Hematology, Department of Translational and Precision Medicine

"Sapienza" University of Rome

PhD School "Innovation in Immuno-mediated and Hematological disorders"

XXXV Cycle

Long-term *in vivo* host immune modulation following CD19-CAR-T cell therapy in diffuse large B-cell lymphoma and B-lineage ALL patients

Tutor: Prof. Sabina Chiaretti

Candidate: Giulia Radice

Academic Year 2022-2023

INDEX

1. ABSTRACT

Chimeric antigen receptor (CAR)-T cells represent a potentially curative strategy for patients with advanced relapsed or refractory (R/R) B-cell malignancies. However, little is known about the *in vivo* effects of this treatment on the patients' immune lymphocyte populations. In this study, we investigated the effects of Axi-cel and Tisa-cel administration in the control of R/R diffuse large B-cell lymphoma (DLBCL) and B-lineage acute lymphoblastic leukemia (B-ALL), with a primary focus on the immunomodulatory changes induced over time on the host immune system of treated patients. Despite the short *in vivo* persistence of CAR^+ cells, we could document a constant and significant increase over time of $CD3^+$, $CD4^+$, $CD8^+$ and NK cells, associated with an increased capacity of T lymphocytes to produce IFNγ and TNFα. The results obtained also highlight the fact that different co-receptors endow T cells with different functions: the presence of 4-1BB or CD28 domain affects T cells function and fate and our results suggest that infusion with Tisa-cel product induces a bigger expansion of T lymphocytes compartment, which is also found to be functionally more active in producing immunomodulatory cytokines, compared to Axi-cel. We, furthermore, confirmed the prominent role of plasmatic IL-6 in mediating CRS, but we also emphasized the involvement of plasmatic IL-8 in ICANS pathophysiology. IL-8, moreover, emerged as a new early indicator of clinical response, as high IL-8 levels at T3d resulted associated with partial or absent response evaluated at day 30 and month 3. Taken together, these findings show that CAR-T cells are capable to exert a modulation and activation of host immune system, highlighted the differences induced by different product phenotypes and shed light on the role of IL-6 and IL-8 in adverse events.

2. INTRODUCTION

2.1 Hematological malignancies

Hematological malignancies comprise a diverse set of neoplasms that affect the bone marrow, blood and lymphatic system. These biologically and clinically heterogeneous disorders account for 6.5% of all cancers around the world. [1]

Cancer blood cells can arise from genetic errors occurring during the finely regulated and multi-step process of hematopoiesis that allows regeneration of blood cells. During this process, hematopoietic stem cells (HSCs), that retain both self-renewal and multipotency capabilities, exert either function following a combination of extrinsic and intrinsic factors, such as niche-associate factors, signal transduction pathways, transcription factors and chromatin modifiers. [2]

HSCs can differentiate and mature following two major lines of commitment: lymphoid lineage, generating T, B and natural killer (NK) cells, or myeloid lineage giving rise to erythrocytes, megakaryocytes, granulocytes and monocytes.

Mutations, deletions and translocations, as well as epigenetic modifications can alter the maturation of these cells across different stages of the process, leading to an uncontrolled proliferation of abnormal immune cells. Hematological malignancies can arise during any stage of blood cell differentiation and can impair the production and function of blood cells. Deregulation of hematopoiesis can result in different types of cancers, classified in leukemia or lymphoma on the basis of the cell of origin and the maturative state in which neoplastic transformation occurs. [3]

2.2 B-acute lymphoblastic leukemia

2.2.1 General overview of leukemia and classification

The term leukemia is derived from the Greek words “leukos” meaning white and “haima” meaning blood and refers to the transformation and clonal expansion of a single hematopoietic progenitor cell in the bone marrow, that can spread to the bloodstream and lymph nodes via a cascade of molecular events entailing extravasation and tissue colonization. [4]

Leukemias are currently categorized according to cell lineage (lymphocytic or myeloid) which from the neoplastic cell arises and stage of maturation arrest (acute or chronic).

In particular, acute lymphoblastic leukemia is an aggressive and rapidly progressive disease unless promptly treated and results in the accumulation of immature, poorly differentiated lymphocytes (i.e., blasts) in the bone marrow, which eventually impair the proper production of healthy blood cells.

2.2.2 Acute lymphoblastic leukemia

The most common form of pediatric malignancy is acute lymphoblastic leukemia (ALL), with an estimated incidence of 1 to 1.5 per 100,000 people, with peak between 2 and 5 years and a second peak after age 50.

ALL itself can be subdivided into two major categories, B- and T- ALL, on the basis of the progenitor cell that undergoes oncogenic transformation.

Symptoms and signs reflect the effects of dysfunctional hematopoiesis (anemia, abnormal white cell counts, fever, thrombocytopenia) and clonal proliferation and infiltration of the leukemia cells (lymphadenopathy, hepatosplenomegaly and bone pain).

Despite limited knowledge on predisposing factors, it is clear that leukemic transformation is the result of multiple events entailing host susceptibility and exposure to physical or chemical agents that may cause chromosomal damage, such as ionizing radiation or benzene. [5]

2.2.3 Diagnostic process of acute lymphoblastic leukemia

The diagnosing of ALL and ALL subtype is a multistep process including cytomorphology, immunophenotype and genetics/molecular genetics.

The suspect of ALL is mainly derived by an altered full blood count, since people with ALL can exert low levels of red blood cells and platelets, as well as high level of white blood count (leukocytosis), or more rarely, leukopenia: the three aberrations can occur concomitantly or, less frequently, a single lineage is involved. Complete blood count is followed by morphological examination of cells, to detect pathologic immature cells, also defined as blasts, in the blood.

The key process for diagnosing ALL is carrying out bone marrow aspirate to perform an appropriate diagnosis. The first step in diagnostic pathway is represented by morphological bone marrow assessment. Morphological features help to distinguish ALL from acute myeloid leukemia (AML) by sorting lymphoblasts from myeloblasts: the discrimination between the two subsets relies on the analysis by cytochemistry, of myeloperoxidase. [6]

The diagnostic gold standard technique to identify cell lineage and to determine ALL subclassification is flow cytometry, a laboratory test that enables examination of pattern of either surface or cytoplasmic proteins on cells. Leukemia cells can have different antigens on their surfaces, depending on the type of leukemia. The evaluation if these antigens, called cluster of differentiation (CD), is crucial for an accurate diagnosis. Phenotypic characteristics of the malignant population reflect the expression of lineage-associated antigens of the stage.

Approximately 85% of cases of ALL belong to the B-cell lineage. Within this type of ALL, the most important markers for diagnosis and sub-classification are CD19, CD22, CD79a (the earliest B-lineage markers), CD10, CD20, IgM and some antigens of immaturity, such as TdT and CD34. According to the immunophenotype as per the European Group of the Immunological Characterization of Leukemias (EGIL), B-ALL can be classified into four subgroups: Pro-B ALL, defined by the positivity for any two of CD19, CD22, CD79a antigens without additional differentiation markers; the "common" ALL subgroup, defined by the presence of CD10; Pre-B ALL cells, expressing cytoplasmic IgM heavy μ chain, and the mature B-ALL, characterized by the presence of surface immunoglobulin light κ and λ chains (Table 1A).

Likewise, the 15% of ALL derived from the T-cell lineage reflects the stages of thymic differentiation. The antigens that define T-ALL include cCD3, CD7, CD4, CD8, CD2, CD5, CD1a, CD99 and TdT. According to the above-mentioned markers, T-ALL can be distinguished in four groups: Pro-T ALL, with the expression of cCD3 and CD7; Pre-T ALL defined by the expression of cCD3, CD7, CD5/CD2; cortical T-ALL, characterized by the expression of cCD3, CD1a and sCD3, and, at last, mature T-ALL, with a typical expression of cCD3, sCD3 and CD1a (Table 1B). [7]

Once established the lineage and the stage of differentiation, the diagnostic work up must be implemented with the cytogenetic and molecular classification of leukemic cells.

Characterizing patients' karyotype allows the identification of prognostic factors and useful markers for therapeutic choices.

The cytogenetic alterations can be classified into numerical and structural aberrations.

The most frequent numerical change (25-30% of pediatric ALL and 9% of adult ALL) is high hyperdiploidy, characterized by the presence of more than 50 chromosomes. Hypodiploidy, or the presence of less than 44 chromosomes, occurs in 5-8 % of cases.

Structural aberrations encompass chromosomal translocations, deletions and inversions.

Chromosomal rearrangements give rise to fusion genes. The most common translocation in pediatric B-ALL patients (25% of cases) encodes for the *ETV6-RUNX1* rearranged gene, perturbing the expression of RUNX1-related genes, [8] while the translocation that results in the formation of the "Philadelphia" chromosome encodes for *BCR-ABL1* rearranged gene causes the constitutive activation of a tyrosine kinase; this aberration is the most frequent within adult ALL, occurring in 25% of cases. [9] Table 2 summarizes the most frequent lesions.

Since conventional karyotype often fails in ALL, it is common practice to use molecular genetics to identify the fusion genes derived by the above mentioned karyotypic aberrations.

Currently, this complex diagnostic process is integrated with several molecular techniques that allow the identification of novel mutations and the stratification of patients onto risk groups.

Within a translational research setting, genome-wide technologies identified many novel recurrent fusion genes in B-ALL that result in the expression of chimeric fusion transcripts and allowed to define novel subtypes of ALL. The most important findings concern the so called "B-other ALL", defined as those cases lacking major recurrent abnormalities. [10] This kind of analyses identified copy number alterations (CNAs), the most common of which affect genes involved in crucial mechanisms such as differentiation, cell cycle regulation, lymphoid signaling, apoptosis, transcription, regulation of chromatin structure and epigenetic. The most frequent target is the lymphoid transcription factor PAX5, a gene involved in maintaining B lineage specificity, which harbors deletions or focal amplifications in almost 30% of B-ALL cases. Other genes frequently affected by deletions and mutations are those involved in cell cycle regulation (*CDKN2A/2B, RB1, TP53*), transcription (*ETV6, TBL1XR1, ERG*), lymphoid signaling (*BLTA, CD200, TOX*), RAS signaling (*NF1, KRAS, NRAS, PTPN11*), epigenetic modifications (*EZH2, CREBBP, SETD2, MLL2, NSD2*), cytokine receptors and tyrosine kinases (*ABL1, ABL2, CRLF2, CSF1R, EPOR, FLT3, ILR2B, IL7R, JAK1/2, NTRK3, PDGFRB*). [11, 12]

A

Antigen	Pro-B	B-common	Pre-B	Mature B
CD22	+	+	+	+
cCD79	+	+	+	+
CD19	+	+	+	+
CD20	-	+/-	+/-	+
CD10	-	+	+/-	-
slgμ	-	-	-	+
clgμ	-	-	+	+
sk/λ	-	-	-	+
CD34	+	+	+	+/-
Tdt	+	+	+	-

B

Antigen	Pro-T	Pre-T	Cortical T	Mature T
cCd3	+	+	+	+
CD7	+	+	+	+
CD5	-	+	+	+
CD2	-	+	+	+
CD1a	-	-	+	-
sCD3	-	-	+/-	+
γ/δ or α/β	-	-	+/-	+
CD34	+	+	-	-
Tdt	+	+	+	+/-

Table 1. Immunophenotypic classification of **A)** B-ALL and **B)** T-ALL.

B-ALL	*BCR-ABL1+* / t(9;22) (q34;q11.2) (Ph+)	Ph-like	*TCF3-PBX1+* / t(1;19) (q23;p13)	*KMT2A-AFF1+* / t(4;11) (q21;q23.3)	*IGH-MYC+* / t(8;14) (q24;q32)	*TCF3-HLF+* / t(17;19) (q22;p13.3)	iAMP21	14q32 transloc	9p13 transloc/ del	7p12.2 focal del/ mut	*DUX4* and *ERG* deregulation	*MEF2D* rearrang	*ZNF384* rearrang
Incidence	20-50 % increasing with age	10-15% of pediatrics; 27% of young adults; 25% of adults	10-15%	5%	1-5%	<1%	~2%	<5%	~25%	15% of pediatrics; 50% of adults	3-7%	3-4%	6-7%
Genetic alterations	*BCR-ABL1* rearrang	ABL rearrang; JAK-STAT pathway mut; *CRLF2* hyperexpr; *IKZF1* del	*TCF3-PBX1* rearrang	*MLL* rearrang	*IGH-MYC* rearrang	*TCF3-HLF* rearrang	-	*IGH* rearrang	*PAX5* rearrang	*IKZF1* del	*ERG* and *IKZF1* del	-	*EP300*, *CREBBP*, *TAF15*, *SYNRG*, *EWSR1*, *TCF3*, *ARIDIB*

Table 2. The most frequent lesions in B-ALL and related incidences. Abbreviations: rearrang, rearrangement; hyperexpr, hyperexpression; transloc, translocation; mut, mutation; del, deletion.

2.2.4 Treatment of B-acute lymphoblastic leukemia

Treatment of ALL has dramatically changed over the last decade, mostly in adult. The standard elements of ALL treatment include different phases: 1) induction phase, whose aim is to eliminate leukemic cells and to induce the so-called complete remission (i.e., <5% blasts), 2) consolidation, based on high doses chemotherapy and aimed at eradicating minimal residual disease (MRD), 3) maintenance therapy to avoid relapse and 4) central nervous system prophylaxis. Optimal dosages of conventional chemotherapeutic agents and schedules for combination chemotherapy were

developed on the basis of tolerability, response evaluation and pharmacodynamic and pharmacogenomic studies. Currently, over the last decade, this backbone has been also applied to adult ALL, ultimately leading to a significant improvement in the survival rates.

Remission induction therapy consists of administration of three or four drugs: a glucocorticoids, vincristine and asparaginase, with the possible addition of anthracycline. This regimen induces complete remission (CR) in more than 95% of pediatric patients. [8]

The subsequent phase of consolidation requires high dose of chemotherapy (cytarabine and methotrexate). In B-ALL patients, the 5-year event free survival (EFS) percentage is about 92% for pediatric patients who achieved negative minimal residual disease (MRD) at the end of these two phases [13]. For patients at high risk, it is necessary to intensify the therapy with allogeneic stem cell transplant (SCT) with compatible donor stem cells or, more rarely, with autologous transplantation if patient achieved negative MRD. [14]

Maintenance therapy typically lasts over 12-24 months and consists of daily mercaptopurine and weekly methotrexate with or without vincristine and steroid pulse. [8]

CNS prophylaxis starts with the induction phase, with treatment with drugs as methotrexate alone or in combination with cytosine-arabinoside/prednisone; this treatment persists during consolidation and maintenance phases and it is proportional to the patients' risk, in accordance with prognostic factors.

Despite significant progresses in treatment of ALL and improvement, survival is largely affected by relapse.

Driven by the urge to improve outcomes and reduce adverse effects, genome-wide analyses have profoundly affected our understanding of ALL genetics. Risk stratification for therapy is indeed based on somatic and germline genetic analysis.

The first example of targeted therapy is represented by the use of tyrosine kinase inhibitors (TKI) Imatinib in *BCR-ABL1* cases, encountered predominantly in adults, that improved substantially CR rates to over 80%. [15] Most recently, a chemotherapy-free induction and consolidation treatment with another TKI, Dasatinib, plus administration of the bispecific monoclonal antibody blinatumomab was associated to 95% of overall survival [16] at a median follow-up of 18 months.

However, novel targets and therapeutic strategies are constantly under investigation in order to effectively manage this challenging malignancy.

2.3 Diffuse large B-cell lymphoma

2.3.1 General overview of lymphoma and classification

Well-differentiated B and T lymphocytes are involved in oncogenic transformation in lymphomas, typically forming malignant masses in lymphatic tissue.

Mature lymphoid neoplasms are classified broadly into three groups: mature B-cell neoplasms, mature T- or natural killer (NK)-cell neoplasms and Hodgkin's lymphoma (HL).

Collectively, B- and T/NK-cell neoplasms constitute non-Hodgkin's lymphomas (NHLs), a group of malignancies characterized by molecular, morphological and clinical heterogeneity, which encompass more than 60 subtypes.

NHL is the most common hematological malignancy in western countries; this subgroup can be further divided in indolent or aggressive on the basis of the likeliness to grow and spread. They are characterized by rapid growth and are commonly accompanied by systemic symptoms such as fever. 80-85% of cases originate from abnormal B lymphocytes, while T-cell lymphomas are much less common and account for about 10-15% of cases. The indolent NHLs mainly comprise follicular

lymphoma (FL), mantle cell lymphoma, marginal zone lymphoma, small lymphocytic lymphoma and cutaneous T cell lymphoma. The aggressive NHLs consist of diffuse large B-cell lymphoma (DLBCL), Burkitt's lymphoma, lymphoblastic lymphoma and various groups of T- and NK-cell lymphoma.

The most frequent subtype is represented by diffuse large B-cell lymphoma (DLBCL), that accounts for 30%-40% of cases. [17]

2.3.2 Diffuse large B-cell lymphoma

DLBCL itself comprises a group of biologically distinct subtypes, resulting from the clonal proliferation of a germinal or post-germinal malignant B cell.

The most commonly used system divides DLBCL cases on the basis of the cell of origin into germinal center B-cell like and activated B-cell like subtypes, with about 10-15% of cases being unclassifiable. [18]

It can occur at any age, but it is rare in children and is more common in adults.

The etiology is still not clear. Factors thought to potentially confer increased risk include immunosuppression (such as AIDS-related), ultraviolet radiation and pesticides. A subset of diffuse large B cell lymphoma is highly associated with the Epstein-Barr virus.

The disease is usually aggressive, with patients showing B symptoms such as fever, weight loss, night sweats and symptoms related to organ(s) involvement. [19]

2.3.3 Diagnostic process of diffuse large B-cell lymphoma

The diagnosis is commonly made by biopsy of an abnormally enlarged lymph node or extranodal sites, such as kidneys, adrenal gland, brain, bones and soft tissues.

Morphologically, the normal architecture is substituted by large cells that show large nucleoli and abundant cytoplasm. These pathologic cells typically stain positive for B cell antigens, such as CD19, CD20 and CD79a.

A consistent number of DLBCL cases shows complex karyotypes; hyperdiploidy can be observed in two third of cases, with an increased frequency of X, 3, 5, 7, 11, 12 and 18 chromosome gains. Losses and deletions are related to chromosomes Y, 4, 6 and 15. The 14q32/IGH rearrangements constitute the most frequent structural anomaly, occurring in 40-50% of cases. The *BCL6* gene is rearranged with a frequency of 30-40%, most often in the form of *IGH-BCL6*, while the gene *BCL2*, in its translocated form of *IGH-BCL2*, can be observed in 15-20% of cases. Rearrangements of *MYC* gene are observed in 5% of adult DLBCL and in 15-20% of pediatric DLBCL patients. [20]

The application of gene expression profiling to the study of DLBCL has greatly helped subtypes identification and therefore classification of the risk.

The transcriptomic studies allowed the identification of molecular clusters that define subtypes of B lymphomas, on the basis of differential expression of signatures relating to proliferation, T cells and lymph-node biology. Differential expression of genes allows classification of DLBCL cases into germinal center B-like (GCB) DLBCL, characterized by the expression of genes that define the germinal center B-signature, activated B-cell-like (ABC) DLBCL, in which are expressed genes characteristic of activated B cells and plasma cells, and primary mediastinal lymphoma (PMBL), that shows low levels of genes of normal germinal center B-cells. Genes that define GCB DLBCL are, indeed, markers of germinal center differentiation, such as the cell-surface proteins CD10 and CD38, the DNA repair protein 8-oxoguanine DNA glycolsylase and BCL-6, which is the most frequent translocated gene in DLBCL. In contrast, genes that define the ABC subtype are not expressed in normal germinal center B-cells, but are preferentially expressed in activated peripheral blood B cells and include *IRF4*, essential for B proliferation in response to signals from

the antigen receptor, and *BCL2*, known to inhibit apoptosis. [20] PMBLs are more difficult to define; they frequently express the genes *MAL*, which encodes an integral membrane protein, and interleukin 4-induced gene 1 (*FIG1*), even though these genes can also be expressed in other types of DLBCLs. [20,21]

2.3.4 Treatment of diffuse large B-cell lymphoma

The high heterogeneity of this disease mirrors the variable clinical course and prognosis.

Concerning treating options for DLBCL, in the past two decades, the introduction of the anti-CD20 monoclonal antibody (moAB) rituximab in addition to chemotherapy substantially improved the outcome and nowadays it has been introduced in standard regimen together with cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP) as the standard-of-care therapy, with 50-60% of patients typically being cured using this approach.

However, the remaining patients are either refractory to treatment with R-CHOP or have relapsed disease after a complete remission (CR).

The clinical management of refractory or relapsed (R/R) patients largely depends on their eligibility for high-dose therapy (HDT) and autologous stem cell transplant (ASCT). However, only 10% of patients with R/R DLBCL can be cured with this approach, whereas the outcome of the remaining 90% of patients remains dismal, suggesting a major unmet therapeutic need.

Over the past 20 years, identification of disease subtypes on either cell-of-origin or molecular and immunophenotypic features led to great progresses and improvements in clinical treatment: biological agents, including ibrutinib, bortezomib or lenalidomide have been combined with R-CHOP, with variable success. ABC subtype is driven by dysregulation and constitutive activation of B-cell receptor (BCR) signaling, leading to activation of the nuclear factor k-light-chain-enhancer of activated B cells (NF-kB) pathway. On these basis, ABC DLBCL patients may benefit from treatment

with ibrutinib, an inhibitor of the BTK kinase in the BCR signaling. Bortezomib, a proteasome inhibitor, is thought to prevent proteasomal degradation of the inhibitory kinase IkB that acts on NF-kB. [22]

Lenalidomide exerts either direct cytotoxic and indirect anti-angiogenetic effects on neoplastic cells and showed promising effects in monotherapy or in association with rituximab in treatment of R/R DLBCL. [23]

Recently, besides rituximab, other monoclonal antibodies have been developed and applied to clinic: the anti-CD20 moAB tafasitamab gained FDA and EMA approval for use in combination with the immunomodulatory drug lenalidomide for R/R DLBCL patients. [24]

2.4 T-cell immunity and immunotherapies

Improvements in the outcome and quality of life rely on novel approaches, the most encouraging being immunotherapies.

In the modern era, fundamental advances in immunology, molecular biology, and virology alongside technological advances in cell manufacturing and genetic engineering have led to exciting progresses in the development of immune cell therapies, with T cell therapies emerging as the most advanced within this therapeutic class.

T lymphocytes have been considered indeed as the core of several immunotherapeutic approaches, due to the well-known anti-tumor activity. [25]

T cells recognize antigens loaded on the major complex of histocompatibility (MHC) molecules, exposed on the surface of antigen-presenting cells (APCs).

Effector functions are elicited by the expression of the co-receptor, CD4 or CD8. $CD4^+$ T cells (or T helper lymphocytes) exert several functions and are all carried out by specific subpopulations. They are central players of the adaptative arm of the immune system and are capable to secrete cytokines, mostly with pro-inflammatory properties, which can, in turn, stimulate both adaptative and innate immune response. At least one $CD4^+$ T cell subclass - the $CD4^+CD25^+$ regulatory T cells - dampens the immune response acting as a negative feedback mechanism. At variance, $CD8^+$ T cells carry out direct cytotoxic reactions that kill infected or neoplastic cells. [26]

The unique transmembrane receptor located on T cells (TR) is responsible for recognition of antigen presented by MHC and such binding, together with co-stimulatory signals, result in the activation of the cell.

The TR molecule is a complex formed by highly polymorphic α- and β-glycoprotein chains, each containing a variable and constant region, and a group of non-polymorphic chains involved in signaling transduction, called CD3 γ, δ, ε and ζ.

Following TR engagement, activated T cells can undergo three major fates: 1) if the stimulus does not persist, T cells can contract through apoptosis; 2) T cells can exhibit a phenotype of exhaustion induced by repeated low-dose and low-affinity stimulation and, 3) lastly, a subset of these effector cells can constitute a pool of long-term immunological memory, primed to react rapidly to a second encounter with the same antigen. Memory T cells are crucial mediators of immune response to pathogens and tumors. [27]

Evidences that paved the way to T-cell therapies are based on studies conducted in early 1990, in which patients affected by metastatic malignant melanoma treated with infusions of *ex-vivo* expanded autologous tumor-infiltrating T lymphocytes (TILs), together with the addition of recombinant human interleukin-2 (rhIL-2), achieved long-lasting CR in 25-50% of cases. [28]

These studies demonstrated the key role of T cells in contrasting cancer cells. The main limitation of this approach in the clinical setting application concerns the difficulty of generating adequate numbers of bioactive TILs from patients with non-melanoma cancers.

Encouraging results and advances in genetic engineering, enabled cloning of tumor-reactive T cell receptors (TRs) from TILs in responding patients and expression of the TR in T cells expanded from peripheral blood of other patients with cancer, thus generating T cells expressing engineered TRs. Translation of this approach into the clinic, however, faced several challenges: at first, the transgenic TR α and β chains paired with endogenous receptors, with risk of off-target toxicity; [29] and secondly, the difficulty to identify safe and effective TRs within the TIL population to be used for genetic transfer. [30]

Continued progress with T cell therapeutic agents incorporating engineered TRs will require technologies to enhance potency, specificity, and safety.

The power of immune system has furthermore been harnessed in therapies that do not target the tumor, but rather the immune system itself, enhancing the anti-tumor function of T cells by interfering with the mechanisms of immunosuppression.

Some of these drugs, called checkpoint inhibitor antibodies, such as anti-CTLA4 and anti-PD1 antibodies, have been included in clinical trials for patients with R/R non-Hodgkin lymphoma. [31]

Another approach that exploits T lymphocytes effector function involves T-cell engager antibodies. These molecules are immunoglobulin fragments able to recognize and connect two antigens: one located on tumor cells and the other on T cells. This interaction recruits and activates T cells, triggering tumor disruption. The first bispecific T cell engager to demonstrate great results in clinic, and thus approved by Food and Drug Administration in 2014, was Blinatumomab, indicated to use in MRD+ and R/R B-ALL patients. [32] Given exciting results, blinatumomab was also involved in trials for non-Hodgkin lymphoma patients, achieving an overall response rate of 69%, but was not

devoid of toxicity. Furthermore, a phase II trial evaluated blinatumomab in R/R DLBCL patients, where overall response rate was 43%. [33]

2.5 CAR-T cells

2.5.1 History and structure

Remarkable success in the field of cancer immunotherapy has been achieved by therapy with chimeric antigen receptor T (CAR-T) cells, an adoptive cell therapy involving autologous immunocompetent cells engineered by gene transfer.

CAR-T cells are autologous genetically modified T cells formed by combining the antigen-binding site of an antibody with the intracellular domain of a T-cell activation receptor.

Genes encoding antigen receptors can be introduced into the T cell genome using a gammaretroviral or lentiviral vector. Engineered T cells are then rapidly expanded to derive memory and effector lymphocytes capable of proliferating robustly *in vivo* and eliciting potent antitumor activity. [34]

The birth of CAR technology dates back in 1989 when Eshhar and colleagues engineered the first functional CAR-T cell for cancer treatment, demonstrating that a single-chain Fv of an antibody fused to the ʒ chain of the CD3 complex can be expressed in T cells as an antigen specific receptor, triggering IL-2 production and mediating target cell lysis. [35]

Furthermore, back to three decades ago, Irving and Weiss determined that a chimeric receptor composed of CD8 and CD3ʒ chain is capable of transducing activator signal in absence of CD3 γ, δ and ε, suggesting the key role of CD3ʒ in coupling the TR to intracellular signal transduction mechanisms. [36]

CARs are indeed typically structured in an extracellular domain for tumor antigen recognition linked to one or more intracellular transduction domains. The antigen binding region (single-chain variable fragment or scFv) consists of the variable heavy and light chains of an antibody, together with a spacer peptide. The intracellular signaling molecule comprises the CD3ʒ chain and other co-stimulatory domains, such as 4-1BB and CD28. [37]

Therapy with CAR-T cells overcomes major issues related to TILs and antibody-based approaches, primarily because the antigen binding site can recognize intact cell surface proteins, meaning that CAR-T cells can interact with target avoiding the process of antigen presentation by MHC molecules. So that, CAR-T cells are not affected by tumor escape mechanisms that result in loss of MHC. Moreover, the chimeric receptor can interact with glycolipids and conformational epitopes.

These chimeric receptors indeed combine the effector functions of T lymphocytes and the ability of antibodies to recognize target antigens in a non-MHC restricted manner, harnessing the power of both innate and adaptive immunities. [38]

Despite encouraging expectations, therapy with CAR-T showed unconvincing results in clinic at first. Patients affected by neuroblastoma underwent infusion with CAR-T cells containing CD3ʒ chain alone but engineered cells showed poor persistence *in vivo.* [39]

CD28 or 4-1BB domains were lately fused to CD3ʒ module to enhance *in vivo* persistence and expansion. [37]

2.5.2 CAR-T cell generations

Currently, CARs are categorized into four ‘generations’ according to the number of intracellular signaling molecules (Figure 1).

First generation CAR is represented by CAR T cell engineered by Irving and Weiss (1991) [36] and contains CD3ζ chain as a single intracellular signaling domain. This molecule however shows low

cytotoxic potential and reduced proliferation. Second generation CAR was developed by combining the CD3ζ chain with an intracellular signaling domain from a co-stimulatory molecule, most commonly, 4-1BB (CD137) or CD28. Both antigens are expressed on T cells lineage, $CD4^+$ and $CD8^+$ lymphocytes, and are demonstrated to transduce signals that promote proliferation of TR-stimulated T cells. [40] Other signaling co-stimulatory molecules that might be engineered into the chimeric receptor are ICOS, OX40 (CD134), CD27, DAP10, and 2B4 (CD244). Due to the additional co-stimulatory molecule, second-generation CARs are able to induce the production of a higher level of cytokines, compared to first-generation CAR. [41] In third-generation CAR, CD3ζ chain is combined with two co-stimulatory molecules fused together. Third generation CARs show augmented potency with stronger cytokine production and killing ability. The disadvantages of third-generation CARs are their risk of signal leakage and their excessive cytokine production. [42] Fourth-generation CARs are engineered by combining second-generation CARs with a cytokine expression cassette, which is called "T-cells redirected for universal cytokine-mediated killing" (TRUCKs) that is constitutively or inducibly expressed. As an example of TRUCKs, tumor-targeted T-cells modified to secrete IL-12 have been shown to activate and attract innate immune cells to the targeted lesion. [43] A fifth generation of CAR is also under investigation. This molecule is structured as a second-generation CAR with a truncated cytoplasmatic IL-2b receptor that carries a binding site for the transcription factor *STAT3*, further enhancing the cytokine signaling via *JAK-STAT* pathway. [44]

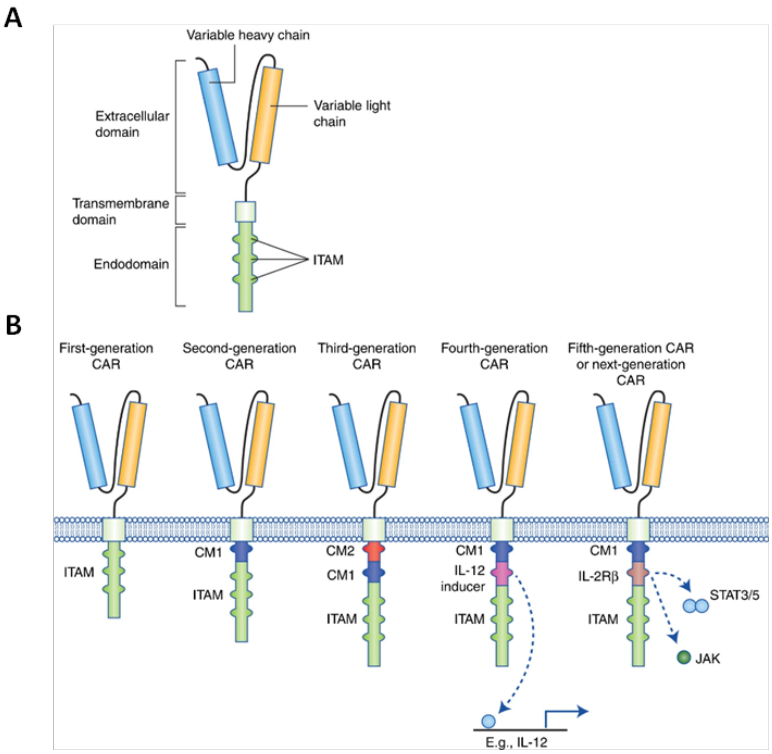


Figure 1. Structural variants of CAR. A) The core structure of CAR that encompass components of the extracellular domain, the transmembrane domain and the intracellular domain. **B**) Structure of different CAR generations, from the first to the fifth generation. (Adapted from Tokarew et al., 2019)

2.5.3 CAR-T cells in the clinic

As a target for CAR-T cells, CD19 transmembrane protein of B-lymphocytes emerged as an ideal candidate, since the antigen has a pattern of expression restricted to B-cell lineage and hypogammaglobulinemia, that can be caused by "on-target-off-tumor" activity of CAR-T cells, is generally manageable.

In light of this, CAR-T cell therapy found its main application in hematological B-cell malignancies. Several trials led to the approval of either CD28- or 4-1BB- based second generation CAR products: the JULIET trial was the pivotal phase II protocol conducted on R/R DLBCL, HGBCL and tFL patients who underwent infusion of the CD19-directed CAR-T cell product Tisagenlecleucel (Tisa-cel)

composed of the co-stimulatory module 4-1BB. Eligible patients were relapsed or resistant to at least two prior lines of treatment or were unfit or relapsed after autoSCT. Median age was 56 (range 22-76). The study reached a best ORR of 52%, with 40% of patients achieving CR. [45]

The same CAR product was involved in another trial named ELIANA, in which seventy-five children and young adults with R/R B-cell ALL were evaluated for efficacy after infusion. Within three months, the ORR was 81%, with all patients who had a response to treatment found to be MRD negative. EFS and OS rates were 73% and 90%, 50% and 76%, at 6 and 12 months, respectively.

This study indicated that Tisa-cel produced high remission rates and durable remissions without additional therapy. [46]

On these basis, Tisa-cel was the first product to gain approval for clinic application. It was approved by Food and Drug Administration (FDA) in August 2017 and later in August 2018 by Agenzia Italiana del Farmaco (AIFA) for treatment of young patients up to 25 years affected by B-ALL refractory or relapsed after at least 2 lines of therapy or after allogeneic SCT or for treatment of DLBCL patients refractory to two or more lines of chemotherapy.

ZUMA-1 was the pivotal phase I/II trial involving the anti-CD19 CAR product engineered with CD28 as a co-stimulatory domain, named Axicabtagene ciloleucel (Axi-cel), for patients with R/R NHL. At first, the phase I aimed to demonstrate safety of manufacturing and administration of the product to 7 patients. Consequent to the positive results obtained, two additional cohorts of patients were enrolled in the phase II arm of the study, totaling 101 patients. Patients enrolled were affected by DLBCL, HGBCL, tFL or PMBCL who had primary refractory disease, disease refractory to second or subsequent lines of therapy, or relapsed within one year after autologous stem cell transplant. Median age of patients was 58 (range 23-76). The best overall response was 74% with 54% of patients in complete remission (CR) and the median progression free survival (PFS) was 5.9 months. [47]

Axi-cel gained FDA approval in October 2017 and AIFA approval in August 2018 for treatment of DLBCL and PMBCL patients refractory to at least two lines of chemotherapy.

Tisa-cel and Axi-cel are commercially available under the names Kymriah®, produced by Novartis, and Yescarta®, produced by Gilead, respectively.

Currently, after years of active research and following favorable responses in clinical studies, four additional CAR products have been licensed so far by FDA: Brexucabtagene autoleucel (Brexu-cel) for R/R MCL and adult B-ALL, according to the phase II clinical trial ZUMA-3, [48] and Lisocabtagene maraleucel (Liso-cel), for treatment of R/R DLBCL, HGBCL, primary mediastinal large B-cell lymphoma and FL, on the basis of the TRANSCEND trial; [49] both Brexu-cel and Liso-cel are CD19-directed products. Idecabtagene vicleucel (Ide-cel) and Ciltacabtagene autoleucel (Cilta-cel) are, otherwise, CAR-T cells that target the B-cell maturation antigen (BCMA), for treatment of MM patients. [50] BCMA is a cell surface receptor that belongs to the tumor necrosis factor (TNF) receptor superfamily that binds the cytokine called B-cell activating factor. This antigen can be expressed by normal B cells and, more excessively, by MM. [51]

Information about CAR constructs and trial design are detailed in Table 3.

The clinical process starts with collection of patient's T cells through apheresis. The apheretic product is then shipped to the manufacturing facility where it is enriched in T cells using magnetic bead separation, to obtain CD4 and CD8 subsets. Cells are then engineered through transduction with a viral vector carrying the chimeric receptor gene. Subsequently, CAR-T cells are allowed to proliferate in a perfusion bioreactor in presence of interleukin-2 (IL-2) for at least 2 weeks; once expanded they are collected and cryopreserved. After cryopreservation, cells are shipped back to the clinical site.

The patient undergoes a period of lymphodepletion, essential to enhance survival and proliferation of newly produced CAR-T cells. Common lymphodepleting regimens consist of cyclophosphamide (Cy), fludarabile (Flu) and bendamustine, alone or in combination.

Thawed CAR-T cell product is finally intravenously infused (Figure 2). [52]

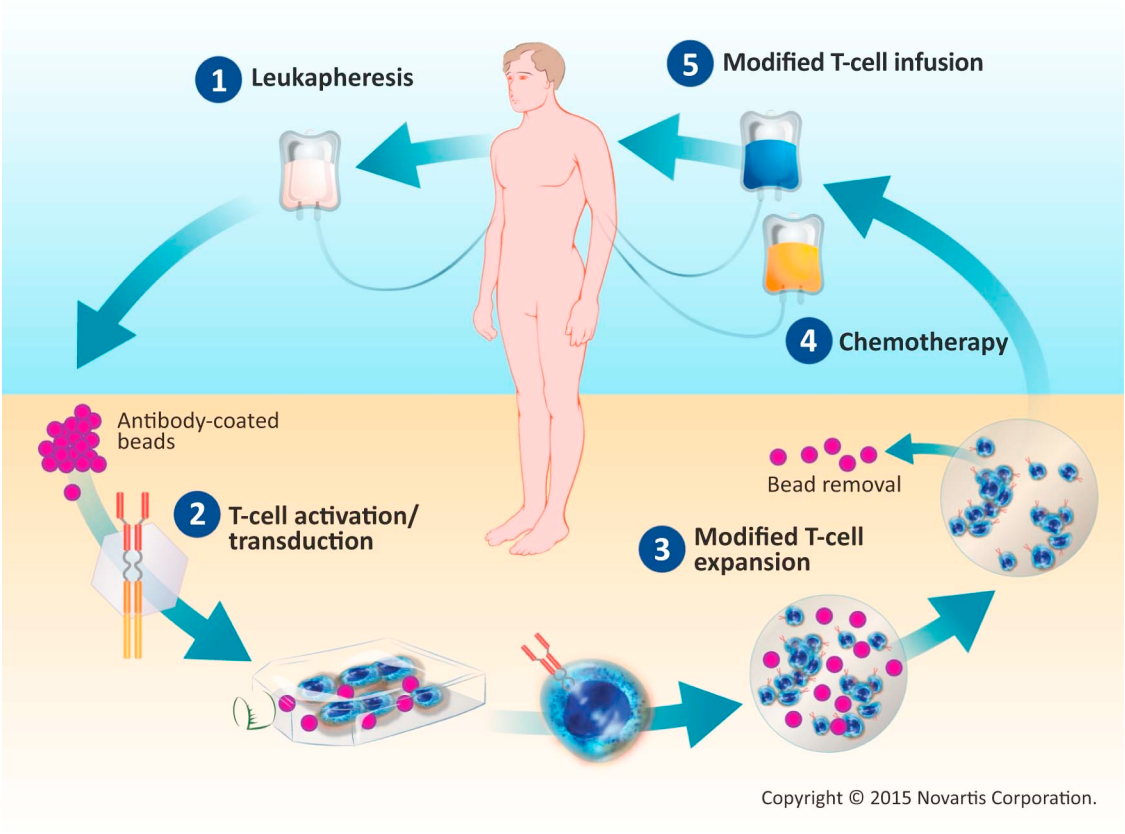


Figure 2. Clinical process of CAR-T cell administration (Adapted from McGuirk et al., 2017)

	Tisa-cel	Axi-cel	Brexu-cel	Liso-cel	Ide-cel
CAR	αCD19	αCD19	αCD19	αCD19	αBCMA
Transmembrane domain	CD8	CD28	CD28	CD28	CD8
Co-stimulatory domain	4-1BB	CD28	CD28	4-1BB	4-1BB
Clinical trial	ELIANA; JULIET	ZUMA-1; ZUMA-5	ZUMA-2; ZUMA-3	TRANSCEND	KarMMa
Disease	B-ALL; HGBCL	HGBCL; FL	MCL; B-ALL	LBCL	MM

Table 3. CAR constructs and trial design. Abbreviations: CAR, chimeric antigen receptor; CD, cluster of differentiation; B-ALL, B-acute lymphoblastic leukemia; HGBCL, high grade B-cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; LBCL, large B-cell lymphoma; MM, multiple myeloma.

2.5.4 Immunological features of CAR-T cell therapy

Upon encountering the target, the engineered T-cells undergo conformational changes to form the core region of the immunological synapse, in which components of the intracellular domain aggregate in microclusters that induce recruitment and phosphorylation of transduction domains and of the downstream cascading proteins. The activation results in proliferation and differentiation of CAR-T cells, that are finally ready to perform effector functions. Primarily, CAR-T cells mediate killing of the tumor cell via perforin-granzyme mediated mechanism. Perforin creates transmembrane pores on the target cells, thereby causing granzyme to penetrate the cytoplasm and induce cytolysis through either caspase-dependent or independent pathway. [53]

CAR-T cells are known to employ other synergistic tumor-killing mechanisms, such as lysis mediated by death ligand FasL, which, upon interaction with the corresponding receptor Fas on the target cell, is capable to induce caspase 8 and, thus, apoptosis. [54]

One of the most interesting features of activated CAR-T cells is their ability to recruit other components of the immune system.

This results in increased production of cytokines and growth factors that eventually can infiltrate in the tumor microenvironment causing inflammation and contributing to tumor eradication. [55]

Cytokines play also an important role in the relevant complications induced by this therapy. Indeed, despite promising results in clinic, this therapy is associated with a number of adverse effects, the most frequent being cytokine release syndrome (CRS), a form of systemic inflammation triggered by hyper-activation of cells involved in antitumor response.

Pathophysiological process of CRS is not only elicited by activated CAR-T cells, but also by monocytes, macrophages and dendritic cells, that participate in the synthesis and release of cytokines. Moreover, cytokines can also be released by the dead cancer cells, further increasing immunological reaction.

Clinical manifestation typically peaks within the first week after CAR-T administration and is hallmarked by fever, sometimes associated to tachycardia, hypotension, high levels of acute-phase proteins and, occasionally, organ toxicity. [56]

CRS is mild in most patients, but symptoms can range to a spectrum of severity from low- to high-grade. CRS is determined on the basis of the evaluation of four vital parameters, being temperature, blood pressure, oxygen saturation and organ toxicity and grading of the adverse event is referred to criteria of the European Society for Blood and Marrow Transplantation (EBMT). [57]

Current management of CRS is based on the use of anti-IL-6 drug Tocilizumab and the efficacy of this treatment as well as biological studies emphasize the key role of IL-6 in the inflammatory reaction; other therapeutic approaches are steroids-based, and anakinra, an anti-IL-1 receptor.

Studies have shown that patients with massive lesions and a high tumor burden are prone to severe CRS. [58]

In most severe cases, elevated cytokine levels can give rise to a form of hyperinflammation that mimics hemophagocytic lymphohistocytosis (HLH) syndrome. HLH is a life-threatening syndrome seen in the context of infection, autoimmunity and immune dysregulation caused by hyper activation of macrophages and lymphocytes. When associated with rheumatological disease, HLH is also termed macrophage activation syndrome (MAS). CRS and HLH have similar clinical features, but CRS is generally manageable through supportive care and anti-IL-6 therapies as well as corticosteroid treatments, while HLH, although rare, is associated with high mortality. Diagnosis of CAR-T cell-related HLH/MAS is usually based on ferritin levels, elevated over 10,000 ng/ml, concurrent with high-grade organ toxicity or haemophagocytosis in the bone marrow or other organs, elevated d-dimers, low NK-cell activity. Patients with suspected HLH should be treated promptly as per CRS indications with additional therapy with etoposide, an antineoplastic drug with myelosuppressive function, if the patient does not present clinical improvement. [59]

Besides, another clinical challenge is immune effector cell-associated neurotoxicity syndrome (ICANS), that typically manifests as a toxic encephalopathy and is marked by symptoms that range from cognitive impairment to altered level of consciousness, disorientation, tremors and in most severe cases, seizures, increased intracranial pressure and cerebral edema. [60]

The underlying pathophysiology is still poorly understood. Increased blood-brain barrier (BBB) permeability probably leads to high concentrations of pro-inflammatory cytokines (e.g., IL-6, IFN-γ and TNF-α) in the cerebrospinal fluid. It is also clearly associated with CRS, since CRS in most cases precedes ICANS; the two forms of toxicity may also temporarily overlap, while it is rare to observe severe ICANS in the absence of severe CRS. [61]

As for CRS, ICANS severity is graded according to criteria of the European Society for Blood and Marrow Transplantation (EBMT). [57]

Anti-IL-6 therapy is recommended for patients with grade ≥1 ICANS with concurrent CRS. Otherwise, If CRS is not present, corticosteroids are preferred treatment for ≥2 ICANS.

Grade 3 ICANS with raised intracranial pressure should be managed with corticosteroids and acetazolamide; patients who develop grade 4 ICANS with cerebral edema should receive high-dose corticosteroids, hyperventilation, and hyperosmolar therapy. [60]

These conditions are the main barriers to safe administration of CAR-T cells, further investigations are needed to define predictors of efficacy and toxicity.

Several evidences report that transmembrane domain might be able to modulate CAR-T cell activation and, consequently, the immune reaction. CAR-T cell products engineered with 4-1BB or CD28 molecules are indeed reported to exert different adverse events; different studies report that CD28 induces more severe adverse events respect to 4-1BB. [62, 63] This might be due to the ability of CD28 to dimerize with endogenous CD28, thus exerting a stronger and faster signal than 4-1BB co-stimulation. [64] Plus, CD28-CD3ζ combination is thought to circumvent CTLA-4 inhibition.[65]

Key mediators of the inflammatory reaction and activation profiles associated with CRS and ICANS are crucial issues to address to adequately define the immunological signatures involved in antitumor responses and to promptly manage these life-threatening conditions.

3. AIM OF THE STUDY

CAR-T cell therapy has shown encouraging results in treating DLBCL and B-ALL patients R/R to previous lines of treatment, providing a chance of remission to patients without other treatment options. However, this therapy is affected by possible adverse events and the underlying biology of the immunologic reaction process is not fully elucidated.

Therefore, the aim of the present study is to investigate how this therapy affects immune populations and how it shapes the tumor microenvironment.

For this purpose, it has been evaluated:

- the clinical outcome of treated patients;
- the numerical variation of the engineered cells over time;
- the numerical changes in the main lymphocytic subpopulations (T4, T8 lymphocytes and NK cells) following infusion with CAR-T cells at various monitoring points;
- the ability of lymphocyte populations to produce cytokines with immunomodulatory function following CAR-T cells infusion at various monitoring points;
- the change in plasma cytokine concentration following CAR-T cells infusion at various monitoring points;
- the differences between the different diseases and between patients treated with the two different CAR-T products.

The final purpose is to better understand the role of main lymphoid populations involved in the immune response in different settings of disease and treatment and the pathophysiology underlying adverse events.

4. MATERIALS AND METHODS

4.1 Patients

The analysis was carried out on a series of 32 patients enrolled in an observational prospective study that involved the Hematology Center at the 'Sapienza' University of Rome (22 patients: 2 B-ALL and 20 DLBCL) and the Department of Pediatrics of Monza (10 B-ALL patients). As inclusion criteria, all patients were relapsed/refractory (R/R) to previous lines of therapy (median of 2.2, range 2-4 for DLBCL patients and median of 2.9, range 2-6 for B-ALL patients). For B-ALL patients, indication for treatment comprised an age limit of 25 years, while there was no age limit for DLBCL patients. Treatment consisted of one infusion with the commercial second generation CD19 CAR-T cell product Tisa-cel or Axi-cel.

Administration of CAR-T cells was preceded by a lymphodepleting regimen, consisting of fludarabine 30 mg/m^2 administered daily for 3 days for DLBCL patients and of fludarabine for 4 days and cyclophosphamide for 2 days for B-ALL patients.

DLBCL patients included 9 females and 11 males with a median age of 52 years (range 21-71), of which 6 females and 7 males were treated with Tisa-cel and 3 females and 4 males with Axi-cel, while B-ALL patients included 4 females and 8 males with a median age of 10 years (range 3-21), all treated with Tisa-cel.

Evaluation of risk was, for DLBCL patients, based on 3 parameters: disease stage, symptoms and LDH values. The presence of an advanced disease stage, symptoms and LDH values at least one and a half times higher than normal, classified patients as "high risk". 8 out of 20 DLBCL patients (40%) were defined as high risk, while 12 DLBCL patients (60%) showed low risk. For B-ALL

patients, risk classification was defined according to blasts percentage. Patients presented a median of 32% of blasts prior to CAR-T cell infusion (range 0%-90%).

Patients' characteristics are summarized in Table 4.

The study was conducted in accordance with the Declaration of Helsinki and with the approval of the local Ethic Committees.

A

Patients	Disease	Gender	Age	Number of previous lines of treatment	Risk classification (multiparametric evaluation)
#1 MS	DLBCL	F	52	2	Low
#2 SM	DLBCL	M	66	3	Low
#3 SM	DLBCL	F	49	4	Low
#4 PM	DLBCL	M	59	3	Low
#5 GM	DLBCL	F	52	3	Low
#6 MM	DLBCL	M	69	4	High
#7 RV	DLBCL	M	70	2	Low
#8 LF	DLBCL	M	43	2	Low
#9 RG	DLBCL	F	45	2	High
#10 CT	DLBCL	F	53	2	Low
#11 CG	DLBCL	M	48	2	Low
#12 NE	DLBCL	F	71	2	High
#13 FA	DLBCL	M	34	2	High

B

Patients	Disease	Gender	Age	Number of previous lines of treatment	Risk classification (multiparametric evaluation)
#1 FAM	DLBCL	F	56	2	High
#2 RS	DLBCL	F	21	2	Low
#3 RC	DLBCL	F	71	2	High
#4 PM	DLBCL	M	69	2	High
#5 VD	DLBCL	M	57	4	Low
#6 VD	DLBCL	M	23	2	High
#7 GV	DLBCL	M	32	2	Low

C

Patients	Disease	Gender	Age	Number of previous lines of treatment	Blasts percentage
#1 PR	B-ALL	F	15	5	90%
#5 SR	B-ALL	M	20	2	0%
#3 BC	B-ALL	F	16	4	75%
#4 CG	B-ALL	M	21	6	0%
#5 SO	B-ALL	M	8	2	0.8%
#6 RK	B-ALL	F	4	3	0%
#7 JA	B-ALL	M	3	3	0%
#8 KJIY	B-ALL	M	9	2	0.1%
#9 GAS	B-ALL	M	7	2	0.01%
#10 HN	B-ALL	M	6	2	0.1%
#11 EL	B-ALL	M	4	2	1%
#12 OP	B-ALL	F	6	2	7%

Table 4. Patients' characteristics before CAR-T cell treatment. A) DLBCL patients that underwent infusion with Tisa-cel product. **B)** DLBCL patients that underwent infusion with Axi-cel product. **C)** B-ALL patients that underwent infusion with Tisa-cel product.

4.2 Immunophenotyping

Peripheral blood (PB) lymphocytes were obtained from whole blood samples collected on day 0 before CD19 CAR-T cell infusion and at different time points after infusion (days 3, 7, 14, 28; months 3 and 6), following the EuroFlow Bulk Lysis protocol. [66] Flow cytometry was performed using an 8-color monoclonal antibody (moAb) combination to monitor CAR-T cells and lymphocytes subsets (T, B and NK cells). CAR^+ cells were detected by incubation with biotinylated CD19 antigen and FITC-conjugated or APC-conjugated anti-biotin protein, according to manufacturers' instructions (Acro Biosystems, Beijing, China and Miltenyi Biotec, Bergisch Gladbach, Germany, respectively). Lymphocyte subsets (T, B and NK cells) were analyzed by staining with a combination of labelled moAbs against the CD3, CD4, CD8, CD16, CD19, CD45 and CD56 antigens (all antibodies from Becton Dickinson, BD, San Jose, CA) Staining details are reported in Table 5. Samples were acquired using the FACSCanto II flow cytometer collecting at least 30,000 events and analyzed using FACSDIVA software (BD). Absolute cell counts were determined by correlation of the percentage of positive populations to WBC.

CAR	CD3	CD4	CD8	CD16	CD19	CD45	CD56
FITC	PE	APC	APC-Cy7	PerCP	V450	V500	PE-Cy7

Table 5. Monoclonal antibodies and related fluorochromes used for immunophenotypic characterization of CAR-T and lymphocytic cells.

4.3 Intracellular T and NK cell cytokine production

Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood samples collected before the CAR-T cell infusion and at different time points after the infusion as detailed above, by Ficoll-Paque density centrifugation (Nycomed Pharma AS, Oslo, Norway). To assess cytokine production, PBMCs were analyzed after 4 h of incubation with phorbol 12-myristate 13-acetate 25 ng/ml (PMA; Sigma–Aldrich) and ionomycin 1 µg/ml (Iono, Sigma–Aldrich) for cell activation, in the presence of the GolgiStop™ Protein Transport Inhibitor solution (BD Biosciences) for cytokine secretion inhibition. For T and NK cell cytokine production, PBMCs were stained with FITC-, PE-, PerCP-, PE-Cy7-, APC-labelled moAbs against CD3, CD4, CD16, CD56, interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα). After washing, samples were acquired using the FACSCanto I flow cytometer (Becton Dickinson) and analyzed using FACSDIVA software (BD Biosciences).
Absolute cells counts were determined by correlating the percentage of cytokine producing populations to the total lymphocytes numbers.

4.4 Plasma cytokines determination

For plasma collection before and at different time points after CAR-T cell infusion, PB samples were centrifuged at 3000 rpm at 20°C for 10 minutes and the supernatants were carefully harvested and stored at -80°C until cytokine analyses. Concentrations of IL-2, IL-4, IL-6, IL-8, IL-10, IL17a, IFNγ and TNFα in the plasma were quantitatively determined using the Cytometric Bead Array (CBA) technique (CBA Human Th1/Th2/Th17 Cytokine Kit, CBA Human Inflammatory

Cytokines Kit and CBA Flex Set, BD Biosciences) according to the manufacturer's instructions. Following the acquisition of samples on the FACSCanto I flow cytometer (Becton Dickinson), the results were generated in a graphical and tabular format using the BD CBA Software FCAP 3.0.1 (BD Biosciences). A set of standard curves (range from 0 to 5000 pg/ml for CBA Kits and range from 0 to 2500 pg/ml for CBA Flex Set) was obtained from one set of calibrators and a set of results was obtained on one test sample, with a range of detection of 2-5000 pg/ml for CBA Kits and 2-2500 pg/ml for CBA Flex Set, respectively.

4.5 Statistical analysis

The 2-sided Student t test and the two-way ANOVA test were used to evaluate the significance of differences between groups. Results are expressed as the means plus or minus SD. Statistical significance was defined as a P value <0.05.

5. RESULTS

5.1 Clinical outcome of CAR-T cell treatment

Patients treated with CAR-T cells were clinically monitored over time, in terms of evaluation of symptoms, adverse events, response at day 30 and month 3, time to eventual relapse and follow-up time.

Among the 20 DLBCL patients, treatment with Tisa-cel (n=13) elicited no adverse reactions in 30.8% of cases (n=4/13); 38.4% showed fever associated to a grade 1 CRS (n=5/13); 30.8% of patients showed symptoms related to a grade 2 CRS (n=4/13). Among this subgroup, one patient also experienced concurrent grade 1 ICANS.

Axi-cel product (n=7), instead, induced adverse events in all of the DLBCL patients, resulting in grade 1, grade 2 or grade 3 CRS in 71.4% (n=5/7), 14.3% (n=1/7) and 14.3% (n=1/7) of cases, respectively. In 42.8% of patients (n=3/7) the adverse reaction was not associated with any form of neurotoxicity, while 42.8% (3/7) also experienced grade1 ICANS and 14.3% (1/7) manifested a severe grade 5 ICANS, that led to death.

Concerning B-ALL patients (n=12), 58.3% (n=7) did not show any symptom related to adverse events; 16.7% (n=2) manifested grade 1 CRS and 8.3% (n=1) of patients manifested grade 2 CRS, while 16.7% (n=2) of B-ALL patients showed a clinical manifestation categorized as grade 4 CRS. None of the B-ALL patients experienced ICANS.

Response was evaluated at day 30 and month 3 and was defined, for DLBCL, on the basis of positron emission tomography (PET) scan, according to the Lugano criteria (2017), while, for B-ALL, through immunophenotypic and, when feasible, molecular blasts monitoring.

5.1.1 Clinical response of DLBCL Tisa-cel patients

At day 30, 76.9% of DLBCL patients treated with Tisa-cel reached a complete remission (CR, n=10/13), meaning that no residual mass was detected. One out of 13 patients (7.7%) only reached a partial response (PR); this patient, although responding favorably to the therapy, never achieved a CR. 15.4% of patients (2/13) had a progressive disease (PD), meaning that no positive response was achieved and the disease was spreading, instead.

At month 3, 46.2% of patients (6/13) maintained a CR, while relapse occurred in 30.8% of cases (n=4/13). Patients that presented PR and PD at day 30, showed PD at 3 months.

For evaluable patients at subsequent follow-up, DLBCL patients treated with Tisa-cel, presented a 53.8% relapse rate (n=7/13), with a median of 3.7 months after infusion. These patients were monitored for a median of 11.5 months.

5.1.2 Clinical response of DLBCL Axi-cel patients

As for DLBCL patients treated with Axi-cel, at day 30, CR was achieved in 57.1% of cases (n=4/7), while 28.6% of patients (n=2/7) achieved a PR. One out of 7 patients (14.3%) presented a PD. At the 3rd month, all the CR patients maintained this condition, while the patient with PD and one patient who presented a PR, did not show a better response and presented a PD. The other patient who achieved a PR at day 28, unfortunately died from toxicity before clinical evaluation at 3 months. These patients were monitored for a median of 5.5 months during which they maintained the response observed at month 3.

5.1.3 Clinical response of B-ALL Tisa-cel patients

B-ALL patients achieved a CR in 66.7% of cases (n=8/12) at day 30. 25% of patients (n=3/12) only reached a PR, while one patient (8.3%) presented a PD. At month 3, 50% of B-ALL patients

(n=6/12) maintained a CR state, while relapse occurred in 25% of cases (3/12). PR patients evolved as follows: one patient achieved a CR, while the other two patients presented a PD. 83.3% of B-ALL patients (10/12) had a relapse to subsequent follow-up, with a median of 6.1 months after the infusion. B-ALL patients were monitored for a median of 16.9 months.

Patients' clinical outcomes are summarized in Table 6.

A

Patients	Disease	CRS grade	ICANS grade	Response at day 30	Response at day 180	Time to relapse after infusion (months)	Follow-up (months)
#1 MS	DLBCL	0	0	CR	CR	7.7	28.3
#2 SM	DLBCL	0	0	CR	CR	-	21.5
#3 SM	DLBCL	2	0	CR	CR	6.9	11.4
#4 PM	DLBCL	0	0	PD	PD	PD	11.7
#5 GM	DLBCL	0	0	CR	CR	-	17.0
#6 MM	DLBCL	2	1	CR	REL	2.1	15.6
#7 RV	DLBCL	2	0	CR	REL	2.9	10.4
#8 LF	DLBCL	1	0	CR	CR	-	10.7
#9 RG	DLBCL	2	0	PR	PD	2.2	5.7
#10 CT	DLBCL	1	0	CR	REL	1.0	5.4
#11 CG	DLBCL	1	0	CR	CR	-	3.0
#12 NE	DLBCL	1	0	CR	REL	3.3	4.6
#13 FA	DLBCL	1	0	PD	PD	PD	3.6

B

Patients	Disease	CRS grade	ICANS grade	Response at day 30	Response at day 180	Time to relapse after infusion (months)	Follow-up (months)
#1 FAM	DLBCL	1	1	PR	PD	1.9	3.7
#2 RS	DLBCL	3	1	CR	CR	-	11.8
#3 RC	DLBCL	1	1	PD	PD	PD	3.8
#4 PM	DLBCL	1	0	CR	CR	-	7.6
#5 VD	DLBCL	1	0	CR	CR	-	5.7
#6 VD	DLBCL	2	5	PR	-	2.1	2.0
#7 GV	DLBCL	1	0	CR	CR	-	3.6

C

Patients	Disease	CRS grade	ICANS grade	Response at day 30	Response at day 180	Time to relapse after infusion (months)	Follow-up (months)
#1 PR	B-ALL	4	4	PR	PD	2.0	2.7
#5 SR	B-ALL	0	0	CR	REL	3.0	21.3
#3 BC	B-ALL	4	0	PR	REL	2.1	17.9
#4 CG	B-ALL	0	0	PR	CR	17.1	24.5
#5 SO	B-ALL	0	0	PD	PD	PD	24.2
#6 RK	B-ALL	1	0	CR	CR	-	21.0
#7 JA	B-ALL	0	0	CR	CR	-	22.2
#8 KJIY	B-ALL	2	0	CR	CR	6.1	15.9
#9 GAS	B-ALL	1	0	CR	CR	13.1	14.6
#10 HN	B-ALL	0	0	CR	REL	1.8	13.9
#11 EL	B-ALL	0	0	CR	CR	12.6	13.6
#12 OP	B-ALL	0	0	CR	REL	1.8	11.5

Table 6. Patients' clinical outcome after CAR-T cell treatment. A) DLBCL patients treated with Tisa-cel product. **B)** DLBCL patients treated with Axi-cel product. **C)** B-ALL patients treated with Tisa-cel product.

5.2 CAR-T cell peripheral monitoring

Infused CAR-T cells were monitored in peripheral blood at different time-points, both in percentage and in absolute numbers.

Engineered cells showed, for DLBCL patients treated with Tisa-cel, a decreasing trend. This trend was comparable in percentage and absolute number and did not show any expansion peak following infusion, but conversely a marked decrease was observed up to the 14^{th} day of monitoring, and a subsequent maintenance of the low concentration up to the 6^{th} month. The decrease was statistically significant for percentage values at days 14 (p=0.02) and 28 (p=0.02) (Figure 3A).

Concerning the Axi-cel product in DLBCL patients, we observed an expansion peak on day 7, going from an average percentage of 3.7%±1.9 on day 3 to 6.1%±12.3 on day 7. However, on subsequent monitoring days, a sharp concentration drop was observed, reaching an average percentage of 0.48±0.15 at day 14. The decrease, compared to the value at day 3, was statistically significant at day 14 (p=0.001), day 28 (p=0.001) and month 3 (p=0.01). The trend of absolute values differed considerably, showing a slight increase, from 0.013±0.008 CAR-T cells $x10^9$/L at day 3 to 0.036±0.05 of month 6. Absolute values were, however, not statistically significant (Figure 3B).

In B-ALL patients, a numerical peak of peripheral CAR-T cells was observed at 14 days after infusion. Average percentage values of 0.97±2.2 at day 3 moved to 2.25±2.01 at day 7 and reached 12.67±20.12 at day 14. A subsequent decrease was observed at day 28, months 3 and 6. The trend was confirmed in absolute numbers: at days 3 and 7 the concentration of CAR-T $x10^9$/L in PB was low (0.018±0.05 and 0.017±0.03, respectively) and showed a peak increase at day 14 (0.10±0.25), followed by a rapid decrease. The variations were not statistically significant, due to high inter-patients' variability (Figure 3C).

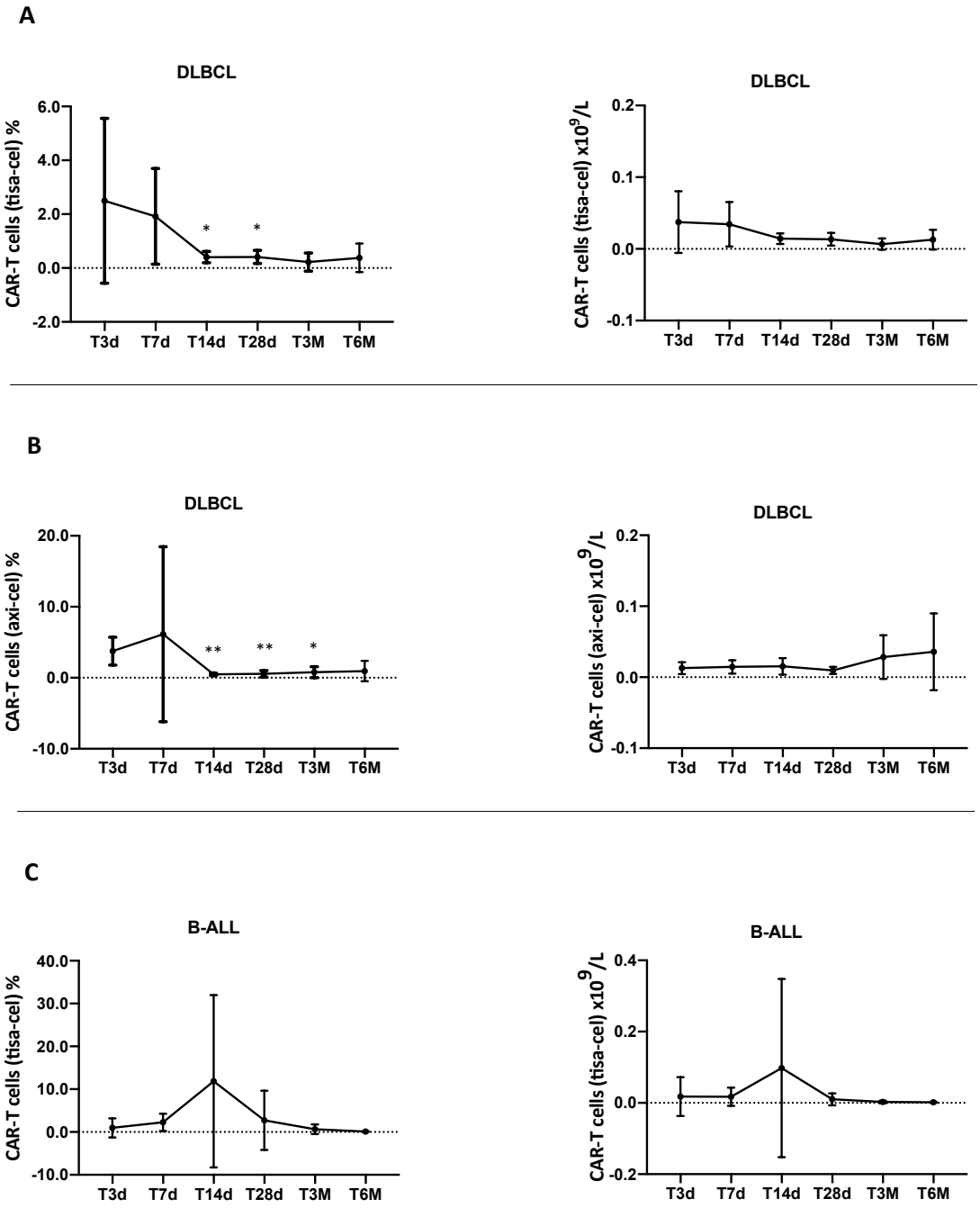


Figure 3. *In vivo* longitudinal monitoring of CD19 CAR-T cells at different time-points after infusion. Data are reported as means and standard deviations of the percentage (left panel) and absolute number (right panel) of CAR^+ cells in DLBCL patients treated with Tisa-cel (**A**), DLBCL patients treated with Axi-cel (**B**) and B-ALL patients (**C**).

5.3 $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ peripheral T-cell subset monitoring

Main lymphocytes subsets were monitored over time, before (T0) and after (T3d, T7d, T14d, T28d, T3M, T6M) CAR-T cells infusion.

A constant increase in the absolute numbers of peripheral CD3 lymphocytes was observed in DLBCL patients treated with Tisa-cel, with a major expansion observed at month 3. More in detail, these patients presented an average absolute value of 0.05±0.04 CD3 lymphocytes $x10^9$/L before infusion and gradually reached the value of 0.97±0.62 cells at month 3. At month 6, we observed a slight decrease, presenting an average absolute value of 0.80±0.35 CD3 $x10^9$/L. All the variations were statistically significant when compared to T0 (Figure 4A red line).

In DLBCL patient treated with Axi-cel, CD3 lymphocytes showed a maximum increasing peak at day 14 (0.58±0.58), followed by a smaller decrease at day 28 (0.32±0.24) and a subsequent further rise (0.42±0.47 at month 3 and 0.44±0.24 at month 6). However, variations compared to T0 were not statistically significant (Figure 4A blue line).

Variations induced by the two types of CAR-T products were compared using a two-way ANOVA test, resulting in the Tisa-cel product being able to induce a significantly greater increase in CD3 lymphocytes than the Axi-cel product (p=0.01) (Figure 4A).

In B-ALL patients the number of circulating CD3 cells ($x10^9$/L) increased significantly from T14d and the highest values were observed at T6M. In detail, CD3 cells were at T0 0.15±0.16, at T3d 0.49±0.59 (NS), at T7d 0.85±1.23 (NS), at T14d 1.12±0.99 (p=0.004), at T28d 0.88±0.72 (p=0.003), at T3M 0.82±0.52 (p=0.0008) and at T6M 1.62±0.90 (p=0.0001) (Figure 4B). A similar behavior was observed for $CD3^+$ $CD4^+$ (T4) lymphocytes.

In DLBCL patients, Tisa-cel induced a constant and significant increase of peripheral T4 cells at all monitoring time-points. T4 lymphocytes $x10^9$/L raised from 0.02±0.02 at T0 up to the maximum

absolute value of 0.40±0.33 at day 14 (Figure 4C red line). In Axi-cel case study, T4 population showed an increasing trend up to month 3, from 0.09±0.16 (T0) to 0.13±0.14 (T3M), although not statistically significant (Figure 4C blue line).

Even for the T4 population, it was evaluated whether the differential variations induced by the two CAR-T products could be statistically significant by the ANOVA test, and, as with CD3, the Tisa-cel product was found to induce a significantly greater increase in T4 lymphocytes with respect to the Axi-cel product (p=0.01) (Figure 4C).

In B-ALL patients, Tisa-cel induced a gradual increase in circulating T4 cells, which resulted significant starting at day 7 up to month 6 of monitoring. In detail, at baseline we found an average absolute value of 0.07±0.06, at day 7 0.21±0.21 (p=0.03), at day 14 0.35±0.31 (p=0.007), at day 28 0.29±0.24 (p=0.009), at month 3 0.30±0.25 (p=0.006) and at month 6 0.56±0.47 (p=0.003) x10^9/L T4 cells (Figure 4D).

A steady increase over time of $CD3^+$ $CD8^+$ (T8) lymphocytes was also observed in DLBCL patients treated with Tisa-cel. Actually, peripheral T8 concentration raised from 0.03±0.04 x10^9/L at T0 to a maximum absolute value of 0.59±0.54 at month 3. All the variations recorded were statistically significant (Figure 4E red line).

On the other hand, in DLBCL patients Axi-cel induced T8 variations that did not result in any statistical significance, even though the trend was steadily increasing (Figure 4E blue line).

ANOVA test, likewise the aforementioned lymphocytic populations, revealed that Tisa-cel CAR-T product induced significantly greater variations in T8 lymphocytes than the Axi-cel product (Figure 4E).

In B-ALL patients, T8 showed a comparable trend to T4 subpopulation, showing a peak at day 14, from 0.07±0.08 at baseline to 0.70±0.86 at T14d, and then a second increase at month 6 (0.90±0.66 x10^9/L T8 cells). Variations were significant starting from day 14 (Figure 4F).

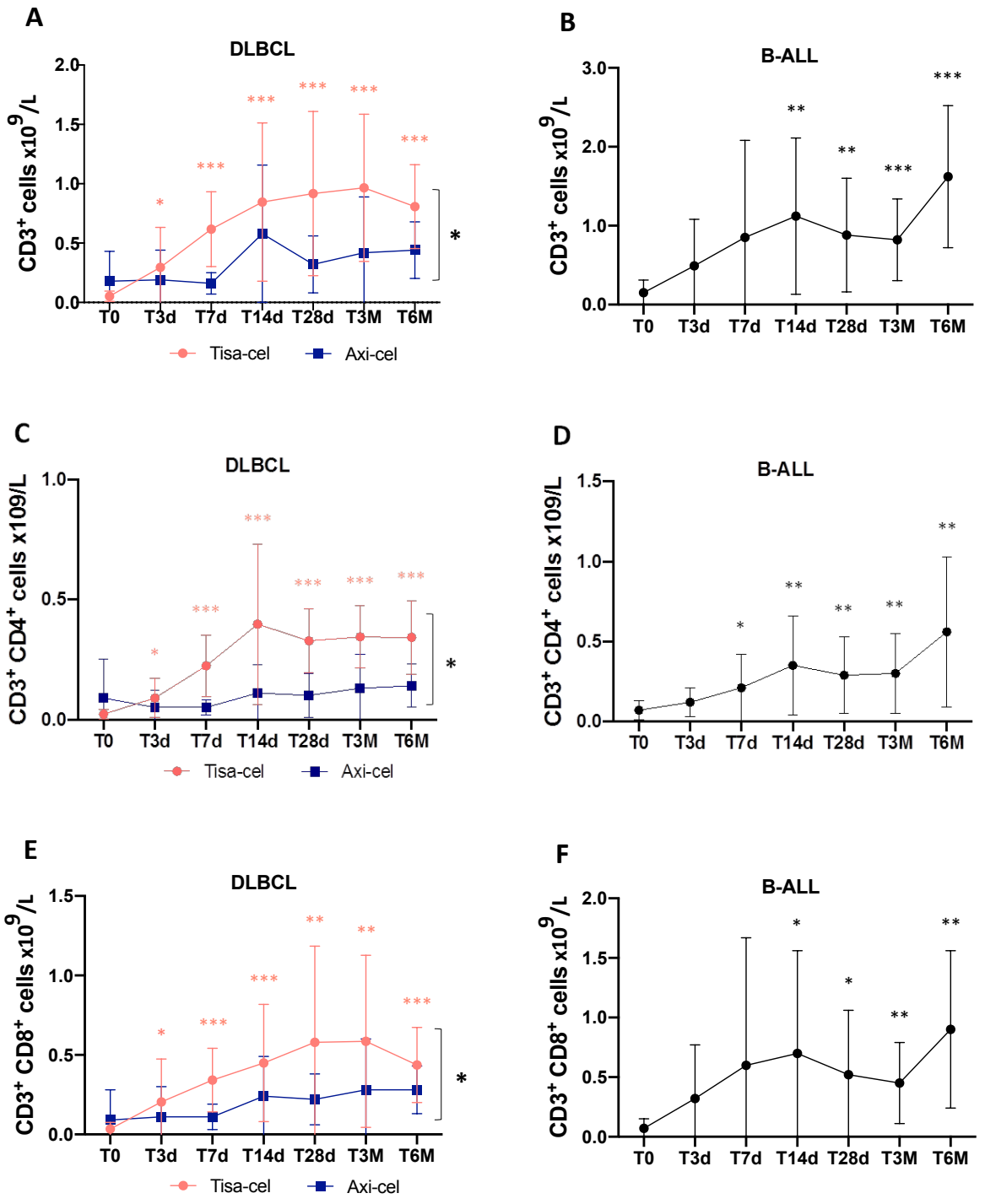


Figure 4. *In vivo* longitudinal monitoring of peripheral blood (PB) $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes at different time-points after CAR-T cell infusion. Patients' PB samples were collected prior to CAR-T cell infusion (T0) and after 3, 7, 14, 28 days and 3, 6 months. Data are reported as means and standard deviations of the absolute number of $CD3^+$ in DLBCL (**A**) and B-ALL patients (**B**), $CD3^+CD4^+$ in DLBCL (**C**) and B-ALL patients (**D**) and $CD3^+CD8^+$ lymphocytes in DLBCL (**E**) and B-ALL patients (**F**). Significant differences are calculated respect to T0 and are indicated as ***p<0.001, **p<0.01, *p<0.05.

5.4 NK cell monitoring

In a subgroup of 20 DLBCL patients, treated at the Hematology Center at the 'Sapienza' University of Rome, circulating NK cells ($CD3^+$ $CD56^+$ $CD16^+$) were evaluated as absolute number $x10^9/L$.

As previously described for other lymphocytic populations, we observed that Tisa-cel CAR-T product induced a constant increase of the peripheral concentration of NK cells. The increase became statistically significant from day 7 and was maintained up to month 6 (Figure 5 red line).

In patients treated with Axi-cel, NK population showed an expansion in the range of 7 days to 3 months, achieving a statistical significance from day 14 to month 6 when compared to baseline (Figure 5 blue line).

Trend comparison analysis of the two case studies treated with two different CAR-T products, performed with the ANOVA test, did not result significant, stating that the two CAR-T products induced similar variations of NK population (Figure 5).

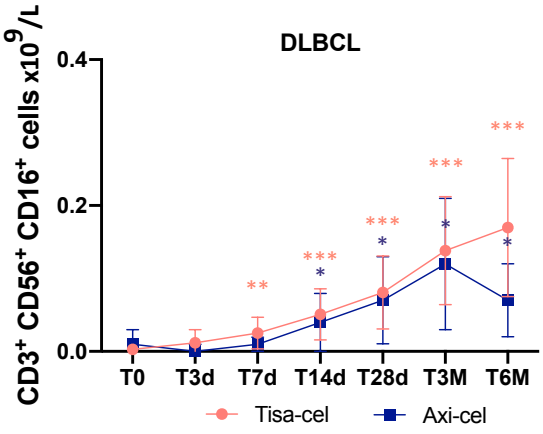


Figure 5. ***In vivo*** **longitudinal monitoring of peripheral blood (PB) NK lymphocytes at different time-points after CAR-T cell infusion.** PB samples from patients enrolled at Sapienza University of Rome (20 DLBCL) were collected prior to CAR-T cell infusion (T0) and after 3, 7, 14, 28 days and 3, 6 months. Data are reported as means and standard deviations of the absolute number of NK lymphocytes. Significant differences are calculated compared to T0 and are indicated as ***p<0.001, **p<0.01, *p<0.05.

5.5 Immunomodulatory cytokine production by stimulated lymphocyte populations

In the same subgroup of 20 DLBCL patients, treated either with Tisa-cel or Axi-cel, numeric variations of main lymphocytic populations able to produce immunomodulatory cytokines (IFNγ and TNFα) following stimulation were assessed.

Concerning IFNγ production, the trend was similar for every lymphocytic population studied. In DLBCL patients treated with Tisa-cel a gradual and constant increase in the absolute number of IFNγ producing cells was observed. For CD3, from 0.02±0.02 $x10^9$/L producing cells at T0, a value of 0.56±0.22 was detected at month 6, with every variation from day 3 to month 6, being statistically significant compared to baseline (Figure 6A, red line, left panel). Likewise, in IFNγ producing T4 population, Tisa-cel infusion induced an increase, from 0.01±0.01 $x10^9$/L cells at T0 to 0.30±0.30 at month 6, with gradual and significant increases at every time-point (Figure 6B, red line, left panel). The IFNγ producing T8 cells also showed a steady increase, from 0.01±0.01 at T0 to 0.38±0.27 at month 6, with all changes being, again, statistically significant (Figure 6C, red line, left panel). Same for IFNγ producing NK cells, from 0.001±0.001 observed at T0, to a gradual and significant increase up to 0.05±0.07 $x10^9$/L cells at month 6 (Figure 6D, red line, left panel).

TNFα producing cells also increased in absolute numbers following infusion with Tisa-cel. More in depth, from values of 0.04±0.03 for CD3, 0.03±0.03 for T4, 0.01±0.01 for T8 and 0.001±0.001

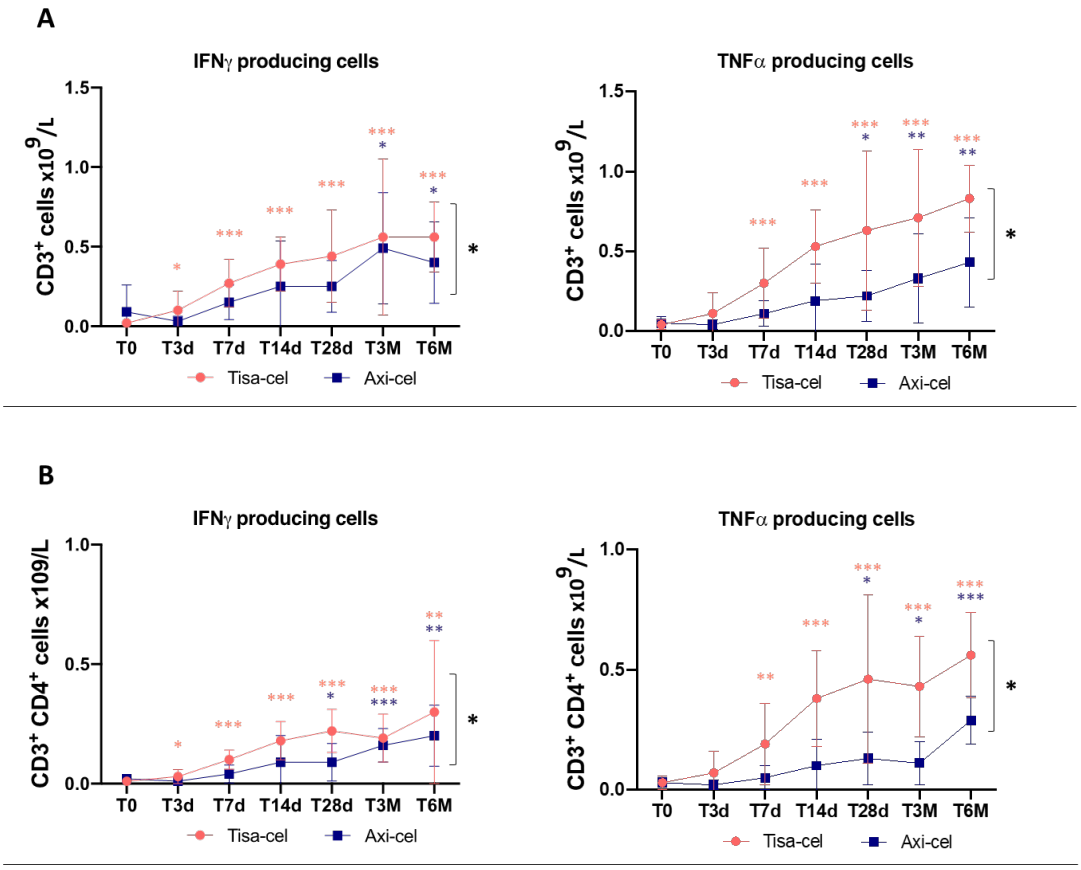
x10^9/L for NK cells at T0, we observed absolute values of 0.83±0.21, 0.56±0.18, 0.38±0.24 and 0.04±0.06 x10^9/L at month 6, for TNFα producing CD3, T4, T8 and NK cells, respectively, with variations being statistically significant from day 7 of monitoring up to month 6 for every population studied (Figure 6A, B, C, D, red line, right panel).

Infusion with Axi-cel in DLBCL patients induced similar variations in lymphocytic populations producing IFNγ and TNFα.

A progressively increasing trend was indeed observed. In IFNγ producing CD3 cells, infusion with Axi-cel induced an increase that went from 0.09±0.17 at baseline to 0.40±0.25 x10^9/L cells at month 6, with a major expansion peak at month 3 (0.49±0.35), gaining statistical significance at months 3 and 6 (Figure 6A, blue line, left panel). The absolute value of IFNγ producing T4 cells grew from 0.02±0.01 at T0 to 0.20±0.13 at month 6, with variations being significant from day 28 to month 6 (Figure 6B, blue line, left panel); IFNγ producing T8 cells increased from an absolute value of 0.08±0.16 at T0 to 0.39±0.13 at month 6, with a numerical peak observed at month 3 (0.50±0.29 x10^9/L cells) (Figure 6C, blue line, left panel); IFNγ producing NK cells grew from 0.0004±0.0002 at baseline to 0.07±0.04 at month 6, with variations at day 14, month 3 and 6 being significant compared to T0 (Figure 6D, blue line, left panel).

TNFα producing cells also showed numerical expansion following Axi-cel infusion during the monitoring period. TNFα producing CD3 and T4 cells showed a very similar trend and increased from 0.05±0.04 and 0.029±0.016 at baseline to 0.43±0.28 and 0.29±0.09 at month 6, respectively, with the last three time-points being significant (Figure 6A, B, blue line, right panel). TNFα producing T8 cells achieved a statistical significance at month 6, with the absolute number of 0.34±0.18 compared to 0.07±0.15 observed at T0 (Figure 6C, blue line, right panel). TNFα producing NK cells also showed an expansion, from 0.0003±0.0002 at T0 to 0.05±0.03 at month 6. Values at days 7, 14 and month 6 were statistically significant (Figure 6D, blue line, right panel).

In order to understand whether the infusion of either CAR-T product could result in a different expansion of immunocompetent cells, the ANOVA test was used. This test revealed a statistically significant difference between the two CAR-T products in CD3 and T4 populations, but not in T8 and NK populations, meaning that Tisa-cel CAR-T product induced a greater expansion of IFNγ producing CD3 and T4 cells, while the two CAR-T products induced a similar expansion of IFNγ producing T8 and NK cells. Same results were obtained concerning TNFα producing cells: Tisa-cel CAR-T product induced a greater expansion of TNFα producing CD3 and T4 cells, while the two CAR-T products induced a similar expansion of TNFα producing T8 and NK cells.



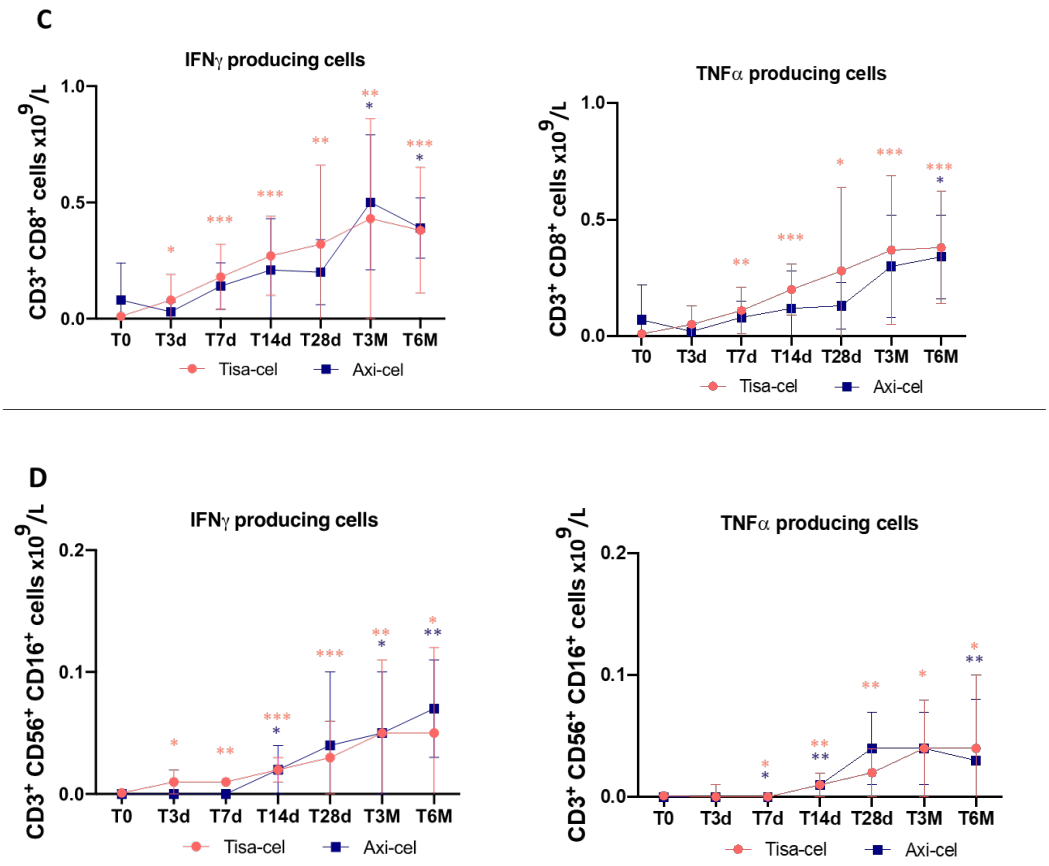


Figure 6. ***In vivo*** **longitudinal monitoring of absolute numbers of IFNγ and TNFα producing CD3$^+$, CD3$^+$ CD4$^+$, CD3$^+$ CD8$^+$ and CD3$^+$ CD56$^+$ CD16$^+$ cells at different time-points after CAR-T cell infusion.** PB samples from patients enrolled at Sapienza University of Rome (20 DLBCL) were collected prior to CAR-T cell infusion (T0) and after 3, 7, 14, 28 days and 3, 6 months and activated *in vitro* to evaluate IFNγ (left panel) and TNFα (right panel) production. Data are reported as means and standard deviations of the absolute number of CD3$^+$ (**A**), CD3$^+$ CD4$^+$ (**B**), CD3$^+$ CD8$^+$ (**C**) and CD3$^+$ CD56$^+$ CD16$^+$ (**D**) cells in DLBCL patients treated with Tisa-cel (red line) or Axi-cel (red line). Significant differences are calculated compared to T0 and are indicated as ***p<0.001, **p<0.01, *p<0.05.

5.6 Plasma cytokine levels

Finally, in the same subgroup of 20 DLBCL patients treated either with Tisa-cel or Axi-cel, plasma cytokine levels were measured before and after CAR-T cell infusion.

An increase in plasma concentration was observed for IL-6, IL-8 and IL-10, while, in contrast, no increases were observed for IL-2, IL-4, IL-17a, IFNγ and TNFα.

The cytokine plasma levels were correlated with the occurrence of a cytokine release syndrome (CRS). It is well known [56] that IL-6 is the main plasmatic cytokine to mediate inflammatory reactions and that this adverse event usually occurs within the first week after infusion.

Indeed, we observed that patients who experienced grade 1 CRS presented an average IL-6 plasmatic concentration of 50.43±78.59 pg/ml at day 3 and 289.08±513.90 pg/ml at day 7, while patients who experienced grade 2 or higher had an average concentration of 592.53±1179.84 at day 3 and 412.79±658.51 pg/ml at day 7, in contrast to the concentration of 11.94±4.78 and 5.26±3.43 pg/ml observed at days 3 and 7, respectively, in patients who did not experience adverse reactions.

On the other hand, we observed that ICANS was mainly mediated by IL-8, since patients who manifested ICANS showed an average IL-8 concentration of 68.59±94.52 pg/ml at day 3 and 53.97±40.86 pg/ml at day 7, in contrast to average values of 29.92±66.27 at day 3 and 22.42±35.43 pg/ml at day 7 showed by patients who did not experience ICANS (Figure 7).

IL-6 and IL-8 levels also showed a correlation with tumor burden: following the categorization of patients into the two categories of high and low tumor burden, trend analysis of these two cytokines revealed that both IL-6 and IL-8 were significantly higher in the high tumor burden category at the different time-points. More in detail, patients with high tumor burden presented an average level of IL-6 of 58.86±52.83 at day 28 compared to 14.77±21.29 pg/ml observed in patients with low tumor burden. IL-8 at days 0, 7 and month 3 presented average plasmatic values of 11.04±4.86, 44.65±43.59 and 8.48±4.23, respectively, in patients with high tumor burden, in contrast to values of 4.63±3.73 (T0), 12.33±16.79 (T7d) and 4.37±1.88 (T3M) observed in patients with low tumor burden.

To further investigate the role of IL-8, plasmatic levels were correlated to clinical response. Interestingly, in regards to the response at day 30, we observed that patients who responded partially (partial response, PR) or did not respond (progressive disease, PD) to CAR-T therapy showed plasmatic levels of IL-8 significantly higher at days 3 and 28 in comparison to patients who achieved a CR. As for response at month 3, patients who still presented a PD showed, likewise, average levels of IL-8 significantly higher than patients in CR at days 3 and 28. Patients who relapsed, on the other hand, showed low levels of IL-8, comparable to levels expressed by patients in CR (Figure 8).

IL-10 showed a trend with an expansion peak at day 7. Although, considering cytokine levels in patients treated with Tisa-cel or Axi-cel separately, we could note that the aforementioned peak resulted primarily from the Axi-cel product (Figure 9C).

When patients were categorized on the basis of the CAR-T cell product infused, we could observe that IL-6 was mainly produced by patients treated with Tisa-cel (Figure 9A), in contrast to the trend of IL-8, that was most expressed by patients treated with Axi-cel (Figure 9B), in accordance to the observations that Tisa-cel induces a bigger expansion of the main lymphocytic populations and that Axi-cel product induces a higher number of ICANS compared to Tisa-cel product (1/13 Tisa-cel, 4/7 Axi-cel).

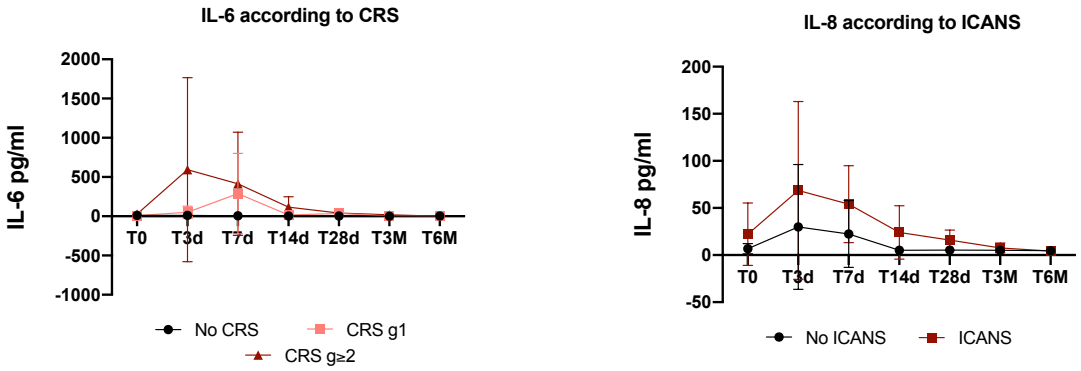


Figure 7. Plasmatic levels of IL-6 and IL-8 in DLBCL patients (n=20) at different time-points after CAR-T cell infusion. Plasma was collected from the patients' peripheral blood (PB) prior to CAR-T cell infusion (T0) and after 3, 7, 14, 28 days and 3, 6 months. Patients were categorized on the basis of the occurrence of CRS (left panel) or ICANS (right panel). Data are reported as cytokine concentration (pg/ml).

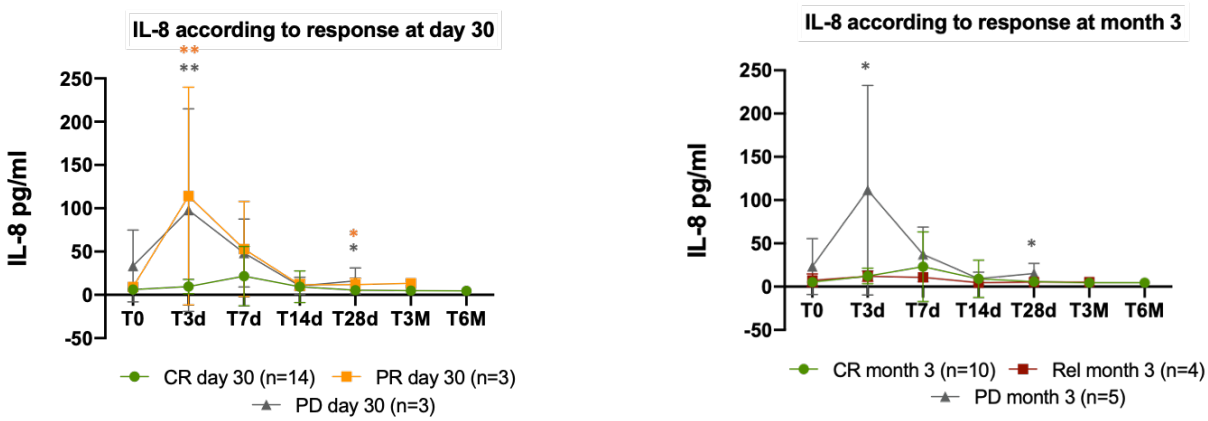


Figure 8. Plasmatic levels of IL-8 in DLBCL patients (n=20) at different time-points after CAR-T cell infusion. Plasma was collected from the patients' peripheral blood (PB) prior to CAR-T cell infusion (T0) and after 3, 7, 14, 28 days and 3, 6 months. Patients were categorized on the basis of the response at day 30 (left panel) and month 3 (right panel) in CR (complete response), PD (progressive disease), PR (partial response) and Rel (relapse). Data are reported as cytokine concentration (pg/ml). Significant differences are calculated compared to T0 and are indicated as ***p<0.001, **p<0.01, *p<0.05.

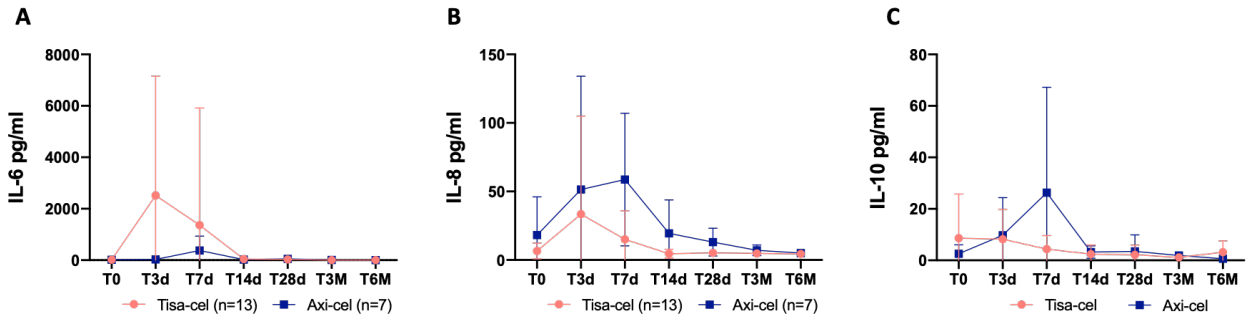


Figure 9. Plasmatic levels of IL-6 (A), IL-8 (B) and IL-10 (C) in DLBCL patients (n=20) at different time-points after CAR-T cell infusion. Plasma was collected from the patients' peripheral blood (PB) prior to CAR-T cell infusion (T0) and after 3, 7, 14, 28 days and 3, 6 months in patients treated with Tisa-cel (red lines) or Axi-cel (blue lines) CAR-T product. Data are reported as cytokines concentration (pg/ml).

6. DISCUSSION

This study aimed at investigating the effects of CAR-T cell administration in the control of R/R DLBCL and B-ALL, and at highlighting the immunomodulatory effects induced over time *in vivo* on the host immune system of the treated patients. This work also intended to investigate the different changes elicited by different co-stimulatory molecules.

The results obtained i) confirmed the ability of anti CD19 CAR-T cells to control the disease at least for a limited period of time; ii) documented a significant increase over time of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ and NK cells coupled to iii) an increased capability of T lymphocytes to produce IFNγ and TNFα; iv) documented that 4-1BB co-stimulation exerts bigger expansion of the lymphocytic compartment; v) confirmed that CD28 co-stimulation elicits higher toxicity but also higher efficacy in comparison to 4-1BB and vi) shed light into the role of IL-6 and IL-8 in adverse events.

While some studies have focused on the characterization of CAR-T infused cells, [67, 68] little is known on the *in vivo* effects of this treatment on the patients' immune lymphocyte populations. Our study demonstrates that CAR-T cells are capable of inducing a bystander effect on the lymphoid populations of patients evaluated from the time of the infusion (T0) up to six months (T6M) after the treatment. We could indeed document a constant, prolonged and significant increase in $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$, as well as in the NK cell population (Fig. 4, 5). Even more importantly, we could show for the first time the ability of T lymphocytes to produce IFNγ and TNFα (Fig. 6), cytokines that are essential in the process of recognition and antitumor cytotoxic capacity. [69, 70]

To the best of our knowledge, these are the first data showing that the infusion of CAR-T cells in patients with DLBCL and B-ALL induce a marked and long-lived modulation of the host immune system in terms of T and NK cell expansion, and cytokine release. So far, we had indirect evidence

from pre-clinical models that pointed towards a possible crosstalk between CAR-T cells and the immune system. Boulch *et al* [71] reported in a mouse B-cell lymphoma model the impact of $CD4^+$ and $CD8^+$ CAR-T cells on the host immune system and the possible role of cytokines released *in vivo*, underlining the importance of a cross-talk between CAR-T cells and the tumor microenvironment in order to enable an optimal anti-tumor CAR-T cell efficacy. Based on data obtained in a syngeneic mouse glioblastoma it has also been reported that CAR-T cells has the potential to establish a cross-talk with the tumor microenvironment, essential to promote endogenous anti-tumor immunity involving the *in vivo* production of interferon-γ (IFNγ). [72] In addition, Pei-Hsuan Chen et al, [73] through the histopathological examination of DLBCL tumor microenvironment in patients treated with the anti-CD19 CAR-T cell product Axi-cel, demonstrated the presence of high levels of activated non-CAR-T cells expressing Ki-67, IFNγ, granzyme B and/or PD-1. These non-CAR-T cells were also the exclusive source of IL-6, the most crucial cytokine associated with the CRS. The authors suggest a role for CAR-T cells in activating non-CAR immune cells within the tumor microenvironment, enhancing the anti-tumoral cytotoxicity as well as the risk of immune-mediated adverse effects. Alizadeh D et al [72] reported that CAR-T cells can boost the activity of resident myeloid cells and endogenous T cells, emphasizing the role of both innate and adaptive host immunity for CAR-T cell therapy of solid tumors.

Taken together, these data indicate that the role of CAR-T cells appears to extend beyond their capacity to kill neoplastic cells, showing that they are also capable of exerting a marked modulation and activation of the patient's immune compartment that persists over time. It is likely that this *in vivo* host immune modulation may play a role in the long-term control of the disease, at a time when CAR-T cells may be barely found in treated patients. [73, 74]

The results obtained also highlight the fact that different co-receptors endow T cells with different functions. It is widely assumed that different phenotypes of engineered products lead to a different

effect on the host immune system, probably as a result of the fact that, upon target recognition, co-stimulatory domains mediate a cell response that differs in terms of strength and kinetics. [75] A rapid and overwhelming immune response can be more commonly observed in patients treated with Axi-cel product, that carries CD28 co-stimulatory domain, that, in fact, outcomes in a higher frequency of adverse events (100% CRS and 57.1% of ICANS compared to 56% CRS and 8% ICANS observed in Tisa-cel treated patients).

Therefore, 4-1BB co-stimulation confers a more favorable safety profile in comparison to CD28 co-stimulation that, on the other hand, in agreement with data in the literature, presents a higher efficacy, since relapse rate observed on our cohort of patients results in 64% for Tisa-cel and 28.6% for Axi-cel product.

The presence of 4-1BB or CD28 domain affects, in turn, T cells function and fate and our results suggest that infusion with Tisa-cel product induces a bigger expansion of T lymphocytes compartment, which is also found to be functionally more active in producing immunomodulatory cytokines. On the basis of the literature, it is possible to speculate that CD28 promotes a fast and strong signaling that is effective in counteracting cancer cells but also prompts T cell exhaustion.

This work also sheds light on the role of plasma cytokines and their involvement in adverse events.

We confirmed the prominent role of IL-6 in mediating CRS, but we also emphasized the involvement of IL-8 in ICANS pathophysiology (Fig. 7).

A work by Gust and colleagues (2017) [76] states that IL-8 might be elevated during neurotoxicity as a consequence of endothelial activation and release of Weibel-Palade bodies in which the cytokine is stored; this process may lead to an enhanced vascular permeability and inflammatory cytokines leakage to blood brain barrier.

Interestingly, plasmatic levels of IL-8 seem to be related to clinical response, as early (T3d) high IL-8 levels are found in patients who presented a partial or no response to CAR-T treatment at day 30 and

month 3 (Fig. 8). This evidence has a considerable clinical relevance, since monitoring plasma levels of this cytokine can help understanding the patients' symptomatology and adapt the clinic accordingly. IL-8, emerges, thus, as a new response predictor marker.

In summary, our results clearly suggest that in patients with DLBCL and B-ALL the administration of CAR-T cells is capable of inducing a marked *in vivo* reshaping of the host immune system, both in children and adults and provide the basis for a better understanding of the immunologic signatures involved in the response and adverse events to promptly manage these life-threatening conditions.

7. BIBLIOGRAPHY

1. Tietsche de Moraes Hungria V, Chiattone C, Pavlovsky M, et al. Epidemiology of Hematologic Malignancies in Real-World Settings: Findings From the Hemato-Oncology Latin America Observational Registry Study. *J Glob Oncol*. 2019;5:1-19.

2. Li L, Clevers H. Coexistence of quiescent and active adult stem cells in mammals. *Science*. 2010;327(5965):542-545.

3. Hu D, Shilatifard A. Epigenetics of hematopoiesis and hematological malignancies. *Genes Dev*. 2016;30(18):2021-2041.

4. Whiteley AE, Price TT, Cantelli G, Sipkins DA. Leukaemia: a model metastatic disease. *Nat Rev Cancer*. 2021;21(7):461-475.

5. Rose-Inman H, Kuehl D. Acute leukemia. *Emerg Med Clin North Am*. 2014;32(3):579-596.

6. Lai R, Hirsch-Ginsberg CF, Bueso-Ramos C. Pathologic diagnosis of acute lymphocytic leukemia. *Hematol Oncol Clin North Am*. 2000;14(6):1209-1235.

7. Chiaretti S, Zini G, Bassan R. Diagnosis and subclassification of acute lymphoblastic leukemia. *Mediterr J Hematol Infect Dis*. 2014;6(1):e2014073.

8. Inaba H, Mullighan CG. Pediatric acute lymphoblastic leukemia. *Haematologica*. 2020;105(11):2524-2539.

9. Chiaretti S, Gianfelici V, Ceglie G, Foà R. Genomic characterization of acute leukemias. *Med Princ Pract*. 2014;23(6):487-506.

10. Lilljebjörn H, Fioretos T. New oncogenic subtypes in pediatric B-cell precursor acute lymphoblastic leukemia. *Blood*. 2017;130(12):1395-1401.

11. Ribera J, Zamora L, García O, Hernández-Rivas JM, Genescà E, Ribera JM. [Copy number alterations in adult patients with mature B acute lymphoblastic leukemia treated with specific immunochemotherapy]. *Med Clin (Barc)*. 2016;147(11):488-491.

12. Messina M, Chiaretti S, Fedullo AL, et al. Clinical significance of recurrent copy number aberrations in B-lineage acute lymphoblastic leukaemia without recurrent fusion genes across age cohorts. *Br J Haematol*. 2017;178(4):583-587.

13. Conter V, Bartram CR, Valsecchi MG, et al. Molecular response to treatment redefines all prognostic factors in children and adolescents with B-cell precursor acute lymphoblastic leukemia: results in 3184 patients of the AIEOP-BFM ALL 2000 study. *Blood*. 2010;115(16):3206-3214.

14. Hamilton BK, Rybicki L, Abounader D, et al. Allogeneic Hematopoietic Cell Transplantation for Adult T Cell Acute Lymphoblastic Leukemia. *Biol Blood Marrow Transplant*. 2017;23(7):1117-1121.

15. Bassan R, Hoelzer D. Modern therapy of acute lymphoblastic leukemia. *J Clin Oncol*. 2011;29(5):532-543.

16. Foà R, Bassan R, Vitale A, et al. Dasatinib-Blinatumomab for Ph-Positive Acute Lymphoblastic Leukemia in Adults. *N Engl J Med*. 2020;383(17):1613-1623.

17. Miao Y, Medeiros LJ, Li Y, Li J, Young KH. Genetic alterations and their clinical implications in DLBCL. *Nat Rev Clin Oncol*. 2019;16(10):634-652.

18. Sehn LH, Salles G. Diffuse Large B-Cell Lymphoma. *N Engl J Med*. 2021;384(9):842-858.

19. Friedberg JW, Fisher RI. Diffuse large B-cell lymphoma. *Hematol Oncol Clin North Am*. 2008;22(5):941-952, ix.

20. Rosenwald A, Staudt LM. Gene expression profiling of diffuse large B-cell lymphoma. *Leuk Lymphoma*. 2003;44 Suppl 3:S41-47.

21. Rosenwald A, Wright G, Leroy K, et al. Molecular diagnosis of primary mediastinal B cell lymphoma identifies a clinically favorable subgroup of diffuse large B cell lymphoma related to Hodgkin lymphoma. *J Exp Med*. 2003;198(6):851-862.

22. Liu Y, Barta SK. Diffuse large B-cell lymphoma: 2019 update on diagnosis, risk stratification, and treatment. *Am J Hematol*. 2019;94(5):604-616.

23. Duell J, Maddocks KJ, González-Barca E, et al. Long-term outcomes from the Phase II L-MIND study of tafasitamab (MOR208) plus lenalidomide in patients with relapsed or refractory diffuse large B-cell lymphoma. *Haematologica*. 2021;106(9):2417-2426.

24. Salles G, Duell J, González Barca E, et al. Tafasitamab plus lenalidomide in relapsed or refractory diffuse large B-cell lymphoma (L-MIND): a multicentre, prospective, single-arm, phase 2 study. *Lancet Oncol*. 2020;21(7):978-988.

25. Demaria O, Cornen S, Daëron M, Morel Y, Medzhitov R, Vivier E. Harnessing innate immunity in cancer therapy. *Nature*. 2019;574(7776):45-56.

26. Laidlaw BJ, Craft JE, Kaech SM. The multifaceted role of CD4(+) T cells in CD8(+) T cell memory. *Nat Rev Immunol*. 2016;16(2):102-111.

27. Yenyuwadee S, Sanchez-Trincado Lopez JL, Shah R, Rosato PC, Boussiotis VA. The evolving role of tissue-resident memory T cells in infections and cancer. *Sci Adv*. 2022;8(33):eabo5871.

28. Rosenberg SA, Yannelli JR, Yang JC, et al. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J Natl Cancer Inst*. 1994;86(15):1159-1166.

29. Sarukhan A, Garcia C, Lanoue A, von Boehmer H. Allelic inclusion of T cell receptor alpha genes poses an autoimmune hazard due to low-level expression of autospecific receptors. *Immunity*. 1998;8(5):563-570.

30. Yee C, Thompson JA, Roche P, et al. Melanocyte destruction after antigen-specific immunotherapy of melanoma: direct evidence of t cell-mediated vitiligo. *J Exp Med*. 2000;192(11):1637-1644.

31. Manson G, Houot R. Next-generation immunotherapies for lymphoma: one foot in the future. *Ann Oncol*. 2018;29(3):588-601.

32. Inaba H, Pui CH. Immunotherapy in pediatric acute lymphoblastic leukemia. *Cancer Metastasis Rev*. 2019;38(4):595-610.

33. Viardot A, Goebeler ME, Hess G, et al. Phase 2 study of the bispecific T-cell engager (BiTE) antibody blinatumomab in relapsed/refractory diffuse large B-cell lymphoma. *Blood*. 2016;127(11):1410-1416.

34. June CH, O'Connor RS, Kawalekar OU, Ghassemi S, Milone MC. CAR T cell immunotherapy for human cancer. *Science*. 2018;359(6382):1361-1365.

35. Gross G, Waks T, Eshhar Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc Natl Acad Sci U S A*. 1989;86(24):10024-10028.

36. Irving BA, Weiss A. The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell*. 1991;64(5):891-901.

37. Finney HM, Akbar AN, Lawson AD. Activation of resting human primary T cells with chimeric receptors: costimulation from CD28, inducible costimulator, CD134, and CD137 in series with signals from the TCR zeta chain. *J Immunol*. 2004;172(1):104-113.

38. Feins S, Kong W, Williams EF, Milone MC, Fraietta JA. An introduction to chimeric antigen receptor (CAR) T-cell immunotherapy for human cancer. *Am J Hematol*. 2019;94(S1):S3-S9.

39. Park JR, Digiusto DL, Slovak M, et al. Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma. *Mol Ther*. 2007;15(4):825-833.

40. Croft M. Costimulation of T cells by OX40, 4-1BB, and CD27. *Cytokine Growth Factor Rev*. 2003;14(3-4):265-273.

41. Dai H, Wang Y, Lu X, Han W. Chimeric Antigen Receptors Modified T-Cells for Cancer Therapy. *J Natl Cancer Inst*. 2016;108(7).

42. Marin V, Pizzitola I, Agostoni V, et al. Cytokine-induced killer cells for cell therapy of acute myeloid leukemia: improvement of their immune activity by expression of CD33-specific chimeric receptors. *Haematologica*. 2010;95(12):2144-2152.

43. Chmielewski M, Abken H. TRUCKs: the fourth generation of CARs. *Expert Opin Biol Ther*. 2015;15(8):1145-1154.

44. Kagoya Y, Tanaka S, Guo T, et al. A novel chimeric antigen receptor containing a JAK-STAT signaling domain mediates superior antitumor effects. *Nat Med*. 2018;24(3):352-359.

45. Schuster SJ, Bishop MR, Tam CS, et al. Tisagenlecleucel in Adult Relapsed or Refractory Diffuse Large B-Cell Lymphoma. *N Engl J Med*. 2019;380(1):45-56.

46. Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. *N Engl J Med*. 2018;378(5):439-448.

47. Neelapu SS, Locke FL, Bartlett NL, et al. Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. *N Engl J Med*. 2017;377(26):2531-2544.

48. Shah BD, Ghobadi A, Oluwole OO, et al. KTE-X19 for relapsed or refractory adult B-cell acute lymphoblastic leukaemia: phase 2 results of the single-arm, open-label, multicentre ZUMA-3 study. *Lancet*. 2021;398(10299):491-502.

49. Abramson JS, Palomba ML, Gordon LI, et al. Lisocabtagene maraleucel for patients with relapsed or refractory large B-cell lymphomas (TRANSCEND NHL 001): a multicentre seamless design study. *Lancet*. 2020;396(10254):839-852.

50. Munshi NC, Anderson LD, Shah N, et al. Idecabtagene Vicleucel in Relapsed and Refractory Multiple Myeloma. *N Engl J Med*. 2021;384(8):705-716.

51. Chekol Abebe E, Yibeltal Shiferaw M, Tadele Admasu F, Asmamaw Dejenie T. Ciltacabtagene autoleucel: The second anti-BCMA CAR T-cell therapeutic armamentarium of relapsed or refractory multiple myeloma. *Front Immunol*. 2022;13:991092.

52. McGuirk J, Waller EK, Qayed M, et al. Building blocks for institutional preparation of CTL019 delivery. *Cytotherapy*. 2017;19(9):1015-1024.

53. Cullen SP, Martin SJ. Mechanisms of granule-dependent killing. *Cell Death Differ*. 2008;15(2):251-262.

54. Nagata S, Tanaka M. Programmed cell death and the immune system. *Nat Rev Immunol*. 2017;17(5):333-340.

55. Silveira CRF, Corveloni AC, Caruso SR, et al. Cytokines as an important player in the context of CAR-T cell therapy for cancer: Their role in tumor immunomodulation, manufacture, and clinical implications. *Front Immunol*. 2022;13:947648.

56. Hay KA, Hanafi LA, Li D, et al. Kinetics and biomarkers of severe cytokine release syndrome after CD19 chimeric antigen receptor-modified T-cell therapy. *Blood*. 2017;130(21):2295-2306.

57. Yakoub-Agha I, Chabannon C, Bader P, et al. Management of adults and children undergoing chimeric antigen receptor T-cell therapy: best practice recommendations of the European Society for Blood and Marrow Transplantation (EBMT) and the Joint Accreditation Committee of ISCT and EBMT (JACIE). *Haematologica*. 2020;105(2):297-316.

58. Giavridis T, van der Stegen SJC, Eyquem J, Hamieh M, Piersigilli A, Sadelain M. CAR T cell-induced cytokine release syndrome is mediated by macrophages and abated by IL-1 blockade. *Nat Med*. 2018;24(6):731-738.

59. Sandler RD, Tattersall RS, Schoemans H, et al. Diagnosis and Management of Secondary HLH/MAS Following HSCT and CAR-T Cell Therapy in Adults; A Review of the Literature and a Survey of Practice Within EBMT Centres on Behalf of the Autoimmune Diseases Working Party (ADWP) and Transplant Complications Working Party (TCWP). *Front Immunol*. 2020;11:524.

60. Sheth VS, Gauthier J. Taming the beast: CRS and ICANS after CAR T-cell therapy for ALL. *Bone Marrow Transplant*. 2021;56(3):552-566.

61. Morris EC, Neelapu SS, Giavridis T, Sadelain M. Cytokine release syndrome and associated neurotoxicity in cancer immunotherapy. *Nat Rev Immunol*. 2022;22(2):85-96.

62. Ying Z, He T, Wang X, et al. Parallel Comparison of 4-1BB or CD28 Co-stimulated CD19-Targeted CAR-T Cells for B Cell Non-Hodgkin's Lymphoma. *Mol Ther Oncolytics*. 2019;15:60-68.

63. Zhao X, Yang J, Zhang X, et al. Efficacy and Safety of CD28- or 4-1BB-Based CD19 CAR-T Cells in B Cell Acute Lymphoblastic Leukemia. *Mol Ther Oncolytics*. 2020;18:272-281.

64. Ferreira LMR, Muller YD. CAR T-Cell Therapy: Is CD28-CAR Heterodimerization Its Achilles' Heel? *Front Immunol*. 2021;12:766220.

65. Condomines M, Arnason J, Benjamin R, Gunset G, Plotkin J, Sadelain M. Tumor-Targeted Human T Cells Expressing CD28-Based Chimeric Antigen Receptors Circumvent CTLA-4 Inhibition. *PLoS One*. 2015;10(6):e0130518.

66. Theunissen PMJ, Sedek L, De Haas V, et al. Detailed immunophenotyping of B-cell precursors in regenerating bone marrow of acute lymphoblastic leukaemia patients: implications for minimal residual disease detection. *Br J Haematol*. 2017;178(2):257-266.

67. Monfrini C, Stella F, Aragona V, et al. Phenotypic Composition of Commercial Anti-CD19 CAR T Cells Affects In Vivo Expansion and Disease Response in Patients with Large B-cell Lymphoma. *Clin Cancer Res*. 2022;28(15):3378-3386.

68. Deng Q, Han G, Puebla-Osorio N, et al. Characteristics of anti-CD19 CAR T cell infusion products associated with efficacy and toxicity in patients with large B cell lymphomas. *Nat Med*. 2020;26(12):1878-1887.

69. Castro F, Cardoso AP, Gonçalves RM, Serre K, Oliveira MJ. Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion. *Front Immunol*. 2018;9:847.

70. Josephs SF, Ichim TE, Prince SM, et al. Unleashing endogenous TNF-alpha as a cancer immunotherapeutic. *J Transl Med*. 2018;16(1):242.

71. Boulch M, Cazaux M, Loe-Mie Y, et al. A cross-talk between CAR T cell subsets and the tumor microenvironment is essential for sustained cytotoxic activity. *Sci Immunol*. 2021;6(57).

72. Alizadeh D, Wong RA, Gholamin S, et al. IFNγ Is Critical for CAR T Cell-Mediated Myeloid Activation and Induction of Endogenous Immunity. *Cancer Discov*. 2021;11(9):2248-2265.

73. Chen PH, Lipschitz M, Weirather JL, et al. Activation of CAR and non-CAR T cells within the tumor microenvironment following CAR T cell therapy. *JCI Insight*. 2020;5(12).

74. Scholler N, Perbost R, Locke FL, et al. Tumor immune contexture is a determinant of anti-CD19 CAR T cell efficacy in large B cell lymphoma. *Nat Med*. 2022;28(9):1872-1882.

75. Salter AI, Ivey RG, Kennedy JJ, et al. Phosphoproteomic analysis of chimeric antigen receptor signaling reveals kinetic and quantitative differences that affect cell function. *Sci Signal*. 2018;11(544).

76. Gust J, Hay KA, Hanafi LA, et al. Endothelial Activation and Blood-Brain Barrier Disruption in Neurotoxicity after Adoptive Immunotherapy with CD19 CAR-T Cells. *Cancer Discov*. 2017;7(12):1404-1419.