REVIEW

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Mechanisms of failure of chimeric antigen receptor T-cell therapy

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Purpose of review
Although chimeric antigen receptor T (CART)-cell therapy is best recognized for its antitumor effect in relapsed/refractory B-cell hematological cancers, it is still associated with a high relapse rate.

Recent findings
We firstly analyzed internal immunological and genetic reasons of CD19+ relapse after treatment for R/R B-cell hematological cancers with CART19 cells. The reasons: murine-derived scFv may limit expansion of CART cells. Repeated antigen exposure leads to T-cell exhaustion. Activation of T cells can cause T-cell senescence and high expression of inhibitive receptors, PD-1, CTLA4, TIGIT, LAG-3, CD244, CD160, TIM3, which might be solved by some external pharmacological intervention methods [for instance, the use of FC (Fludarabine, Cyclophosphamide) lymphodepletion regimen, lenalidomide, PD-1 inhibitor, ibrutinib and humanized CD19-CART cells. Secondly, mechanism of CD19 relapse can be attributed to the preexisting of $CD19^-$ subclone, the loss or alternative RNA splicing on exon 2 of chromosome 16 on which *CD19* gene is located, B-cell transcript factors – paired-box 5 (PAX5) and early B-cell factor 1 (EBF1) are down-regulated to cause lineage-switch from lymphoid to myeloid.

Summary
Although different preparation techniques generates various entities of CART 19 cells, these problems could be conquered by novel agents and novel CAR system.

Video abstract
Although Chimeric Antigen Receptor T (CART) cell therapy is best recognized for its antitumor effect in Relapsed/Refractory B-cell hematological cancers, it still shows a high relapse rate. We review mechanisms of failure of CART therapy. http://links.lww.com/COH/A18.

Keywords
B-cell hematological cancers, novel agent and novel chimeric antigen receptor T-cell system, the relapse mechanism after CART19 cells

INTRODUCTION

The chimeric antigen receptor T (CART)-cell therapy was most recognized by its antitumor ability in relapse/refractory (R/R) hematological cancers to achieve a high complete remission rate. It thus led us into a new era of immunotherapy. Although CART19 cell therapy has achieved striking curative effect in B-cell hematological cancers in recent years, it still shows a high relapse rate.

The four generations of CART cells with different structures of co-stimulatory domain, identical T-cell amplification degree *in vitro* and CART19 cells infusion dose, heterogeneity of the diseases, as well as the different chemotherapy and lymphodepletion regimen, have been considered as the confounding factors of the research results of CART cell immunotherapy. At present, there are a series of clinical studies on the relapsed B-cell hematological cancers at home and abroad. Patients who relapse after CART cell treatment have been divided into two categories, $CD19^+$ relapse and $CD19^-$ relapse, providing clues for the further

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KEY POINTS

- CD19 positive relapse after CART-cell therapy is mainly because of internal immunological and genetic reasons: limitation of expansion and amplification of CART cells; T-cell exhaustion caused by repeated antigen exposure; T-cell senescence caused by T cells over activation and over expression of inhibitive receptors (such as PD-1, CTLA4, TIGIT, LAG-3, CD244, CD160, TIM3).
- These immunological and genetic impact factors might be solved by some external pharmacological intervention methods, for instance, lenalidomide, PD-1 inhibitor, ibrutinib, and some novel CD19-CART cells.
- CD19 negative relapse can generally be attributed to external reason as preexistence of $CD19^-$ clones and internal molecular biology and cytogenetic factors, such as lineage convert and the RNA splicing leading to loss or down-regulation of CD19 expression.
- The feasible avenue to conquer CD19 negative relapse could be dual/tandem CART cell infusion, SUPRA CART system and other antibody-based therapeutics.

exploration of the complicated relapse mechanism after CART cell treatment.

Mechanisms of activation of CART cells *in vivo*: because of the co-stimulatory molecules of CART19 cells, major histocompatibility complex (MHC) is not imperative for antigen presentation in T-cell stimulation and activation. The activated T cell can process a series of proliferation and differentiation into $CD8^+$ cytotoxic T cells (CTLs). Once encountered and combined with $CD19^+$-expressed lymphoblastic cells, CART19 cells can be activated by the dual signaling pathways, secreting perforin, cytokines and granzyme, thus synergistically kill tumor cells with various mechanisms.

Mechanism had been studied by numerous researches. The mechanism of relapse after the treatment for R/R B-cell hematological cancer with CART19 cells (Fig. 1).

CONFOUNDING FACTORS

External objective reasons

Different co-stimulatory molecules

The antitumor effects of different co-stimulatory molecules in CART19 cells are different as well. CD19 CAR-T cells in ZUMA-1, JULIET and TRANSCEND studies have the similar structure containing same single-chain variable fragment (FMC63) and use CD3 for intracellular signaling but different combinations of transmembrane and costimulatory domains, leading to disparity on efficacy (Table 1).

Long *et al.* [4] selected PD-1, LAG-3 and TIM-3 suppressor receptors as the detection markers for T-cell senescence. Through animal experimentation, it was found that CD28-CART19 cells had a strong tumor killing effect, whereas 4-1BB-CART19 cells was proven to be less potent but increased antitumor persistence.

Distinct manufacture methods

As lentivirus transfection is prone to give rise to insert mutations, *CRISPR/Cas9* gene editing technology has become a prospective method in the manufacturing of CART19 cells [5]. However, recent research [6] found that *CRISPR/Cas9* system causes genomic damage and complex rearrangements, which may lead to pathogenic consequences. The *CRISPR/Cas9* was not as precise and accurate as we expected. Recent study indicates that CART19 cells exhibits better differentiated ability and effector function when harvested from cultures at day 3 or 5 rather than at the routine period of 9–14 days *in vitro* [7].

Various categories and dosage of CART19 cells

Even in conventional CART19 cells, different preparation methods are taken in different centers, leading to distinct T-cell amplification. Furthermore, combinatorial antigen sensing developed to enhance tumor specificity [8▪], dual-CAR, tandem CAR and bi-epitopic CART cells [9▪], that targeted two tumor-specific antigens or epitopes, which can reduce tumor antigen escape rate and tumor relapse rate was also applied to clinical use [10–12]. There are also novel CAR-modified cell varieties in treating other hematological malignancies, such as multiple myeloma and acute leukemia with armored CART [13▪,14], CD44v6-targeted T cells [15], CAR-NK [16▪], CS1 CAR-Redirected T cells [17], anti-BCMA CART cell and anti-CD138-Kappa-light-chain CART cell, and so forth, making it hard to fully understand the relapse mechanism of post-CART cell treatment on B-cell malignancies.

Internal immunological and genetic reasons

Tumor heterogeneity

Different B-cell malignancies have distinct tumor cells and are treated by different chemotherapy regimens. Even in homologous tumors, nontumor cells impact factors, such as somatic cells, transcriptional alterations, epigenetic modifications and molecular interactions can cause diverse disease attributes and lead to distinct prognosis. In addition, given the heterogeneous nature of the

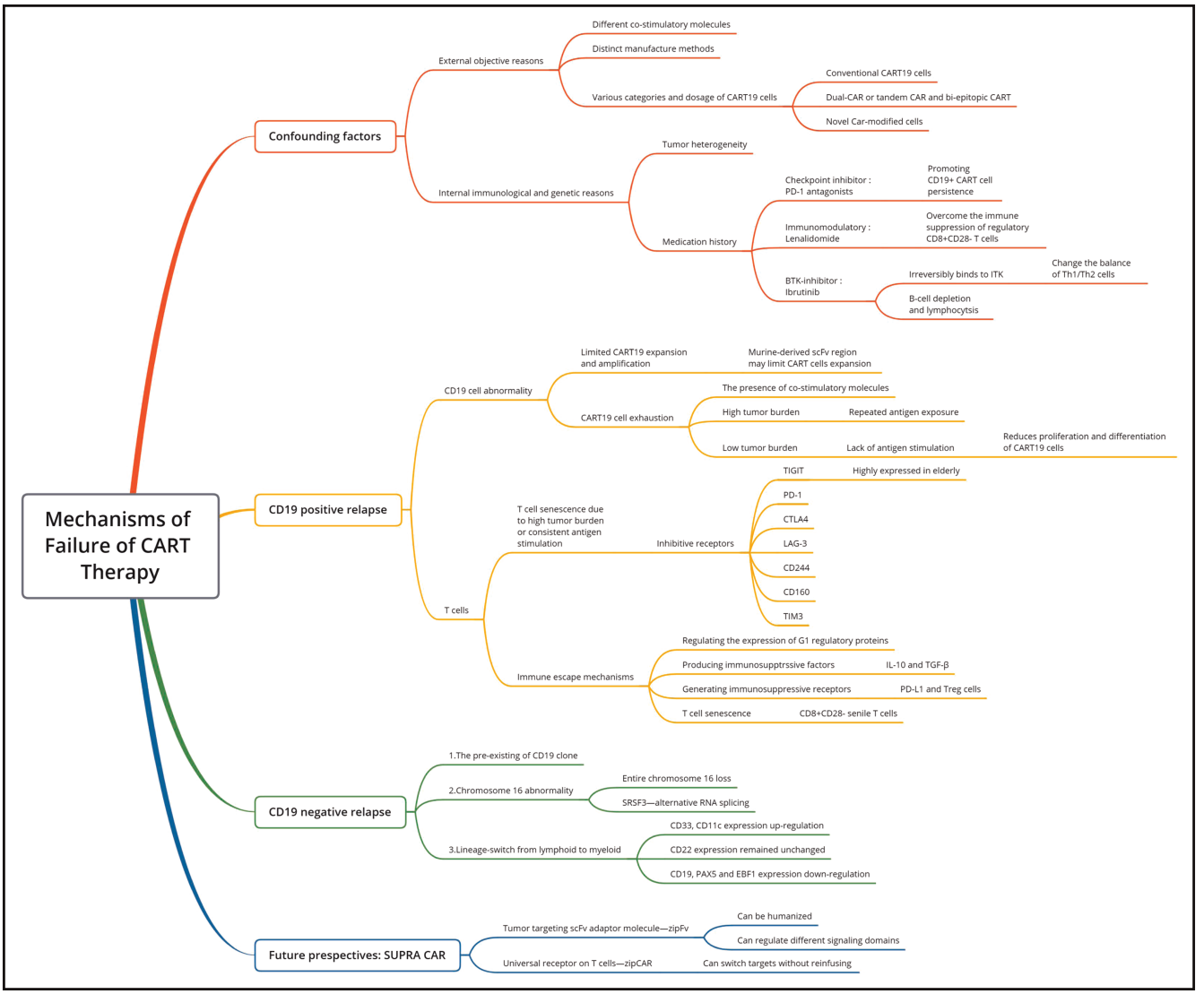

FIGURE 1. The mechanism of relapse after the treatment for R/R B-cell hematological cancer with CART19 cells.

Table 1. CD19 chimeric antigen receptor T-cell structure and efficacy in ZUMA-1, JULIET and TRANSCEND studies

Clinical Trial	CART cells	ClinicalTrials.gov number	Transmembrane domain + costimulation	Tumor category	ORR%	CR%
ZUMA-1 [1▪▪] ($n = 108$)	Axicabatgene ciloleucel	NCT02348216 PHASE 1-2	CD28	R/R DLBCL PMBCL transformed FL	83%	58%
JULIET [2] (n = 93)	Tisagenlecleucel	NCT02445248 PHASE 2	CD28 + 4-1BB	R/R DLBCL transformed FL DHL/THL	52%	40%
TRANSCEND [3] (n = 91)	Lisocabtagene maraleucel	NCT02631044 PHASE 1	CD28 + 4-1BB	R/R DLBCL PMBCL FL3B MCL	74%	52%

Lisocabtagene maraleucel in TRANSCEND study has precise dose and ratio of CD8 and CD4 cells. DHL, double hit lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; FL3B, follicular lymphoma 3B grade; MCL, mantle cell lymphoma; PMBCL, primary mediastinal large B-cell lymphoma; R/R, relapse/refractory; THL, triple hit lymphoma.

patients' baseline conditions including age, disease and risk stratification, prior chemotherapy regimens and lines, whether using targeted drugs or not, curative efficacy evaluation pre-CART therapy are factors that need to be taken into consideration.

Medication history of targeted drugs and immunomodulatory drugs

Checkpoint inhibitor

As immune checkpoints were proved to have a critical role in immunotherapies and tumor microenvironment, antiprogrammed death-1 (PD-1) and programmed death ligands 1 (PD-L1) are currently widely used in relapsed/refractory B-NHL exhibiting high PD1 expression by T cells. Studies [18] reported increased expression of co-inhibitory molecular PD-1 in CART cells after infusion, and the obvious increasing of PD-1-expressed CAR19 T cells occurred between the time of infusion and the time of reaching peak CAR19 blood levels. As well, PD-1 expression is weaker in the CD19-negative CART cells than in CD19-positive CART cells. Zhang *et al*. [19▪] demonstrated that the combination of CD19 CART cells with a dose-adjusted PD-1 inhibitor shows synergistic antitumor capacity in a mouse trial, so the PD-1 inhibitor treatment before CART cell therapy might affect the efficacy positively.

Immunomodulatory lenalidomide

IKZF1 and IKZF3 are transcription factors that are critical to the differentiation of B cells, lenalidomide can increase serum IL-2 level *in vitro* by down-regulating the expression of IKZF1/3 [20], thereby promoting the proliferation of natural killer (NK) cells, NK/T cells and $CD4^+$ T cells. In-vitro studies showed that lenalidomide can decrease the amount of IL-6 that was secreted by monocytes and recede the immunosuppression on CART19 cell through the mechanism of reducing the quantity of $CD8^+CD28^-$ Treg cells [21].

Bruton Tyrosine Kinase inhibitor ibrutinib

Due to the significant sequence and functional homology between BTK (Bruton Tyrosine Kinase) and ITK (IL-2-inducible kinase) [22], ibrutinib can inhibit the ITK signal pathway that is expressed on the surface of NK cells, NK-T cells and especially T cells including CART cells. There is another hypothesis about the interaction between ibrutinib and CD19 CART cell therapy as ibrutinib could cause depletion of targeted B cells in peripheral blood, the consequence of low-tumor burden might cause the loss of immunogenicity, thereby impact the CART cell expansion and proliferation. On the contrast, Ruella *et al*. [23] conducted experiments of combining CTL019 CART cells and ibrutinib to treat mantle cell lymphoma (MCL). Although ibrutinib changes the balance of Th1/Th2 cells *in vitro*, it has lower PD-1 expression and been proved to increase T-lymphocyte counts without changing T subsets by triggering T cells' mobilization into peripheral blood in-vivo experiments. Ibrutinib—CART-cell interaction is complex and remains a controversial issue. We are looking forward to the results of ZUMA-2 study (NCT02601313) [24].

CD19-POSITIVE RELAPSES

CART19 cell abnormality

Limited CART19 expansion and amplification

Application of humanized CART cells: as the immune response induced by murine-derived single chain fragment variable (scFv) region may limit the continuous expansion of CART cells *in vivo* and increase the risk of leukemia relapse, Maude *et al*. [25] developed a humanized scFv, which was derived from mouse FMC63 antibody. Results showed that hCART19 cells therapy was effective for R/R ALL patients and those who relapsed after conventional CART-cell therapy.

CART19 cell exhaustion

T-cell exhaustion is the specific stage of T-cell differentiation caused by repeated antigen exposure, which weakens the function of effector T cells. CART cells, however, will inevitably be consumed because of the presence of co-stimulatory molecules, even without sustained antigen exposure. CART19 cells will be exhausted rapidly because of a high tumor burden, whereas a low-tumor burden reduces proliferation and differentiation of CART19 cells because of the lack of antigen stimulation. Many patients with a low tumor burden as well as low normal blood B-cell level were less likely to obtain remission after CART19 cell infusion [18]. This phenomenon indicated that endogenous $CD19^+$ cells could enhance proliferation of CART19 cells and speculated that $CD19^+$ cellular vaccines might be another avenue to overcome immunological unresponsiveness in CART-cell therapy.

T cells

T-cell senescence

The continuous activation of T cells can cause T-cell senescence that is considered irreversible and is

mainly related to age. T_{reg} cells have been proven to be capable of enforcing $CD8^+$ cytotoxic T cells, $CD4^+$ helper T cells and effector T cells into senescence. The degradation of the effect function of senile T cells is accompanied by high expression of inhibitive receptors [26], such as PD-1, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4, or CD152), T-cell immunoglobulin and ITIM domain (TIGIT), lymphocyte activation gene-3 (*LAG-3*), CD244, CD160, T-cell immunoglobulin and mucin domain-containing-3 (TIM3), and so forth. At the same time, high expression of CD57 can damage the proliferation capacity of T cells, while increasing the ligand number of Killer Cell Lectin-like Receptor Subfamily G1 (KLRG-1) can increase the proliferation of T cells [27]. If these two above-mentioned biomarkers have high expression, both can cause the CART cells lose the co-stimulatory signals, such as CD27 and CD28, whereas the down-regulation of CD28 expression is related to the loss of human telomerase RNA component (hTERC), which causes the loss of telomerase activity and leads to subsequent telomere damage, with a consequence of T cells duplicative senescence [28].

In summary, the mAb targeting the inhibitory receptor TIGIT can administrate immunotherapy by strengthening the antitumor function of NK cells, which is also of great significance to improve the efficacy of existing tumor immunotherapy. We envisage that CART-cell therapy, when combined with mAb to TIGIT, might further increase immune responses to cancer.

Immune escape mechanisms

Malignant tumors utilize various strategies to avoid the antitumor immunological effects of the adaptive immune system by establishing a microenvironment of immunosuppression. Immune escape mechanisms include regulating the expression of G1 regulatory proteins, producing immunosuppressive factors IL-10, TGF-β and IDO, and generating immunosuppressive receptors, such as the recruitment of PD-L1 and Treg cells.

CD19-NEGATIVE RELAPSES

It is hard to find a breakthrough in the complex immune system, so we turned our attention to the mechanism of $CD19^-$ relapse category. However, $CD19^-$ relapse is resistant to CART19-cell reinfusion [29] and cannot be prevented by extending the persistence of T cells. According to the recent estimates, the $CD19^-$ ALL relapsed after blinatumomab ranging between 10 and 30% in retrospective studies [30], another statistics showed that $CD19^-$ relapse accounts for 10–20% of post-CART19 therapy ALL patients [31,32]. Therefore, physicians should maintain a high level of suspicion for the evolution of post-CART malignancies.

The preexistence of $CD19^-$ clones

Preexistence of a minor $CD19^-$ population in the leukemia bulk has been proposed as a mechanism of resistance to blinatumomab and subsequent emergence of a $CD19^-$ relapse [33]. Grupp *et al.* [34] compared the samples of one pediatric case before CART19 therapy and $CD19^-$ relapse after CART19 therapy by flow cytometry, the results which was coincident with Fisher *et al.* [35] and Ruella *et al.* [11] demonstrated that rare $CD19^-$ blasts were existing in some samples before treatment in patients with $CD19^+$ ALL. They hypothesized that these preexisting cells might be the trigger of $CD19^-$ relapse which developed as the dominant clone under the selective pressure of CART19 therapy and eventually resulted in $CD19^-$ relapse.

The loss or down-regulation of CD19 expression and the intervention

CD19 is not an essential condition of survival and proliferation of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cells [36]. It was found as a common phenomenon of $CD19^-$ relapse after CART19 therapy. As CD19 is located on chromosome 16p11.2, experiments conducted by Sotillo *et al.* [37] found that entire chromosome 16 loss or alternative RNA splicing on exon 2, which was induced by serine and arginine-rich splicing factor 3 (SRSF3) occurred on $CD19^-$ xenograft tumor mice models. In their experiments, the *CD19* gene was tested with the methods of whole exome sequencing (WES) and RNA-sequencing, finding de novo frameshift and missense mutations in exon 2 of CD19. The mutations did not result in the silencing of CD19 expression, but expressed the truncated protein with the presence of alternative exon 2 splicing of CD19, thus it could escape from the tumor killing effect as the CD19 epitope could not be recognized by CART19 cells. As the result, future CARs and other antibody based therapeutics should be designed to target essential exons, as a way to prevent escape [38].

Importantly, another mechanism of rapidly relapsing leukemia, especially in *MLL* gene rearranged pediatric leukemia, is lineage-switch from lymphoid to myeloid that results from reprogramming by down-regulating the B-cell transcript factors – PAX5 and EBF1 [39,40]. $CD19^-$ relapse was not only found to have occurred through lineage switch of B-precursor cells from the lymphoid lineage to a

Table 2. Controlling split, universal and programmable chimeric antigen receptor activity *in vivo* through zipFv

	zipFv dosage		zipFv affinity		Competitive zipFv	
	Low	High	Low	High	Low	High
Antitumor effect	–	–	Low	High	–	–
Cytokine release	Low	High	Low	High	High	Low

$CD14^+$ myeloid lineage in 4% of B-precursor ALL [39,41] but also reported that CD22 expression was maintained in the $CD19^-$ phenotype relapses [40], reminding us that dual/sequential CART cell infusion may play a role in preventing $CD19^-$ relapse.

CD22: Jacoby *et al.* [40] suggested that simultaneous pressure on CD19 and CD22 might be an avenue to reduce the possibility of lineage switching, but anti-CD22 CART cells seemed to have only limited activity when B-cell malignancies was $CD19^-$ relapse.

CD123: CD123 is the IL-3 receptor expressing on hematopoietic progenitor cells. The studies [11,42] proved that combining CART19/123 cells could effectively prevent relapse caused by the loss of CD19 phenotype and the patients who developed $CD19^-$ relapse could be treated by CART123 cells.

FUTURE PERSPECTIVES: SPLIT, UNIVERSAL AND PROGRAMMABLE CHIMERIC ANTIGEN RECEPTOR

To alleviate the various limitations of CART cells, Cho *et al.* [43▪▪] presented a split, universal, and programmable (SUPRA) CAR system, which has the ability to switch targets without reinfusing other antigen-specific T cells, and can logically respond to multiple antigens by tuning T-cell activation precisely.

Conventional CART has a fixed structure of invariable antigen-specific scFv and intracellular signaling domains. This SUPRA CAR is composed of a universal receptor on T cell (zipCAR) and tumor targeting scFv adaptor molecule (zipFv). The zipCAR universal receptor is generated from the fusion of intracellular signaling domains and a leucine zipper as the extracellular domain. The zipFv adaptor molecule is generated from the fusion of a cognate leucine zipper and a scFv. The scFv of the zipFv binds to the tumor antigen and the leucine zipper binds and activates the zipCAR on the T cells [43▪▪].

When one of the controllable region is over activated and causes severe cytokine release syndrome (CRS), we can mitigate these toxicities by controlling other variable regions to regulate T-cell activation level. In order to reduce the extent of CRS, a competitive zipFv, which can prevent zipCAR from being activated by binding to the rest of zipFv has been developed (Table 2).

This SUPRA CAR system can also combat the antigen escape and achieve the antitumor effect equal to conventional Dual CART cell therapy. Of note, different antigens can easily be targeted without re-manipulation because of the SUPRA CAR platform.

In addition, SUPRA components have been proven to be effective in reducing immunogenicity while being humanized. Furthermore, the experiment also used orthogonal SUPRA CARs to regulate different T-cell signaling domains and T-cell subtypes independently to increase the range of the immune responses.

CONCLUSION

The SUPRA CAR system is a prospective product with inducible and logical control capabilities that can improve the safety and efficacy of current immunotherapy. However, further research is intensively needed to explore the toxicity and side-effects, the interaction of which with novel agents and the immune system affects the persistence and expansion of these SUPRA CART cells.

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Conflicts of interest

There are no conflicts of interest.

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A prospective novel CART cell entity with logical operated system that simultaneously encompasses multiple critical 'upgrades', such as the ability to switch targets without re-engineering the T cells, finely tune T-cell activation strength, and sense and logically respond to multiple antigens.