

Review Article**Toxicities Associated with CAR-T Cell Therapies**

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Abstract. Chimeric antigen receptor (CAR) T-cell therapy has improved the outcomes of patients with relapsed/refractory B-cell lymphomas, B-cell acute lymphoblastic leukemia, and multiple myeloma. However, CAR-T cell therapy is also associated with distinct toxicities that contribute to morbidity and mortality. A large number of studies now define the different toxicities associated with CAR-T cell therapy and have, in part, clarified their mechanisms. In particular, cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) are the two main acute toxicity events that occur after CAR-T cell infusion. Other CAR-T-related toxicities occur later after CAR-T cell infusion and include B-cell aplasia, hypogammaglobulinemia, infections, and cytopenias. Infections represent the main cause of non-relapse death observed in patients undergoing CAR-T cell therapy. Second primary malignancies are rare and are mainly represented by myeloid malignancies.

Keywords: Heparin-binding protein; Systematic review, Meta-analysis, Diagnosis, Prognosis, Infectious disease, Sepsis, Organ failure, Mortality.

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Introduction. Chimeric antigen receptor T-cells (CAR-T) are T-cells engineered with fusion proteins to target specific antigens present on tumor cells and generate an antitumor immune response. CAR-T cells now represent an established treatment for patients with relapsed/refractory large B-cell lymphomas (LBCL), B-cell acute lymphoblastic leukemia (B-ALL), and multiple myeloma (MM).

Five CD19-directed, namely Axicabtagene autoleucel (Axi-Cel), Brexucabtagene autoleucel (Brexu-Cel), Tisagenlecleucel (Tisa-Cel) Lisocabtagene maraleucel (Liso-Cel), and Obecabtagene autoleucel (Obe-cel), and two BCMA-directed CAR-T, namely Idecabtagene vicleucel (Ide-Cel) and Ciltacabtagene autoleucel (Cilta-Cel), have been approved against B-NHL, B-ALL, and MM so far.

The therapeutic efficacy of CAR-T reaches a plateau

of progression-free survival ranging from 25-45%, mainly differing according to CAR-T product and type of disease. However, CAR-T is associated with unique early (within the first month) and late toxicities that require careful monitoring and prompt interventions. (**Figure 1**)

Non-relapse mortality (NRM) after CAR-T cell therapy. A recent systematic meta-analysis extended to 7,604 patients enrolled in 18 clinical trials, and 28 real-world studies analyzed the NRM observed after CAR-T in LBCL and MM patients.¹ NRM significantly differed for various diseases, varying across mantle cell lymphoma (10.6%), multiple myeloma (8%), LBCL (6.1%), and indolent lymphoma (5.7%).¹ CAR-T products impacted NRM in a disease-specific manner: for LBCL, Axi-Cel was associated with higher NRM

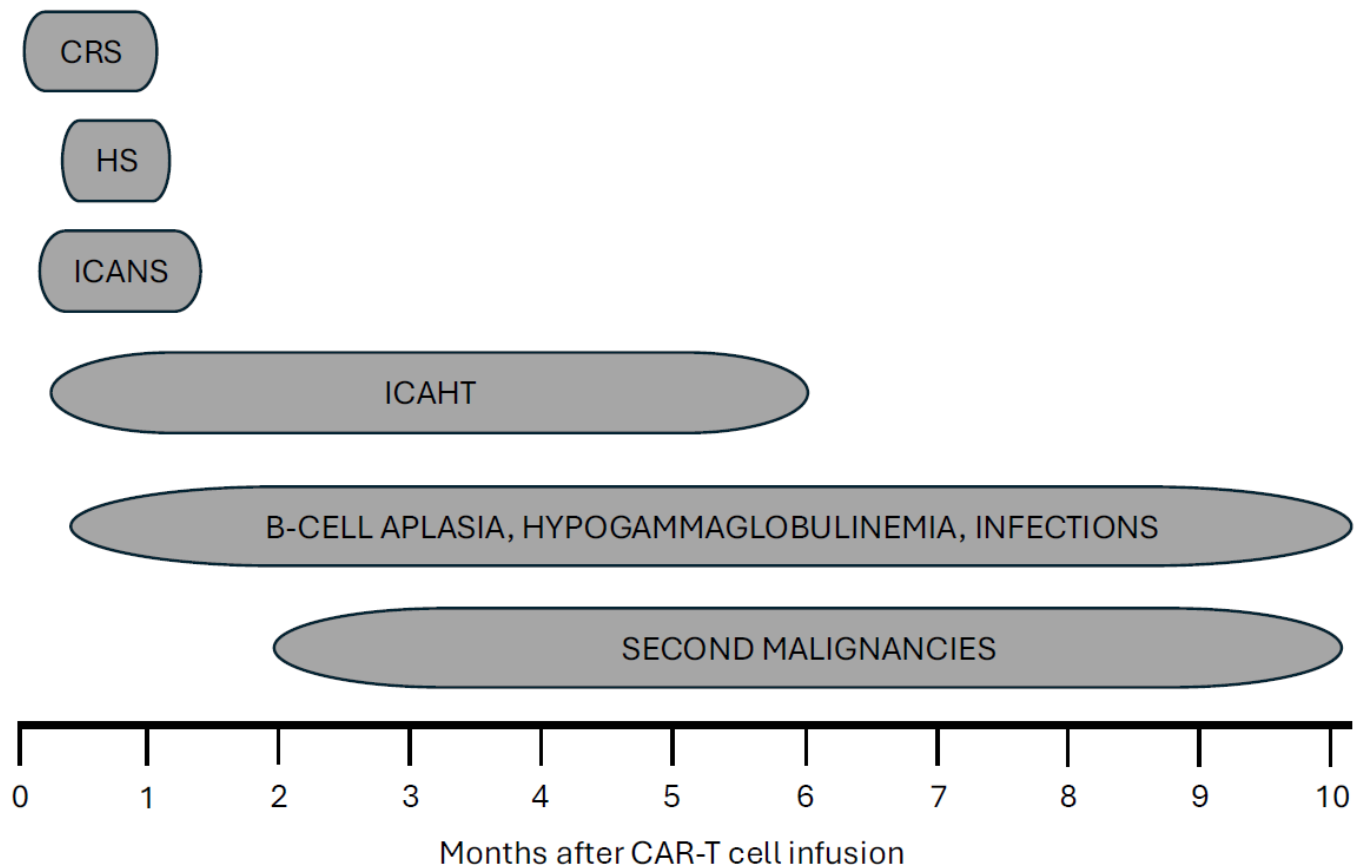


Figure 1. Timing of the main CAR-T cell-related adverse events. Approximate timeline of occurrence of main toxicities related to CAR-T cell infusion; for each of these toxicities, the typical time of onset is underlined by the start of each bar, the time to resolution by the end of the bar, and the duration by the length of the bar.

compared with Liso-Cel and Tisa-Cel (7.4% vs 3.8% vs 4.1%, $p=0.004$); for MM, Cilta-cel was associated with significantly higher NRM compared with Ide-Cel (15.2% vs. 6.3%, $p<0.001$).¹ 50.9% of NR deaths were attributed to infections, 7.8% to the development of a second malignancy, 7.3% to cardiovascular or respiratory events, 4.7% to cytokine release syndrome (CRS), 5.2% to immune effector cell-associated neurotoxicity syndrome (ICANS), and 1.5% to hemophagocytic lymphohistiocytosis.¹

Cytokine Release Syndrome (CRS). The most prominent toxicity of CAR-T cells is CRS, a clinical syndrome affecting multiple organs that is characterized by a multitude of systemic symptoms. Usually, initial symptoms of CRS consist of fever and tachycardia. In more severe cases, CRS is associated with hypotension, hypoxia, capillary leak syndrome, multiple organ failures, and disseminated intravascular coagulation. CRS can onset in the very first days after CAR-T infusion, with a duration of some days in most cases. According to a recent consensus paper, CRS can be graded as follows:²

- Grade 1: Fever $\geq 38^{\circ}\text{C}$ without hypotension or hypoxia.
- Grade 2: Fever $\geq 38^{\circ}\text{C}$ with hypotension responsive to

fluids or requiring low-dose vasopressors and/or hypoxia requiring low-flow supplemental oxygen (up to 6 L/min).

- Grade 3: Fever $\geq 38^{\circ}\text{C}$ with hypotension requiring high-dose vasopressors and/or hypoxia requiring high-flow oxygen (>6 L/min) or non-invasive ventilation.
- Grade 4: Fever $\geq 38^{\circ}\text{C}$ with hypotension requiring multiple vasopressors or inotropic support and/or hypoxia requiring invasive mechanical ventilation.
- Grade 5: Death related to CRS.

Morris and coworkers have described five key phases in the development of CRS.³ Phase 1 involves CAR-T cell infusion, tumor site trafficking, and antigen recognition. Phase 2 includes CAR-T activation, proliferation, cytokine release, and tumor killing, defining the onset of CRS. Phase 3 is characterized by a systemic inflammatory response, corresponding to the peak of cytokines, endothelial cell injury with consequent vascular leakage, and organ dysfunction. Phase 4 involves damage to the blood-brain barrier (BBB), associated with the diffusion of cytokines and transmigration of CAR-T cells in the cerebrospinal fluid (CSF), leading to neurotoxicity. Phase 5 concludes with CAR-T apoptosis, a decrease of serum cytokine levels, and resolution of systemic inflammation, with long-term

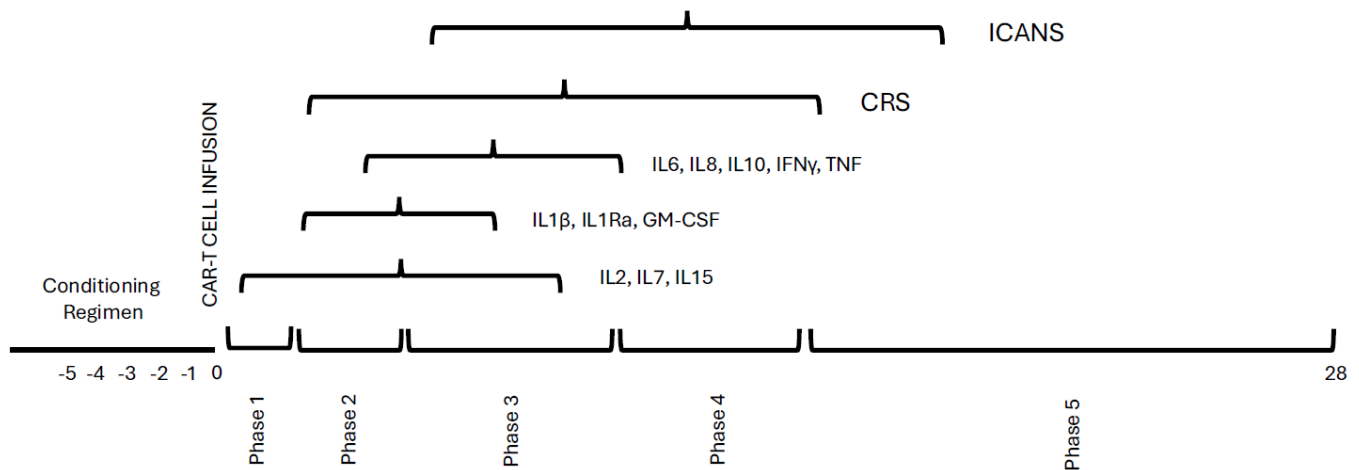


Figure 2. Acute toxicities (CRS and ICANS) occurring after CAR-T cell therapy. The period of first months after CAR-T cell infusion is subdivided into 5 phases, according to Morris et al.² The timing of CRS and ICANS development is outlined, indicating the most frequent times of CRS and ICANS peaks. Also, the kinetics of the production of groups of cytokines at different times after CRA-T cell infusion are shown.

memory CAR-T persistence in responders (**Figure 2**).³

The pathogenesis of CRS presupposes a complex interaction between activated T cells, tumor antigens, and tumor microenvironment involving IL-6, IL-10, IL-8, IFN- γ , and TNF- α .³ Monocytes and macrophages have been identified as the main source of IL-6 and IL-1, which represent major drivers for the induction and progression of CRS.⁴⁻⁵ IFN- γ released by activated CAR-T cells is a potent activator of monocytes/macrophages and was also associated with severe CRS.⁶ In xenograft models, the inhibition or genetic deletion of IFN- γ induced decreased macrophage activation and inflammatory cytokines production, with reduced immune checkpoint expression,⁷ also observed with pharmacological inhibition of IFN- γ by Emapalumab.⁸ Interestingly, IFN- γ blockade did not affect CAR-T cells against CD19⁺ hematologic malignancies but possibly reduced immune-related adverse effects.^{6,7,9}

CD4⁺ T-cells play a major role in mediating the CRS.¹⁰ Circulating CD4⁺ CAR-T cells, but not CD8⁺, could be major drivers of CRS manifestations, together with a high tumor burden.¹⁰

Activated CAR-T cells also release proteolytic enzymes to trigger the induction of target cell death. CAR-Ts rapidly activate caspase-3 through the release of granzyme B, which induces the cleavage of gastrin E (GSMDE), a pore-forming protein highly expressed in B-leukemic cells, resulting in the induction of an extensive pyroptotic process.¹¹ Differently from apoptosis, pyroptosis is an inflammatory cell death. The activation of pyroptosis determines caspase-1-mediated cleavage of GSDMD in macrophages, with consequent release of cytokines and induction of CRS.¹¹ Knocking out GSMDE, depleting macrophages, or inhibiting caspase 1 abrogates CRS occurrence in murine models. High expression of GSMDE in tumor cells determines preferential pyroptosis and associates with CRS, despite apoptosis and pyroptosis are two cell death processes

mediated by caspases.^{11,12}

Another player in the pathophysiology of CRS is the activation of vascular endothelium. Endothelial cell activation is characterized by decreased Angiopoietin-1 (Ang-1) and increased levels of Ang-2 and von Willebrand Factor (VWF), sE-selectin, soluble intercellular adhesion molecule (sICAM-1) and soluble vascular cell adhesion molecule (sVCAM-1).¹³ Coagulopathy can also be observed, with elevated prothrombin time, activated partial thromboplastin time values, D-dimer levels, and decreased fibrinogen levels and platelet counts in patients developing CRS, despite kinetics of fibrinogen is not clear in reason of its role as an acute phase protein. Particularly, the alteration of endothelial cell activation biomarkers, such as increased VWF levels and Ang-2/Ang-1 ratio, correlates with the development and degree of CRS.¹⁴

The modified version of the Endothelial Activation and Stress Index (mEASIX) is a transplant-derived score that associates with endothelial stress considering platelets, C-reactive protein, and lactate dehydrogenase. In patients with lymphoproliferative diseases treated with CAR-T, prothrombin time and activated thromboplastin time, fibrinogen, D-dimer, factor VIII, VWF level increased during CRS graded 2 or more, while platelet count and antithrombin levels decreased. A higher mEASIX score correlated with altered coagulopathy markers.¹⁵ Baseline EASIX score combined with ferritin stratified patients in three groups with low, intermediate, and high risk of developing CRS.¹⁶

Interleukin 6 (IL-6) is one of the most significantly elevated cytokines during CRS occurring after CAR-T cell therapy. IL-6 is secreted by monocytic cells in response to CAR-T cell activation in a contact-independent mechanism following T-cell engagement of target cells.¹⁷ IL-6 produced during CRS does not impact CAR-T cell cytotoxicity or transcriptional profiles, and

inhibition of IL-6 signaling in patients undergoing CAR-T cell treatments is a procedure not affecting CAR-T cell efficacy.¹⁷

Nowadays, current guidelines recommend administering Tocilizumab for grade ≥ 2 CRS, with steroids required for refractory or ICANS-associated forms. Hypotension and hypoxia require specific treatment. Further treatments for severe and unresponsive forms involve anakinra, ruxolitinib, or high-dose steroids.¹⁸

IL-6 signaling contributes to many of the symptoms characterizing CRS, such as vascular endothelium activation and leakage, activation of complement, and coagulation cascade inducing disseminated intravascular coagulation. Blocking IL-6 Signaling using the monoclonal antibody Tocilizumab (targeting IL-6R) has dramatically improved the treatment of patients developing grade 2-4 CRS after CAR-T cell therapy, despite showing two disadvantages: the first is related to the cost and the second is that binding IL-6R, may result in the peak of serum IL-6, which could cross BBB and contribute to neurotoxicity. In order to obtain a more efficacious strategy for blocking IL-6 signaling, CD19- or BCMA-CAR-T cells were engineered to secrete an anti-IL-6 scFv and IL-1 receptor antagonist.¹⁹ Next-generation CAR-T-secreting cytokine antagonists have been conceived, aiming at neutralization of IL-6 storm and block of IL-1 signaling or silencing the IL-6 gene (ssCAR-19) with a small hairpin RNA (shRNA).²⁰ Standard CD19 CAR-T and modified CAR-T (SSCAR-19) obtained similar complete remission rate (91.5% CR with median OS not reached with SSCAR-19, compared to 85% CR and median OS of 32.9 months in the standard CAR-T). Investigators observed grade 1-2 CRS in 53%, grade 3-4 CRS in 14.9%, and grade 1 ICANS in 4.2% of SSCAR-19 patients, compared to grade 1-2 CRS in 47.5% and grade 3-4 CRS in 37.5% and grade 1-2 ICANS in 10% of patients treated with stCAR-19.²⁰ Importantly, peak levels of serum IL-6, IL-2, and TNF- α levels were significantly lower in SSCAR-19 than in stCAR-19 patients.²⁰

An alternative anti-IL-6 approach has been proposed. This approach exploits temperature-sensitive hydrogel conjugated with antibodies for IL-6, subcutaneously injected before the infusion of CAR-T cells. This hydrogel is efficient in reducing IL-6 levels and CRS.²¹

An optimal debulk of the disease may mitigate CRS; in this direction, Sakamura et al. have proposed treating patients with B-ALL with the anti-CD19 mAb Tafositamab before administering CD19-CAR-T cells.²² In preclinical *in vivo* models, this two-step treatment reduced the incidence and severity of CRS and improved antitumor effects with better OS compared to CD19-CAR-T cells alone.²²

CAR-T related neurologic events: ICANS.

Neurotoxicity (ICANS) is the second most common adverse event associated with CAR-T cell therapies, defined as a disorder “resulting in the activation or engagement of endogenous or infused T-cells and/or other immune effector cells”.²³

The occurrence of ICANS stands around 30%, according to different reports and studies.

Usually, ICANS in CAR-T cell-treated patients is a mild entity associated with common symptoms, such as dysgraphia, word-finding difficulties, tremors, confusion, and somnolence. ICANS normally occurs at least 4 to 7 days after CAR-T infusion, with a duration limited to a few days in most patients; however, in a minority and variable proportion of patients, these symptoms progress in a few hours up to fatal outcomes due to fulminant cerebral edema.

ICANS can occur concomitantly with CRS or shortly after CRS subsides; being ICANS and CRS are etiologically related, there is often a correlation between their occurrence (**Figure 3**). This was also confirmed in a wider analysis of CD19, BCMA, and CD22-directed CAR-T cells.²⁴ The incidence of overall and grade ≥ 3 ICANS was more frequent in studies involving CAR-T containing CD28 costimulatory domains compared to those with 4-1BB costimulatory domains (**Figure 3**).

Neuroimaging of the CNS in patients with ICANS shows a symmetric distribution of abnormalities, particularly involving the thalamus (indicative of interstitial or vasogenic edema) and deep grey matter. These findings support the view of ICANS as promoted by a systemic inflammatory process.²⁵

Several studies measuring cytokine levels in serum/plasma and cerebrospinal fluid (CSF) have supported their possible role in the pathophysiology of ICANS. Increased serum/plasma levels of IFN- γ and IL-15, as well as IL-6, IL-10, GM-CSF, IL-2, IL-1RA, and CXCL10, were associated with ICANS.²⁴

Endothelial activation and disruption of the BBB are major mechanisms in the development of ICANS.

In 133 patients treated with CD19-CAR-T, high CD19⁺ cells in bone marrow, high CAR-T cell dose, CRS, and preexisting neurologic comorbidities were associated with an increased risk of neurologic events.²⁶ Patients with severe ICANS exhibited evidence of endothelial cell activation, including disseminated intravascular coagulation, capillary leak syndrome, and increased BBB permeability.²⁶ The endothelial activation is also supported by higher Ang2 levels and lower Ang1/Ang2 ratio in patients with high-grade ICANS.²⁷ These observations are paralleled by the observation of increased serum concentrations of von Willebrand factor released by activated endothelium in patients with severe ICANS.²⁶

Severe ICANS was associated with BBB disruption. High levels of proinflammatory cytokines such as IL-6, IL-8, MCP1, and IP10 were observed in the CSF of

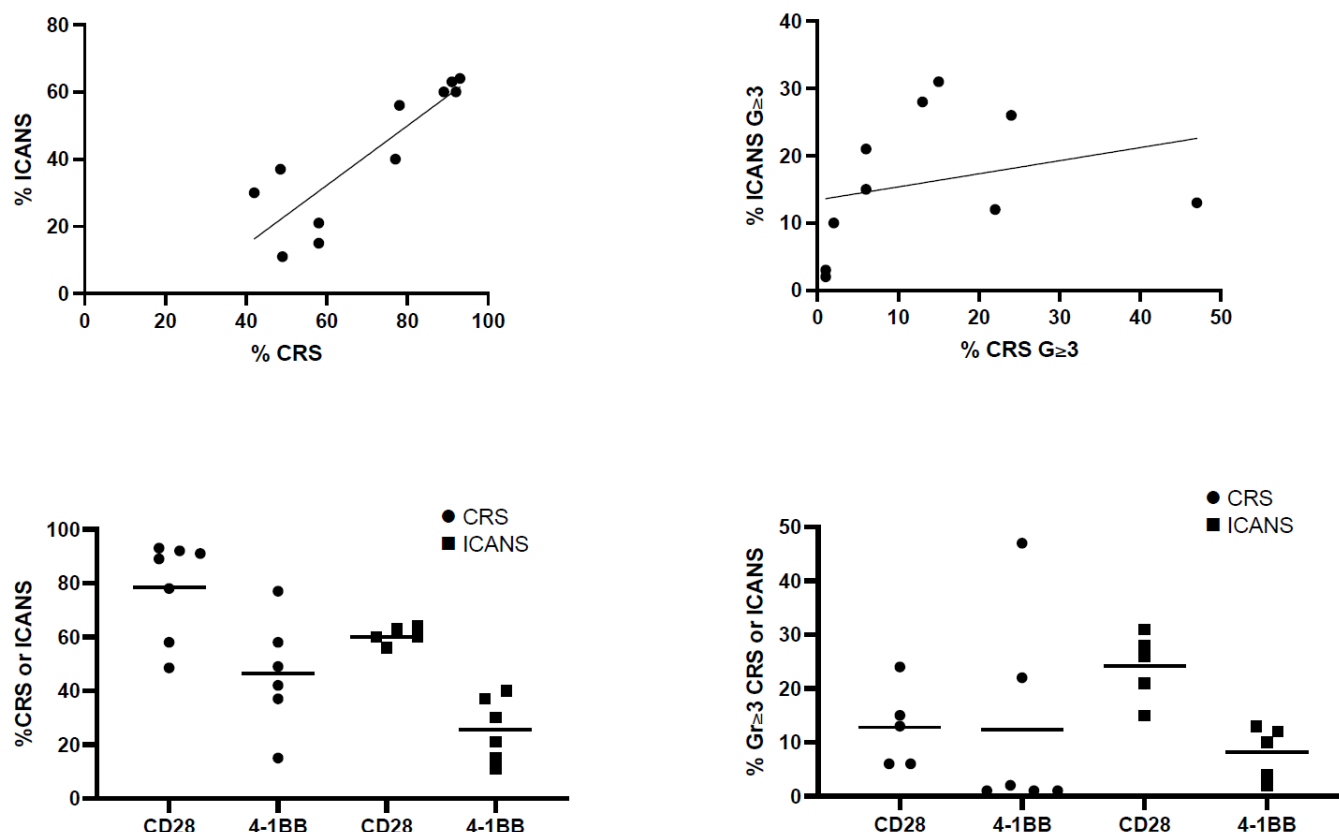


Figure 3. Correlation between the frequency of the occurrence of ICANS and CRS observed in the major clinical trials carried out with commercial CD19-CAR-T cells: left up panel: all ICANS and CRS events; right up panel: grade ≥ 3 ICANS and grade ≥ 3 CRS. Incidence of CRS and ICANS in clinical trials involving CD19-CAR-T cells containing CD28 or 4-1BB costimulatory domains: left down panel: all CRS and all ICANS; right down panel: grade ≥ 3 CRS and ICANS events.

patients with severe ICANS, suggesting a CNS production of these cytokines or passage through the BBB.²⁷

Recent studies suggest that microglia activation also plays a relevant role in the development of ICANS. Studies by translocator protein positron-emission-tomography (PET) and imaging mass cytometry showed a shift from resting to activated microglia in patients with ICANS.²⁸ Accordingly, an animal model of ICANS showed that the TGF β -activated kinase-1 (TAK1)-NF κ B-p38 MAPK pathway is activated in microglia after CD19 T cell transfer, while its pharmacological inhibition improved neurocognitive activity in ICANS mice.²⁸

CAR-T cells can release extracellular vesicles (EVs) carrying CAR constructs; in vivo, CART EVs are detectable as early as 1hr after CAR-T cell infusion, and their level in the plasma preceded the instauration of ICANS.²⁹

A meta-analysis of 6 different clinical trials, as well as a real-world comparison, showed that axi-cel had a higher incidence of both CRS and ICANS compared to tisa-Cel, with ORs of 3.84 and 4.4, respectively.^{30,31}

CAR-T related neurologic events: movement disorders. One case of progressive movement disorder resembling parkinsonism was reported in a patient with

MM from the CARTITUDE-1 study approximately three months after BCMA-CAR T infusion.³² In this patient, CAR-T cells persisted in circulation and in cerebrospinal fluid; basal ganglia showed lymphocyte infiltration.³² Immunohistological studies documented BCMA expression on neurons and astrocytes of the patient's basal ganglia. Analysis of transcriptomic data evidenced BCMA mRNA expression in the caudate region of the normal human brain, thus suggesting that it could represent an on-target off-tumor effect of therapy.³² Twelve out of 97 patients from the CARTITUDE-1 trial reported non-ICANS neurotoxicity, with five patients' having movement and neurocognitive adverse events grade ≥ 3 parkinsonism in three of them.³³

Overall, movement and neurocognitive disorders occurred in 5% of patients from CARTITUDE-1.³⁴ Potential factors associated with movement disorder were high baseline tumor burden, high baseline IL-6 levels, grade ≥ 2 CRS, the incidence of ICANS, high CAR-T cell expansion and persistence, and high absolute lymphocyte counts after CAR-T cell infusion.³⁴ Of these 5 patients, one recovered, one maintained persisting symptoms, and three died.³⁴ Importantly, the onset of movement disorders was delayed with respect to the onset of ICANS (27 days vs 5-9 days, respectively).³⁴ Additional neurologic toxicities observed in the CARTITUDE-1 study included also altered mental

status, concentration impairment, facial paralysis, diplopia, nystagmus, cranial nerve palsy, ataxia, sensory loss and peripheral motor and sensory neuropathies, occurring after resolution of CRS and ICANS.³⁴

In another report from Karschnia et al., cells; 41% of patients with MM treated with BCMA-directed CAR-T developed ICANS, with two (2.6%) patients developing extra-pyramidal symptoms after the resolution of acute ICANS. In detail, one patient exhibited confusion and bradykinesia with increased muscle tone on day 22 post-CAR-T cell therapy with FDG-PET, showing bilateral hypometabolism localized at the level of the basal ganglia.³⁵ The second patient developed confusion, postural instability, mild tremor, bradykinesia, and hypophonia on day 19 after CAR-T cell infusion, with frontal region hypometabolism at FDG-PET.³⁵

One more MM cilta-cel treated patient was reported by Gudera and coworkers, with Parkinson-like symptoms (hypomimia, oppositional paratonia, right-predominant rigidity, decreased arm swing, and dysgraphia), at day 24 after CAR-T infusion [36]. In this case, MRI displayed new bilateral hyperintensities located at the caudate head, putamen, and globus pallidus with concomitant lymphocytosis in the CSF and diffuse hypometabolism at the level of cerebral cortex in FDG-PET.³⁶

The treatment of patients developing movement disorders after CAR-T cell treatment is challenging, with one experience proposing a role for cyclophosphamide.³⁷

It is important to note that a more recent study, carried out using both immunohistochemistry and *in situ* RNA hybridization, questioned the expression of BCMA in normal human brain.³⁸ The mechanism of BCMA-CAR-T cell therapy-related movement disorders remains unclear.³⁸

Hemophagocytic Lymphocytosis. Hemophagocytosis lymphocytosis (HLH) or macrophage activation syndrome (MAS) is a severe syndrome deriving from pathologic immune activation (characterized by fever, hyperferritinemia, hepatosplenomegaly, multi-organ failure, coagulopathy, neurologic toxicities, cytopenias and/or hypertriglyceridemia).³⁹ Secondary HLH may be observed both after CAR-T cell therapy and hematopoietic stem cell transplantation in 3%- 4% of cases.⁴⁰ Immune-effector-associated HLH, named IEC-HS, is typically described with no direct correlation with CRS, typically occurring 10-20 days after CAR-T cell infusion.⁴¹

Hyperferritinemia (normally above >10,000 ng/ml), cytopenia, hypertriglyceridemia, hypofibrinogenemia, coagulopathy, hepatic transaminitis, hyperbilirubinemia, severe neutropenia, elevated lactate dehydrogenase, and in some patients, hemophagocytosis are typical features of the syndrome. However, in many instances, the distinction between severe CRS and IEC-HS remains

difficult.

Hemophagocytosis may be increased in patients treated with CD22-CAR-T, with an incidence of up to 40% in some reports.⁴²⁻⁴⁴ A higher ratio of T cell/ NK cells in bone marrow and elevated inflammatory cytokine levels, including IL18m, may also be associated with the syndrome.⁴² Increased IL-18 levels could contribute to the deregulation of the inflammasome observed in an HLH.⁴³

Serum ferritin can be considered a biomarker related to hematologic disease. It is higher than 1,000 ng/ml during the early phases of the disease and increases to more than 10,000 ng/ml in correspondence with fully symptomatic HLH, with concurrent disseminated intravascular coagulation.⁴⁵

In some instances, HLH is difficult to distinguish from severe CRS.

The pathophysiological mechanisms responsible for HLH remain largely undefined. Some observations suggest that IL-6 plays a less central role in HLH than in CRS: in fact, peak IL-6 levels did not differ substantially between patients who did not develop HLH. The majority of patients developing CAR-HLH had received prior Tocilizumab and developed HLH despite clinical amelioration of CRS.⁴² Alternative cytokine pathways were responsible for the development of HLH. Other observations suggest that IL-1 could play a relevant role in HLH. IL-1 β serum levels were markedly more elevated in patients developing HLH compared to those with CRS alone.⁴² Anakinra and ruxolitinib are now proposed for the treatment of patients with IEC-HS.^{42,46}

Second primary malignancies after CAR-T cell therapy. CAR-T patients share a high risk of developing treatment-related adverse events, including secondary primary malignancies. In 2023, the FDA released a statement concerning some reports on the occurrence of lymphomas and leukemias of the T-cell lineage observed in patients treated with CAR-T cells, either anti-CD19 or anti-BCMA in the context of clinical trials or post-marketing adverse events reporting.⁴⁷ According to these reports, the FDA extended the risk of the development of T-cell malignancies to all commercial CAR-T cell products.⁴⁷ In detail, these reports concerned 22 cases of T-cell malignancies within 2 years after CAR-T treatment, variously presenting as T-cell lymphomas, T-cell large granular lymphocytosis, peripheral T-cell lymphoma, and cutaneous T-cell lymphoma.⁴⁸ Importantly, in three cases in which DNA sequencing was performed, the CAR transgene was detected in the malignant clone.⁴⁸

An exact evaluation of the number and type of adverse events occurring after CAR-T cell therapy remains difficult due to underreporting. Out of the more than 34,000 patients who have been treated, only 8,000 cases have been officially reported in the FDA-adverse

Events Reporting System (FAERS) database.⁴⁹

The FAERS database allows to evaluate the type of SPMs occurring after CAR-T cell therapy. A wide registry analysis by Elsallab and coworkers evaluated 12394 CAR-T adverse event reports included in the FAERS database and observed that 536 (4.3% of SPM events).⁵⁰ The most frequent SPMs concerned leukemias, including myelodysplastic syndromes (38.8% of SPMs), acute myeloid leukemia (19.8% of SPMs), and T-cell large granular lymphocytic leukemia (0.4% of SPMs); hematological neoplasms excluding lymphomas and leukemias were less frequent (4.9% of SPMs); T-cell non-Hodgkin lymphomas were identified in 3.2% of all SPMs. Other tumors involved skin, mostly nonmelanomas (10.1%) CNS, respiratory tract, and gastrointestinal tract (3.9%, 3.7%, and 3.5% of the whole SPMs, respectively).^{50,51} Analysis of SPM disproportionality in the CAR reports showed significantly higher reporting adding ratio (ROR) for myelodysplastic syndromes in patients treated with CAR-T cells.⁵⁰

In order to explore if there was a higher risk of SPM after CAR-T cell treatment compared to chemotherapy alone, a subgroup analysis compared within FAERS the incidence of SPM between patients who received CAR-T therapy and those who received chemotherapy alone: CAR-T recipients seemed to experience higher incidences of some SPMs, such as T-cell lymphomas (ROR 5.0), squamous cell carcinoma of the skin (ROR 3.08) and MDS (ROR 1.50),⁵¹ despite these data needs to be carefully contextualized. In this sense, a subgroup analysis of four clinical trials, which randomized CAR-T cell therapy versus standard of care, showed a similar risk of SPM with either treatment strategy.⁴⁵ In conclusion, these observations, although they raise awareness of SPM as a clinically relevant long-term adverse event in patients undergoing CAR-T cell therapy, do not support the view that CAR-T cell therapy increases the frequency of SPM compared to standard-of-care treatments.⁵² It is worth noticing that solid tumors occurred in post-CAR-T cell therapy as SPM with a delayed manifestation compared to hematological malignancies.⁵¹

In a paper from Ghilardi and coworkers, SPM occurred in 3.6% out of 448 patients treated with commercial CAR-T cells for hematological malignancies at a median follow-up of 10.3 months. Only one patient developed a T-cell lymphoma.⁵³ Similar incidences were reported in other reports.⁵⁴

It is important to underline that patients with non-Hodgkin lymphoma have an approximately five-fold higher incidence of second primary T-cell lymphoma over time compared to the general population;⁵⁵ and that chemotherapy itself determine an increased risk of SPM, particularly of MDS and AML;^{56,57} the same is true for patients with MM treated with immunomodulatory

agents.^{58,59}

A recent paper identified the role of preexisting clonal hematopoiesis, age, and peripheral blood count impairment as possible risk factors for subsequent development of secondary myeloid neoplasia.⁶⁰

B-cell aplasia, hypogammaglobulinemia and infections. B-cell aplasia is frequently observed in patients receiving CD19-directed CAR-T cell therapy and is the result of a direct on-target off-tumor toxicity, which can vary according to CAR-T product and underlying disease ranging between 60% and 80%.⁶¹ B-cell recovery after Tisa-Cel tend to be slower may constitute a predictor of disease relapse. DoR was shorter in patients with recovery at <6 months after CAR-T cell infusion compared with those with B-cell recovering at 6-12 months or >12 months.^{55,62}

Similar observations were not concordant when considering lymphomas.⁶³

Hypogammaglobulinemia is frequently observed in patients after both CD19-directed and BCMA-directed CAR-T cell therapy.

Sutherland et al. reported that 60% of patients had hypogammaglobulinemia before CAR-T infusion, and 37% of patients developed a serious infection after CAR-T.⁶⁴ Preexisting hypogammaglobulinemia worsened after CAR-T therapy; the presence of hypogammaglobulinemia after CAR-T was associated with an increased risk of severe infections post-CAR-T cell therapy.⁶⁴ Risk factors for mortality included also hypogammaglobulinemia <400 mg /dL, together infections and related hospitalizations.⁶⁴

Continuous long-term B-cell aplasia after CAR-infusion may be the surrogate of persistent CAR-T cell activity against normal B cells; in this sense, recovery from B-cell aplasia (BCA) could predict the loss of functional CAR-T cells against target antigen. However, B-cell aplasia could also derive from poor hematopoietic reserve.⁶⁵ In 57 LBCL patients undergoing CD19-CAR-T cell therapy, lower baseline CD4+, neutrophil counts and worse CAR-HEMATOTOX score (see later) predicted the development of long-term BCA, which was observed in 51% of patients.⁶⁶

Infectious complications represent the main cause of non-relapse mortality after CAR-T, with risk factors varying according to the time elapsed since CAR-T infusion.

Early events occur from day 0 to day 30, are mainly represented by bacterial infections, and may be mostly related to the severe neutropenia typical of those timeframes.⁶⁷ These early events may be also exacerbated by the immunosuppressive treatments used to mitigate CRS and ICANS. Late infectious events occur after day 30 and are related to the prolonged depletion of B and T cells, and hypogammaglobulinemia. CD19 and BCMA CAR-T cells induce different immune

deficits due to the expression of their respective targets on normal B cells at different stages of maturation, being extended in the case of BCMA also to mature plasma cells.⁶⁷ Late infectious events may be typically related to viruses, bacteria, or fungi.⁶⁷

Hematologic toxicities. Hematologic toxicities are frequently observed after CAR-T cell therapy of hematologic patients and include a spectrum of adverse events, such as cytopenias, B-cell aplasia and coagulopathies. The European Hematology Association (EHA) and the European Society for Blood and Bone Marrow Transplantation (EBMT) classified cytopenias occurring after CAR-T cell therapy as Immune Effector Cell-Associated hematological toxicity (ICAHT).⁶⁸ Prolonged ICAHT can be subdivided according to their timing of occurrence into early (0-30 days) or late (after 90 days). Despite ICAHT only takes into account absolute neutrophil counts, a deficiency of hemoglobin and platelets count can frequently be associated.

Cytopenias usually last less than 6 months. Some risk factors have been identified for predicting the development of cytopenias.

CAR-T induced inflammation, prior lines of chemotherapy, baseline hematopoietic reserve, and CRS are the most-known key determinant in the development of hematologic toxicities.⁶⁹

The CAR-HEMATOTOX score, introduced by Rejeski et al., represents a well-validated prognostic tool for predicting the risk of enduring cytopenias and is based on the evaluation of some inflammatory markers, such as ferritin and C-protein reactive levels, together with blood cell counts.⁷⁰ The CAR-HEMATOTOX index predicts long-term hematotoxicity and severe infections in patients receiving CAR-T cell therapy for the majority of current indications.

ICAHT occurrence and severity was documented in LBCL, MCL and MM patients undergoing CAR-T cell therapy, despite could be partially missignificant for patientst with B-ALL, where involvement of bone marrow could have some more relevant role.⁷¹ Thus, the ALL-HEMATOTOX was developed, replacing ferritin with medullary disease burden. The ALL-HEMATOTOX score associated with severe prolonged neutropenia and appropriately identified 47% of patients as high-risk patients.⁷¹

Strati and coworkers have explored a large group of LBCL patients who have undergone treatment with Axi-Cel; 53% of these patients developed CAR-T -related prolonged cytopenia;⁷² patients with prolonged cytopenia experienced more frequently CRS and ICANS.⁷² Single cell analysis of bone marrow cells suggested that a subset of IFN- γ -expressing CD8⁺ T cells may be responsible for prolonged cytopenia, together with an associated enrichment of IFN- γ signaling within bone marrow HSCs.⁷²

Treatment of cytopenia may safely include granulocyte colony-stimulating factors (G-CSF), erythropoietin, or thrombopoietin receptor agonists, together with transfusions of irradiated blood products or, more episodely, with stem cells-boosts.⁷³⁻⁷⁵

Generation of CAR-T cells with a lower toxicity profile. The reduction of toxicities associated with CAR-T cell therapies could be achieved by modifying some constituents of CAR-T cells.

Several strategies have been explored in this sense. Obecabtagene autocel (Obe-Cel) is a CD19-directed CAR-T cell product based on a single chain variable fragment (scFv) with a low antigen affinity owing to rapid dissociation.⁷⁶ Obe-cel was evaluated in R/R B-ALL patients, showing a good safety profile with no patient experiencing $\geq$ grade 3 CRS and 15% displaying grade 3 ICANS, rapidly regressing to $\leq$ grade 1 after glucocorticoid treatment.⁷⁶ Complete responses were achieved in 78% of cases after Obe-cel.⁷⁷

Another study was based on the development of a CD19-directed CAR construct containing a CD28 costimulatory domain and CD8 α hinge and transmembrane domain instead of CD28 hinge, transmembrane, and costimulatory domains, as observed in Axi-Cel.⁷⁸ Those CAR-T cells (named Hu19-CD8282) were infused in patients with LBCL, resulting in lower serum cytokine levels and markedly reduced incidence of ICANS compared to fully CD28 co-stimulated CAR-T (5% vs 50%, respectively).⁷⁸

An alternative approach consisted in the interposition of an intracellular domain from Toll-like receptor 2 (TLR2) between CD28 and CD3 ζ ; preclinical studies have shown that a CD19-CAR T cell generated with this modified structure resulted in reduced production of cytokines such as GM-CSF and IFN- γ , with maintained production of IL-7.⁷⁹ The third generation of CAR-T cells developed with this CAR construct was evaluated in 21 LBCL patients (ENABLE clinical trial), resulting in a good safety profile with grade 1-2 CRS in 62% of cases and no grade 3 CRS and no ICANS of any grade.⁷⁹

Since redundancy of CD28 and CD3zeta signaling accelerates T cell differentiation and promotes t cell exhaustion, a further strategy (1XXCARs) consisted of inactivating point mutations in the two distal immunoreceptor tyrosine-based activation motifs (ITAMs) and retaining one functional proximal ITAM; 1XXCARs could exert a significant antitumor activity at lower doses, with reduced incidence of G3-4 CRS and ICANS (3% and 4%).⁸⁰

Another strategy involves designing CARs that incorporate into their structure molecules inhibiting inflammatory cytokines, such as introducing in the CAR-T construct an inductor of an anti-IL6 scFv, an IL1 receptor antagonist (IL-1RA), or an IFN γ antagonist.⁸¹⁻⁸²

Yoshikawa and coworkers developed a CAR (G6/7R)

composed of extracellular IL-6 receptors and intracellular IL-7 receptors with a constitutive mutation.⁸³ G6/7R-expressing CAR-T cells significantly reduce IL-6 levels *in vitro* and demonstrate durable antitumor efficacy in multiple tumor models.⁸³

Mice treated with CAR-T cells secreting Tocilizumab-derived single-chain variable fragments were described as having reduced CRS-related toxicity and enhanced antitumor efficacy.⁸⁴

Another approach was based on selecting T cell populations for the generation of CAR-T cells. In this context, Aldoss et al. reported the evaluation of the safety and efficacy of memory-enriched CD19-CAR-T cells in R/R B-ALL.⁸¹ The results observed in 48 R/R B-ALL patients showed a CR rate of 87%, with a good safety profile (7% grade I CRS and 17% of grade ≥ 3 ICANS).⁸⁵

Conclusions. CAR-T cell therapies are effective for many hematological malignancies, but toxicities may still limit outcomes. Thus, considerable improvements have been made in the treatment of these toxicities, such as CRS and iCANS.

Inflammation is still the major determinant of many toxicities. CRS and ICANS are the most frequent CAR-T-related acute toxicities, depending on CAR-T lytic action, cytokines, and endothelial activation. Treatment

of CRS has considerably improved, particularly through the use of the IL-6 receptor antagonist Tocilizumab and glucocorticoids.

Movement disorders are observed in a minority of patients treated with BCMA-CAR-T cells and represents a severe subtype of ICANS, with difficult so far.

B-cell aplasia and hypogammaglobulinemia are related to an on-target toxicity mechanism and require adequate therapeutic management.

Infectious events represent the most frequent cause of nonrelapse mortality in patients treated with CAR-T, resulting from immunosuppression consequent to cytopenias and BCA.

Late-onset hematological toxicities are due to the exposition of bone marrow to logistic flares and preexisting conditions, resulting in impairment of bone marrow function.

Secondary malignancies can be observed after CAR-T cell and can involve myeloid therapy related malignancies. Patients undergoing CAR-T cell therapies should be monitored for secondary malignancies indefinitely after CAR-T cell treatment.

The development of new constructs and products could reduce the inflammatory-related toxicities of CAR-T cells, resulting in a more favorable toxicity profile.

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