioned data.

	Viral-methods		RNA-based methods
	Lentivirus	Gammaretrovirus	
Gene expression	Permanent		Transient
Long-term disease control	Single infusion		Repeated infusion
Risk of mutagenesis	Low: integration away from cellular promoters	High: near transcriptional starting sites	None

Table 2. Comparison between viral and RNA-based methods encoding CAR expression on T-cells.

Of note, in clinical trials and tisagenlecleucel (i.e., FDA approved CART) only uses viral vectors. It consists of RNA introduction, reverse-transcription to DNA permanently integration into the cell genome, CAR expression in the surface of the cell with its machinery and the maintenance among divisions. After several days of incubation, the vector is washed out, and the medium exchanged. The vector selection is an essential point because optimising the T cell transduction is vital before the continuation with a large production to reduce variability and increase efficiency. Sometimes even using the same method but varying the supplier might lead to different yields (15).

4. **T cell expansion** in bioreactors providing required optimal conditions such as gas exchange and culture mixing for enough expansion. The medium includes some cytokines to favour the growth, such as IL-2. After the period of exponential growth lasting 9-11 days, the final volume of expanded CAR T cells arrives at 5L approximately. Then cells are washed, concentrated until a possible volume that could be infused, cryopreserved and shipped back to the clinical centre where the patient is. Quality controls performance occurs during the whole manufacturing protocol. qPCR is used to measure the construct integration although correlation with efficacy needs flow cytometry measuring (15). *Figure 5* illustrates the last steps of the process.

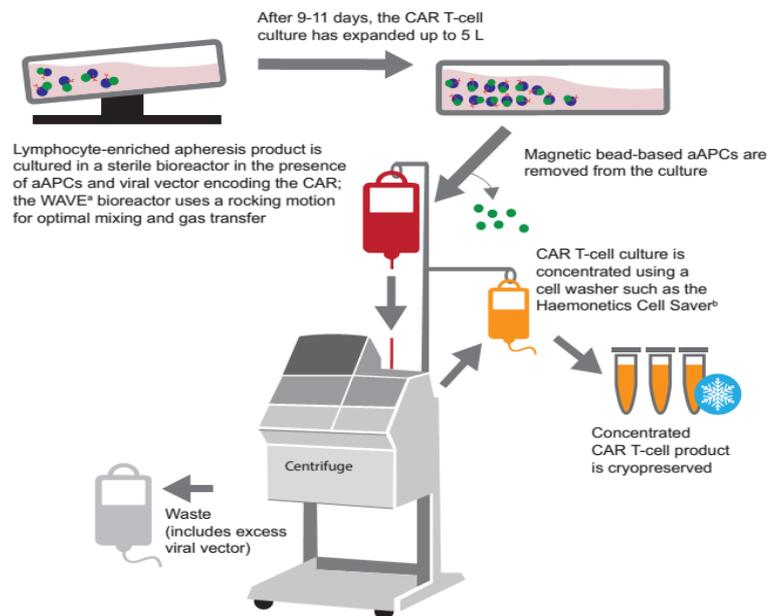


Figure 5. T-cell expansion and final product preparation (15).

Meeting the first advent of CARTs was simple because the same academic centre designing the chimeric antigen receptor to express in T cells could lately clinically assess it in patients assisted by qualified physicians in the same facilities, where interaction between developers and practitioners was practically instantaneous. With the consolidated growth of the therapy and its expansion through geographical barriers, the commercial manufacture supposes an obstacle to overcome and continue offering the same efficiency in treatment. Currently, the majority of hospitals offering the therapy and the manufacturing centre are physically away from each other. Additionally, the lack of guidance documents harmonised between the leading producing countries difficult the procedure as well. The establishment of a typical basement reuniting all the different authorities is of significant concern for assured traceability and product quality. On October 11, 2012, nine members of the global regulatory community, including FDA and EMA, were reunited to discuss gene and cell-based therapies regulation (15). *Figure 6* shows a scheme comparing academic and commercial differences.

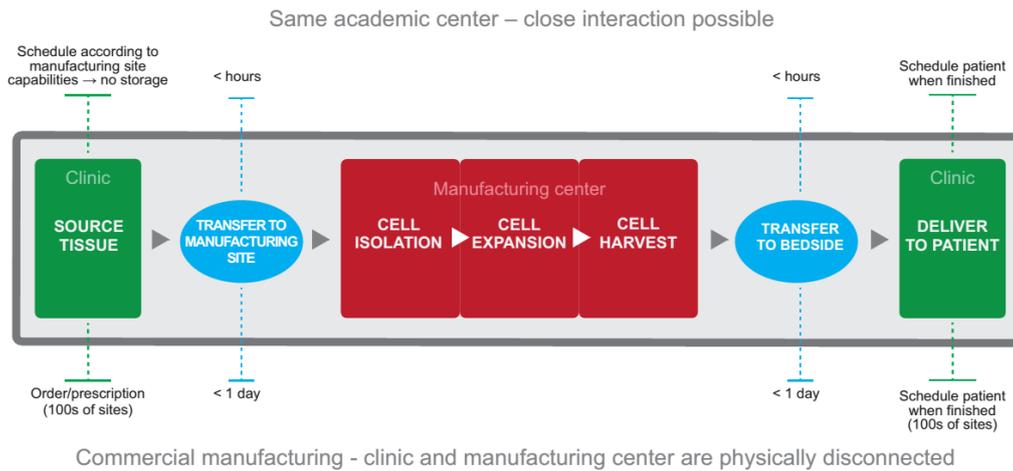


Figure 6. Transition from academic centres to commercial manufacturing (15).

Lymphodepletion regimen administration consisting of given cyclophosphamide alone or in combination with fludarabine before the start of the treatment and while the manufacturing process occurs is beneficial. Such an approach decreases the competition for stimulatory cytokines and diminishes the regulatory T cells, which can prevent CAR T-cell from complete proliferation and activation. Furthermore, it can decrease the graft-versus-host disease (GVHD) prompted by the immunogenicity of the murine CAR components; understanding it as the attack of the host's immunity to an unknown introduced graft that the body interprets as something strange (11). Manufacturing time is vital for the patient, as well as for leukaemia control; both required for satisfactory outcomes. Occasionally, bridging chemotherapy allows a decrease in the disease burden, which lately prevents severe toxicities from appearing (17).

Novartis announced the cost of therapy arriving at 475.000\$ for every infusion. Contextualising the price, allo-SCT costs about 200.000\$, less than a half. Of note, apart from the cost of the therapy itself, there is the price of hospitalisation the patients while monitoring, treatment of adverse effects and even intensive care unit admission. Furthermore, it must fight to additional barriers such as geographic obstacle; in the US, less than 40 countries are authorised to administer the therapy, and, for instance, Novartis has only 2 manufacturing plants, in New Jersey and Germany (16).

The forthcoming product referred to as "off-the-shelf" CAR T-cells, consists of the expression of the chimeric antigen receptor in an allogenic donor cell instead of in an autologous. It avoids the bespoke manufacturing and leukapheresis necessity from each treated patient. It can minimise costs and time and along these lines simplify manufacturing, make it more accessible and efficient. However, as in the case of allo-SCT, it might display some limitations such as GVHD (9).

6.3 CAR-T Cells Toxicity

Therapy using the infusion of CAR T cells for the treatment of relapsed and refractory disease has toxicity associated, so do other cancer therapies such as chemotherapy, transplantation or radiation. The advantage of CART therapy above the others mentioned is that the toxicity is reversible after therapy is over in most of the cases. Besides, it is usually on-target toxicity; meaning that the treatment is efficacious to the patient and it is not attacking healthy structures without acting against tumour cells. In contrast, its main disadvantage is the unpredictability associated, since antigen distinction between cancerous and healthy cells is essential for safety (7).

6.3.1 B-Cell Aplasia

B-cell aplasia was an expected side effect since therapy first designed, resulting from the presence of CD19 in B-cell lineage of healthy cells in the organism. Moreover, it had been observed before with rituximab, a monoclonal antibody against the same targeted molecule, CD19. However, it has resulted in significant severity in the case of CART cells; perhaps due to the more effective against leukaemia cells, the more toxic is at an endogenous level. B-cell aplasia not only consists of an on-target effect because it acts against the desired antigen but also is an off-tumour effect since it affects the normal cells. Even though it disappears after CAR T cells removal, it can be successfully managed by intravenously immunoglobulin supplementation (IVIg) while the adverse effect occurs. Each patient needs an individualised therapy according to serum antibodies. Some serious infectious problems could derive, but they usually resolve successfully. Interestingly, B-cell aplasia postulates as a pharmacodynamic marker for CAR T-cells persistence; owing to an association between B-cell cleavage and sustained remission (12).

6.3.2 Cytokine Release Syndrome

Differing from B cell aplasia, cytokine release syndrome is an on-target but on-tumour adverse effect characterised by an elevated level of IL-6, TNF- α , IL-2 and IFN- γ cytokines in serum caused by a systemic inflammatory response, owing to, mostly, the lysis of the tumour cells. CRS appears typically within the first two weeks after onset of the treatment and rarely happens when the therapy is ineffective, meaning there is a correlation between its development and response to therapy. It typically presents a combination of symptoms including fever, hypotension and hypoxia; sometimes referred to as a mild flu-like process. Although it could seem trivial, it can develop in a life-threatening situation, including capillary leak and multi-organ dysfunction, which can progress to fatal outcomes such as distributive shock and organ failure respectively. The severity of these potential events reveals the necessity for predictable signs before CART infusion that can give physicians some idea. The severity in CRS is associated with a notable tumour burden, meaning that if the patient's load of tumour cells is lofty, the possibility of developing the syndrome is higher. In some cases, the use of pre-infusion chemotherapy could help to deal with the tumour burden and decreases not only the risk but also the severity of CRS (8).

CRS treatment is tedious because the understanding of the role of cytokines is crucial to determine which one is a useful target to block without hampering the efficacy of the CAR treatment, in which cytotoxic mechanism against tumour cell is imperative. For instance, IFN- γ has an essential role in macrophage activation, MHC induction and T-helper cells differentiation; thereby it cannot be regardless suppressed to ameliorate CRS symptomatology because it implies therapy ineffectiveness as well (13). Thus far, CRS management lies in an available monoclonal antibody: tocilizumab. It consists of an anti-IL-6 receptor against both, soluble and membrane-bound forms of the IL-6 receptor, which has also recently been approved by the FDA for this purpose. Its approval came right after tisagenlecleucel was accepted too for r/r-ALL treatment. Because CRS is an almost natural side effect to the therapy, tisagenlecleucel could not have satisfactory approval without a valid solution for its problematic. Apart from tocilizumab, steroids are second-line drugs when patients refractory to the antibody. Steroids could impair T cell function and induce its apoptosis, thus preventing its use for the management of CRS because of its possible negative contribution to the efficacy of the treatment (20).

Due to the possibility of this fatal outcome, there is a remarkable necessity of not only the existence of some biomarkers that could predict it happening but also the consensus on a grading scale. That tool could facilitate the creation of a clinical treatment algorithm and the comparison of the reactions observed across different institutions and trials. Regardless of the publication of different guidelines for treatment, they are not in consensus between different institutions in charge. Additional topics to be discussed in the future affecting CRS are (21):

- How and for how long monitoring patients so they can rapidly go to the intensive care unit whether hypotension or respiratory insufficiency prompting
- Tocilizumab administration prior CART infusion to prevent CRS without compromising the efficacy
- Correlation between hallmark proteins and severe CRS quickly checked: C-reactive protein and ferritin
- CRS grading and agreed guidelines

6.3.3 Neurotoxicity

Neurotoxicity is identified as a possible side effect as well. It develops as confusion, disorientation, visual hallucinations, encephalopathy and sometimes seizures. It is usually reversible and resolves without apparent sequelae; however, fatal outcomes in a few patients evolving cerebral oedema have appeared. Exactly underlying pathophysiology is still unknown, but it could have some connection with severe CRS since it sometimes occurs following its onset and produces after a high peak of cytokines. The finding of T cells and cytokines in CSF are thought to be causal facts, but the reason why only some patients suffer from it is unexplained. Even though unclarity remains and it is a considerable hurdle associated with therapy because it is not a standard but lethal side effect (12).

Current management is driven to damp the inflammatory response with tocilizumab when CRS occurs. In some cases when CRS has not preceded neurotoxicity, steroids have been given. Besides, risk patients with previously epileptic attacks can have levetiracetam as a prophylactic drug. Analogously to CRS, further studies to unmask pathophysiology accompanied by predictors will ease treatment (22).

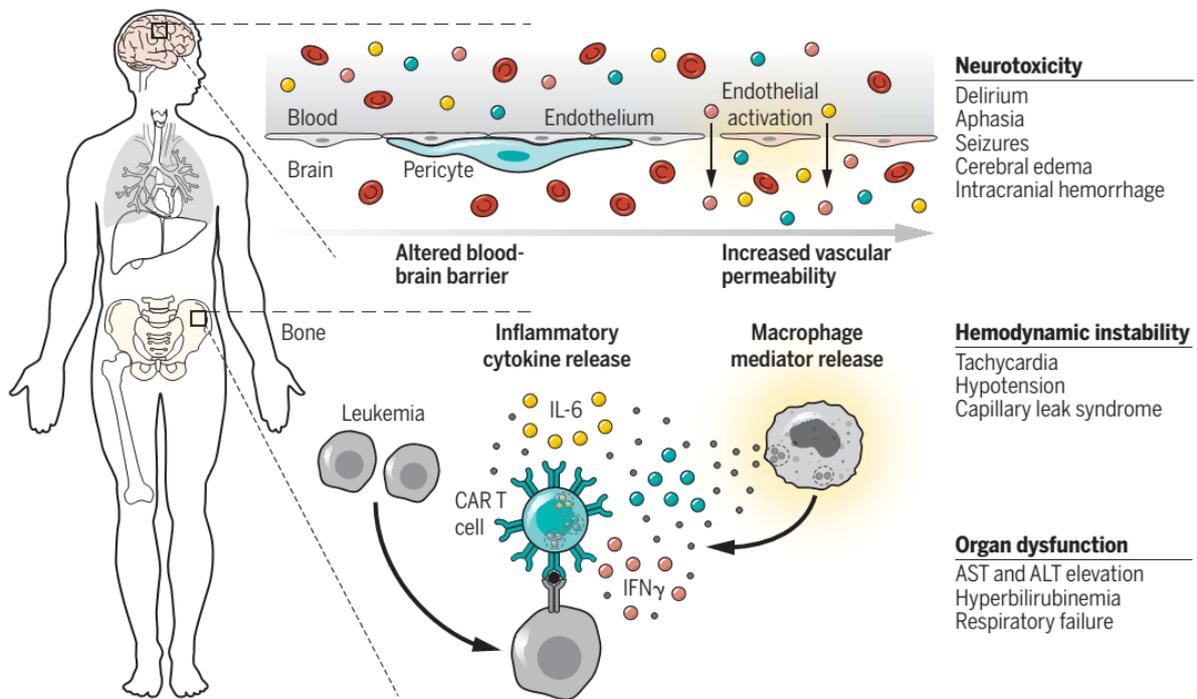


Figure 8. Implicated agents and triggered problematic for neurotoxicity and CRS. AST, aspartate aminotransferase; ALT, alanine aminotransferase (9).

Thus, toxicity management is still a challenging point before the safe implementation of CAR T cells therapy. Furthermore, the cost associated with supportive care to deal with the adverse effects suffered while CAR T cell treatment is still to be estimated and predictive markers to be assessed. The truth is that the more used the therapy is, the more data is known about effectivity, pharmacodynamics and toxicity as well. All this gathered information would make more accessible the improvements in outcomes and safety of the therapy for future patients. *Figure 8* gives a general vision of locus and general factors associated with CRS and neurotoxicity.

6.4 Clinical Trials and remarks on activity and toxicity: from lab to clinic

Clinical trials are essential for promising therapies to evaluate their transition from the laboratory to the clinics. Pre-clinical findings with experimental models are not enough to set all the basis of therapy and require clinical testing in patients suffering from the disease under evaluation. All studies herein considered uses anti-CD19 expressing CARs. The first three studies (23, 24, 25) confirmed previous outcomes on efficacy and possible toxicities. Those served as a first knowledge that afterwards allowed the anti-CD19 T cells to be considered for FDA approval. The pivotal study named ELIANA (26) is a phase II clinical trial that assesses tisagenlecleucel. The last study considered (27), focuses on long-term follow up results for 19-28CARs, a different construct from tisagenlecleucel but against the same antigen and with the same application. All patients included in clinical trials had r/r disease and carried an extremely dismal prognosis due to previous therapies failure. The following sections summarise significant findings and emphasise possible transcendent breakpoints. A comparative table (*Table 3*) compile all data, although indirect comparison as performed lacks significance, due to the assumption of all studies occurring in a homogeneous manner, which was not. Direct comparison is only possible within the same clinical study, in which different arms considered. Each arm is a group of patients with the same features treated with different approaches, including the one tested and the first-line option.

Maude et al. study (23) assessed the persistence and durability of remission. Furthermore, they suggested the correlation between the baseline disease burden before CART infusion and the severity of the CRS developed. Additionally, they observed full recovery of symptoms in all patients with tocilizumab or glucocorticoids. No apparent long-term sequelae were reported. B-cell aplasia occurred in all patients who had a response and persisted for up to 1 year after bioengineered cells were no longer detectable. Investigators postulated association between sustained remission and B-cell aplasia, posing it as a possible pharmacodynamic parameter.

Davila et al. (24) evaluated the potency of CART therapy as a bridge stage for patients to achieve CR and then be eligible for transplantation. Currently, allo-SCT continues being the only therapeutic approaches with curative potential. Patients successfully undergoing transplantation increased from 5% in those treated with salvage chemotherapy to 44% when receiving CART therapy. Besides, no toxicities associated with CARTs prevented patients from transplantation; contrarily to chemotherapy.

Lee et al. (25) suggested tocilizumab as the first-line drug to manage CRS due to ablation of bioengineered T cells when corticosteroids used instead. Furthermore, this study provided the first evidence of CNS leukaemia elimination, which remains a problematic parameter due to inaccessibility and long-term CNS toxicities associated with chemotherapy. Additionally, as explained in 6.5.2 *CD19⁺ relapse*, described the loss of CD19 expression as one of the Achilles heel of the therapy.

Maude, Laetsch et al. (26) determined pharmacokinetic parameters such as maximum time of response and the median duration of cells persistence in blood. They observed that cell expansion and clinical response was indifferent of infused dose. The high number of patients (47%) needing for intensive care unit (ICU), mainly treated with vasopressors, oxygen supplementation and mechanical ventilation, was a derived fact to consider when CART treatment. Additionally, they associated neurologic events presentation with high-grade CRS. However, the significant contribution of this study was the corroboration of effective distribution of the newly approved drug (tisagenlecleucel) across four continents, owing to a global supply company (Novartis). Results across the multi-centres were homogenous in efficacy and safety and similar to the ones observed when single institution study. All the previous manufactured CART cells were on academic centres with limited manufacturing sites, which made reproducibility undetermined.

Median follow-up of 29 months allowed **Park et al.** (27) to distinguish between two main event predictors. A high peak of CART cells predicts short-term response and rapid CR achievement. However, substantial long-term outcomes marker is the disease burden before CARs infusion; resulting in the most useful for overall survival. Furthermore, they established that CR rates do not differ in patients with different status at the time of enrolments, such as the number of previous therapies, conditioning chemotherapy or previous transplantation. This last announcement is significantly favourable, since any patient suffers rejection at first, as there is no limiting factor to receive therapy but the necessity of T cell expansion.

Currently, there are about 250 clinical trials assessing CARTs all over the world. Clinical studies regarding CARs mainly located in China and the United States of America, followed by far from Europe (*Figure 9 left*). This is not the common scenario observed in the rest of clinical trials worldwide (*Figure 9 right*), which suggests that the difference can respond to less restrictive legislation concerning gene therapies (9).

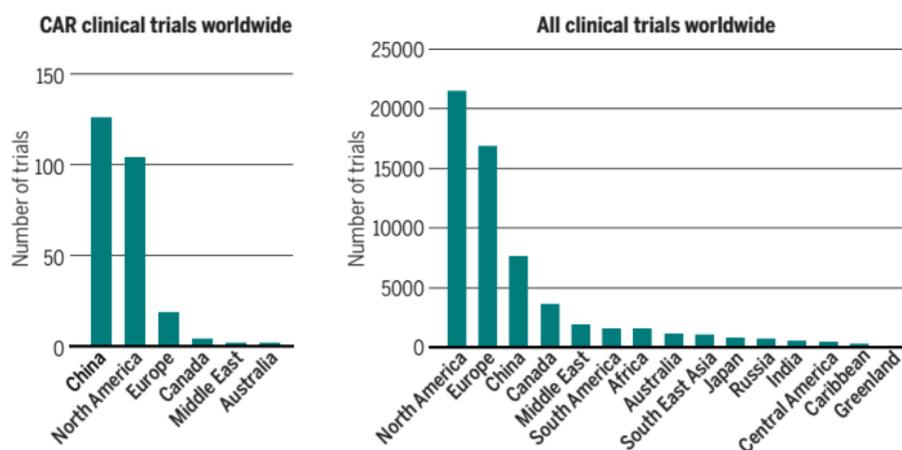


Figure 9. The disparity between geographic localization of CAR trials and all trials (9).

	Maude et al. (23)	Davila et al. (24)	Lee et al. (25)	Maude, Laetsch et al. (26)	Park et al. (27)
Time	April 2012 – February 2014	N/A but prior 2014	July 2012 – June 2014	April 2015 – April 2017	February 2010 – June 2016
Institute	Children’s Philadelphia Hospital of the University of Pennsylvania	Memorial Sloan-Kettering Cancer Center (MSKCC)	Pediatric Oncology Branch of the National Cancer Institute (NCI)	Multi-centre	Memorial Sloan-Kettering Cancer Center MSKCC
Trial name	NCT01626495	NCT01044069	NCT01593696	NCT02435849	NCT01044069
Number of patients	n= 30	n= 16	n= 21	n=75	n= 53
Adults	5: 26 to 60	16	-	-	-
Children	25: 5 to 22	-	21: 1 to 30	75: 3 to 23	-
Phase	I/IIA	I	I	II	I
Vector	Lentiviral	retroviral	Retroviral	Lentiviral	retroviral
CAR T cell	2 nd generation	2 nd generation	2 nd generation	2 nd generation	2 nd generation
T-cell activation signal	CD3-ζ	CD3-ζ	CD3-ζ	CD3-ζ	CD3-ζ
Costimulatory domain	4-1BB (CD137)	CD28	CD28	4-1BB (CD137)	CD28
Dose (CTL019 cells per kilogram of body weight)	0.76x10 ⁶ to 20.6x10 ⁶	3x10 ⁶	1x10 ⁶	3,1x10 ⁶	N/A
Complete remission at first assessment	90%	88%	68%	82%	83%
6-months event-free survival rate (MRD ⁻)	67%	75%	60%	73%	67%
Overall survival	78%	78%	52%	90%	N/A
Persistence of CTL019 at 6 months/median time	68%	Limited to 3 months.	Undetected after day 68	Median duration of persistence 168 days	74 days
Patients undergoing HSCT	50%	44%	48%	10%	39%
Relapse	23%	N/A	24%	23%	50%
CD19+ relapsed	13%	N/A	14%	3%	43%
CD19- relapse	10%	N/A	10%	20%	7%
Cytokine Release Syndrome (any grade)	100%	100	76%	77%	85%
Severe CRS	27%	44%	19%	N/A	26% and 1 death
B-cell aplasia at 6 months	73%	N/A	N/A	83%	N/A
Neurotoxicity	63%	N/A	20%	40%	44%

Table 3. Indirect comparison between structural, efficacy and toxicity parameters regarding the five studied clinical trials. N/A: Not Available

6.5 Relapses after CD19 CAR treatment

Approximately 35% of patients who achieve CR eventually relapse. Patient monitoring allows differentiating two main types of ALL relapse after CAR T-cell therapy. Flow cytometry assessment of CD19 surface expression on B-ALL tumour cells at the time of relapse can distinguish between CD19⁺ and CD19⁻ relapse. CD19⁺ relapse is due to the lack of persistence of the engineered T cells and its cleavage from the patient's body. In contrast, CD19⁻ relapse occurs because of mutations in the targeted antigen from tumour cells surfaces, as a way of avoiding CAR recognition despite CAR T cell persistence (28).

6.5.1 CD19⁺ relapse

CAR T cells survival is crucial for protection against the recurrence of the disease. To enhance this survival, conditioning lymphodepletion utilisation promotes proliferation of the infused cells, since balances homeostatic mechanisms toward effector T cells. The probability of relapse increases whether infused cells cleavage from patients' blood before long-term disease control is conducted. Further infusions or optimal designer techniques may prevent this from happening. Addition of co-stimulatory domains in 2nd and 3rd-generations paved the way for enhanced stimulation and proliferation, preventing its exhaustion. In this scenario, an optimised CAR design may also help in decreasing relapse numbers (29).

6.5.2 CD19⁻ relapse

Resistant tumour cells can bear genetic mutations for the CD19 protein, which results in modified proteins (Figure 10). The lack of surface antigen recognition by the chimeric antigen receptor leads to the ineffectiveness of the engineered T cells, and thus CD19⁻ relapse. Many of the mutations identified in screened patients were throughout exons 2-5 and consisted of frameshift insertion, deletion or missense single nucleotide variants. Besides, different B-cell genes such as CD22, CD20, CD10, CD38 or CD45 were assessed for mutations to ensure the relapse was attributed explicitly to CD19 mutations. Selective pressure may occur to originate the loss-of-function mutations, which were not present before the relapse. A feasible alternative strategy involves the combination with an anti-CD22 CAR T cell, ensuring efficacy even though CD19 alterations produced by tumour cells (28,30).

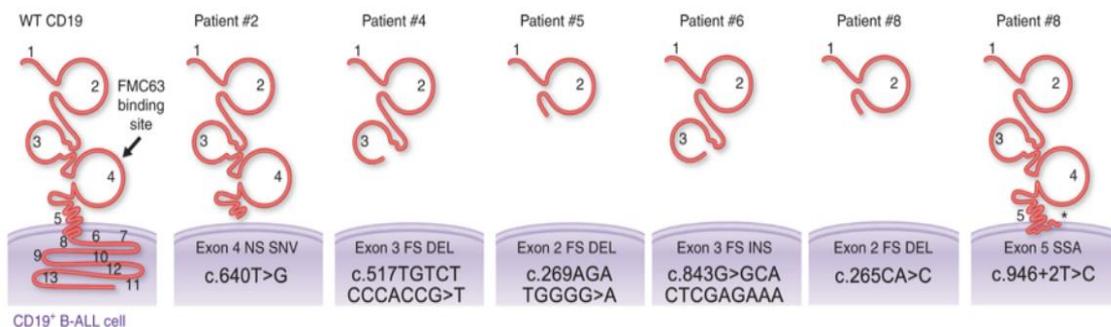


Figure 10. Wild type CD19 protein structure when CD19⁺ B-ALL cell and mutated CD19 found in relapsed patients (28).

6.6 Challenges and Perspectives: novel approaches solving limitations

Thus far, engineered T cells have proved a considerable potential for the treatment of r/r ALL. Conversely, solid tumours have not reported this clinical success with even some deaths related to its use. Furthermore, toxicity is still a principal drawback to overcome for the positioning of the treatment. Herein, there is a discussion of the main hurdles and proposed strategies.

6.6.1 Tumour vs. healthy cells recognition: redirecting CAR T cells

Antigen discrimination is indispensable not only for the efficacy of the therapy but also for safety. These two essential points strengthen binding more than one tumour antigen in the same immunologic synapse for complete activation of the CAR T cell. By this means, off-tumour recognition is nearly impossible. Two approaches referred to as dual CARs and split CARs are herein both explained and depicted in *Figure 11* (31).

Dual CARs: the combination of two identical receptors differing only in the targeted antigen they bind. Complete activation only occurs when both bind their antigen. These CARs go beyond specificity, assuring elimination of the cells that exclusively express both antigens and, thus, reduce off-tumour toxicity.

Split CARs: one of the two receptors contains the co-stimulatory domain (CD28 and 4-1BB), and the other expresses the TCR-CD3 ζ domain. Therefore, activation only happens when there is an engagement of both. They can target the same antigen or a different one.

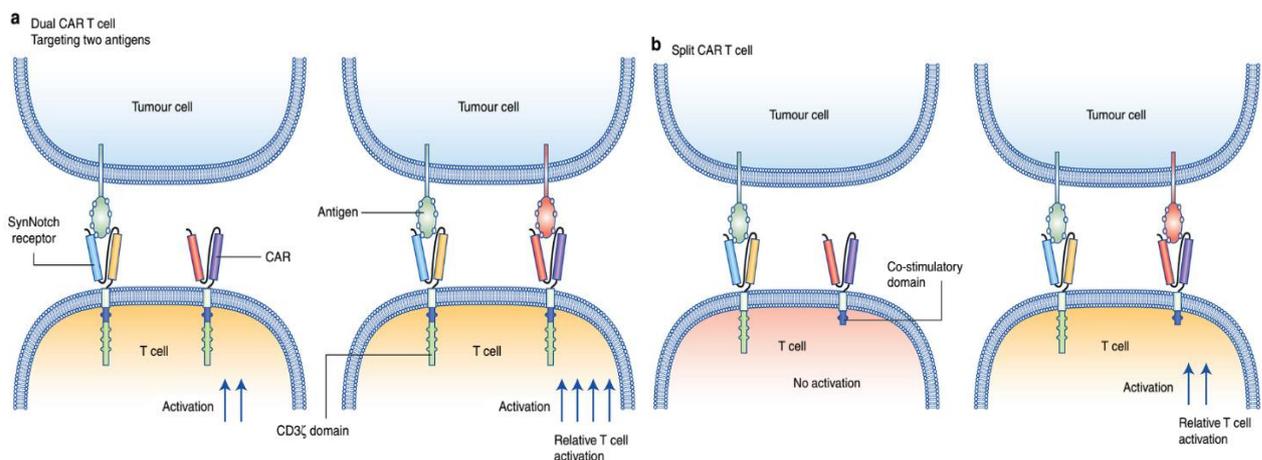


Figure 11. Dual CAR T cells and Split CAR T cell mechanism of action when one vs. both antigens are engaged (31).

6.6.2 Expansion and Survival: fighting tumour immunosuppressive microenvironment

The proliferation of the manufactured T cells is essential for arriving in enough concentration to the targeted cell. T cells require three signals allowing to adequately proliferation: CD3 ζ and co-stimulatory domain but also cytokine signalling, which is not considered in the first three CAR generations (31).

4th generation or TRUCKS: empower the construction of an immunopmissive microenvironment by expressing inflammatory cytokines such as IL-12, IL-18 or IL-15. Cytokines effects synergise CARs' mechanism of action by attracting innate and adoptive immune cells.

6.6.3 Regulation: a switch-off mode for uncontrolled T cells

Once T cells infusion to the patient, they start acting as biologically active compounds and whether production of adverse effects, the elimination of the T cells represents null effectivity. Along these lines, it appears the necessity of CARs with a "switch off" option against healthy cells but preventing its total inactivation (31).

iCARs: apart from an expressing a regular CAR, includes a second receptor linked to an inhibitor domain such as PD-1 or CTLA-4 that recognises an antigen present in those healthy cells that share the same targeted antigen with tumour cells and results in its inactivation.

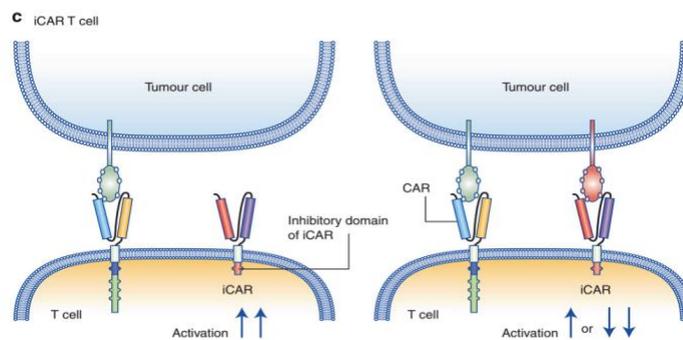


Figure 12. iCAR construction (31).

6.7 Current Status in National Health System

After EMA approval of tisagenlecleucel for paediatric r/r B-ALL indication, the Spanish regulatory agency has also suggested a positioning report to evaluate its viability in our health system and its therapeutic indications, published on 25th February 2019.

Currently, r/r patients have limited options; mainly reduced to salvage chemotherapy followed by allo-SCT when MRD- disease. Even in those better cases of CR and MRD-, patients' associated comorbidities may preclude them from allo-SCT, which reduces survival expectations dramatically. In this situation and after more than one relapse event, clofarabine and blinatumomab are the only approved therapeutic agents apart from tisagenlecleucel. In Spain, none of them has funding currently. Tisagenlecleucel is only indicated in patients younger than 25 years old. The dose is established according to body weight. It is a colourless suspension ready for intravenous infusion in a single-dose. The main drawback of therapy progression is its cost: 356.000€/patient. Considering Spanish incidence: 641 newly diagnosed patients/year, 15% of them suffering r/r ALL and only 39% achieving CR, the targeted population would be reduced to 58 candidate patients/year. Lack of comparative studies between available therapies assessing cost-effectivity difficult the determination of solid conclusions (32).

7. Discussion

Current strategies with astonishing results for ALL patients are lacking, especially for those with refractory or relapsed disease, where long-term survival is dramatically decreased. After chemotherapy variants already tested, new approaches seem to come from immune-oncology. This option usually involves a better quality of life for survivals and less long-lasting toxic effects. CAR T cells offer a straightforward refreshing way of understanding cancer treatment. Furthermore, it feels that conventional chemotherapy understood as a chemical structure against a metabolite or receptor with a defined dose, protocol to administer and addressed to a determined population is stuck. CARs have rebuilt that entire concept by bringing adoptive cell therapies closer to clinicians and strong data.

Although it has been almost 30 years since CART theoretical conception, clinical studies proving efficacy and toxicity information has been conducted recently, by which we can consider it as a relatively new option with fundamental aspects still to be determined and unanswered questions. A hinder factor in integrating it into cancer management is the need for well-trained clinicians, prepared for all possible unstudied outcomes. They must be able to entirely understand cancer type features, immune cells interactions and the biological systems. As said above, it is not limited to administer a tablet or intravenously infuse a standard preparation. Same as in all new launched approaches, the balance between main advantages and inconvenient must be conducted to evaluate therapy progression. It is undeniable the improvement it has supposed for all those patients suffering from r/r ALL. It offers a remission induction in patients with relapsed, refractory or MRD+ disease and can represent a bridge for allo-SCT in patients that were previously excluded. Handicaps considered are its price and geographical barriers for manufacturing. Furthermore, remaining undetermined aspects needs future assessment to improve the following list of undefined points:

- Optimal structure: co-stimulatory domain, CAR-generation or antigen selection.
- Manufacturing: transductor vector or T cell subtypes selected.
- Lymphodepletion and chemotherapy pre-treatment
- Toxicity management: predictable markers, grading or treatment.
- Addressed population
- Regimen: alone or in combination

A noteworthy fact about this new approach is the latest interest of pharmaceutical companies in this type of genetic- and cell-based therapy not seen before. It is understandable that they do not want to bear the risks of such bespoke manufacturing. CARTs are not in this moment a stockable product but a complex manufacturing patients' bioengineered cell product. They must be individually manufactured for each patient and at the same time in a scale up production allowing the pharmaceutical company efficiency.

Pharmaceutical companies must fight not only with manufacturing but also with regulatory landscape concerning all the different countries. Harmonisation regarding the leading regulatory agencies is needed to globalise and overthrow barriers to widespread. A high number of clinical trials occurring means that more and more hospitals are considering it as an option and want to have experts' evaluation for viability. In our national health system, Spanish Hospital Pharmacy Society (SEFH) has recently published the positioning inform evaluating the costs, viability and main pros and cons.

Currently, the majority of clinical trials are taking place in the USA and China. This fact can be due to the legal gap existing in those countries about ethics and transgene compounds used in human medicine. It could also be because of less restrictive legislation than it is in Europe. Although striking efficacy has been proved through clinical trials for hematologic cancers, and especially in herein discussed ALL, currently exist over 100 types of cancer and haematological ones only comprise a small amount of them. The number of deaths associated with ALL, despite being a dreadful disease, is quite a few in comparison with other solid tumours. Inherent difficulties regarding how to approach solid tumours or encapsulated ones are how to arrive at the zone where the tumour controls all immunosuppressive microenvironment and select the tumour associated antigen. CARs have to redirect T lymphocytes against an antigen that can exclusively be a surface molecule due to the impossibility to enter the cell. It implies the study of the surface molecules expressed in the concrete cancer type postulated. Until the moment, reach this point has been the most challenging event. Sometimes, there is no particular TAA without expression in healthy cells or whose destruction results in manageable off-tumour toxicity, as it is in B-cell aplasia for anti-CD19.

As far as I am concerned, the gist of the matter is that the more completed articles used dates from 2018. In them, fundamental questions are resolved, explained and pathways to be assessed determined to follow an exponential enhancement. It gives us the idea that we will hear much more about the whole topic and who knows, maybe include it into academic studies or easily see it in hospitals in a few years. It seems to me that the future of nowadays incurable disease falls on biological drugs. Chemicals have allowed complete disease control, lengthen life-expectancy and even life-saving in many cases. However, in those diseases, particularly in cancer, where after the past century of chemotherapy development, they have not achieved curable capacity is why new investment in other areas of knowledge and science must be made. As I believe, gene therapy, immunology and adoptive cell therapies have the potential to suppose a breakpoint in the concept all physicians have about medicine and how to manage diseases. Forthcoming therapeutic agents will belong to the field of biology and will be applied in different disciplines such as solid tumours, autoimmunity and infectious disease.

8. Conclusion

CARTs have opened the window for cell engineering and its capability. They have literally transformed the management of hematologic malignancies but there are still many hurdles to overcome to successfully broad this approach to solid tumours. Main headlines of conclusions extracted are:

- Both paediatric and adult patients, especially those suffering r/r B-ALL, have benefited from CARTs due to the impressive outcomes of CR rates. Long-term survival is still to be assessed and follow-up of patients must confirm those rates.
- Although several studies have been done, there is always a level of uncertainty when a new approach is launched, and it needs a large number of patients to assess the effectivity and define all possible toxicities.
- CD19-CART therapy needs to consider cost-effectiveness balance to become a real and remarkable strategy for hospitals to consider applicability.
- Biological treatment, including adoptive cell transfer therapies, will bring the solution to unmanageable diseases since today when complete research and experience fulfilled.

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