HHS Public Access

Author manuscript

Am Soc Clin Oncol Educ Book. Author manuscript; available in PMC 2024 December 03.

Published in final edited form as:

Am Soc Clin Oncol Educ Book. 2018 May 23; 38: 830–837. doi:10.1200/EDBK_200773.

The Landscape of CAR T cells beyond ALL for Pediatric Solid Tumors

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Abstract

Adoptive cell therapy with genetically modified T cells holds the promise to improve outcomes for children with recurrent/refractory solid tumors, and has the potential to reduce treatment complication for all patients. While T cells expressing chimeric antigen receptors (CARs) specific for CD19 had remarkable success for B-cell derived malignancies leading to their FDA approval, CAR T cells were less effective for solid tumors and brain tumors. Lack of efficacy is most likely multifactorial, but heterogeneous antigen expressing, limited migration of T cells to tumor sites, and the immunosuppressive, hostile tumor microenvironment have emerged as major roadblocks that need to be addressed. In this review we summarize the clinical experience with CAR T-cell therapy for pediatric solid tumors including brain tumors. In addition, we will review strategies that have and are being developed to enhance their anti-tumor activity.

INTRODUCTION

Immunotherapy for pediatric malignancies holds the promise of improving outcome and reducing treatment-related complications. Among different forms of immunotherapy that are actively being pursued, the adoptive transfer of T cells expressing chimeric antigen receptors (CARs) has garnered significant excitement due to the success of CAR T-cell therapy for CD19-positive malignancies.[1–11]

CARs are synthetic molecules that combine the specificity of monoclonal antibodies (MAbs) with the effector function of T cells.[12–14] The prototypic CAR consists of an antigen binding domain encoded by a single chain variable fragment (scFv) derived from a MAb, a hinge and transmembrane domain, and signaling domains derived from the CD3.ζ chain, and costimulatory molecules such as CD28 and/or 41BB (Fig 1A). The majority of CAR T-cell products are generated by viral transduction using replication incompetent retro- or lentiviral vectors (Fig 1B).

Since submission of the first investigational new drug application (IND) for CD19-CAR T cells, the field as moved rapidly, culminating in the FDA approval of two CD19-CAR T-cell products in 2017.[15] The interested reader is referred to recent reviews, which summarize

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the clinical results of CD19-CAR T cells in detail.[1,2] Here, we will only highlight key findings, which need to be considered as we develop and improve current CAR T-cell therapy approaches for pediatric solid tumors.

First, lymphodepleting chemotherapy with cyclophosphamide and fludarabine is critical to allow engraftment and expansion of adoptively transferred CD19-CAR T cells. Second, CD19-CAR T cells can eradicate B-cell malignancies regardless of their underlying genetic alteration. Third, CD19-CAR T cells, can eradicate B-cell malignancies, which are refractory to chemotherapy and/or radiation, highlighting that T cells kill their target cells through different cytotoxic mechanism than conventional therapies. Fourth, CD19-CARs have to encode a costimulatory signaling domain to be effective. While CD19-CAR.CD28.ζ and CD19-CAR.41BB.ζ T cells have not been compared directly in a single clinical study, results of published studies indicate that CD19-CAR.41BB.ζ T cells persist longer.[1–11] However, at present it is unclear if this translates into improved antitumor activity.[1–11] Fifth, targeting a single antigen, can result in antigen loss variants, and lastly, CD19-CAR T-cell therapy can be associated with significant clinical side effects including cytokine release syndrome (CRS), and neurotoxicity.[1–11]

In this educational review, results of early phase clinical studies with CAR T cells for pediatric solid tumors and brain tumors are summarized (Table 1). In addition, strategies how to improve their antitumor activity, and current challenges are discussed.

CLINICAL STUDIES WITH CAR T CELLS FOR PEDIATRIC BRAIN AND SOLID TUMORS

Neuroblastoma

Two clinical studies have been conducted with first generation CAR T cells in neuroblastoma patients. In the first study, patients received up to $10^9/m^2$ CD8-positive CD171-specific CAR T-cell clones.[16] Adoptive transfer of T cells was well tolerated, but T cells persisted only for 6 weeks, and only 1 out of 6 patients had a partial response. One strategy to improve the persistence of adoptive transferred T cells takes advantage of the specificity of the endogenous αβTCR expressed by CAR T cells. For example, virus-specific T cells receive appropriate stimulation by viral antigens presented by professional antigen-presenting cells. This concept was explored in the second clinical study, in which investigators expressed a 1st generation GD2-specific CAR in polyclonal Epstein Barr virus (EBV)-specific T cells. The *in vivo* fate of GD2-CAR EBV-specific T cells was directly compared to activated T cells expressing the same CAR in individual patienst.[17,18] While GD2-CAR EBV-specific T cells did not expand, they persisted longer than GD2-CAR activated T cells. Five out of 11 patients with active disease showed tumor responses or necrosis. Three of them had complete responses.

More recently, activated T cells expressing a third generation GD2-CAR with a CD28.OX40.ζ endodomain were evaluated in neuroblastoma patients.[19] Three cohorts of patients were infused. The first cohort (4 patients) only received CAR T cells, the second cohort (4 patients) received lymphodepleting chemotherapy (cyclophosphamide and

fludarabine) prior to CAR T-cell infusion, and the third cohort (3 patients) also received 2 doses of pembrolizumab given day-1 and day+21 in relationship to the CAR T-cell infusion. Lymphodepleting chemotherapy induced expansion of CAR T cells for up to 3 logs, which peaked at one to two weeks post infusion. Addition of pembrolizumab did not further improve T-cell expansion or persistence. There was a significant increase in the frequency of circulating myeloid cells in the peripheral blood with an immunosuppressive M2 phenotype post CAR T-cell infusion in all three cohorts. Further studies are needed to investigate the significance of this finding.

In addition to these published studies, several clinical trials are in progress including one study evaluating 2nd generation (41BB.ζ) and 3rd generation (CD28.41BB.ζ) CAR T cells targeting CD171[20] in patients with neuroblastoma and ganglioneuroblastoma (NCT02311621). In addition, studies continue to explore GD2-CAR T cells for neuroblastoma including NCT02107963, NCT02761915, and NCT03373097.

Sarcoma

T cells expressing 2nd generation HER2-CARs (CD28.ζ) have been evaluated in 19 patients with refractory HER2-positive sarcoma (16 osteosarcoma, 1 Ewing sarcoma, 1 primitive neuroectodermal tumor, 1 desmoplastic small round cell tumor). HER2-CAR T-cell infusions were well tolerated with no dose limiting toxicity.[21] HER2-CAR T cells persisted for at least 6 weeks in patients who received greater than $1\times10^6/m^2$ HER2-CAR T cells, and were detected at tumor sites. Of 17 evaluable patients 4 had stable disease. Three of these had their tumor removed with one showing ≥90% necrosis. Subsequently, six patients received lymphodepleting chemotherapy prior to HER2-CAR T-cell infusion. Significant HER2-CAR T-cell expansion was observed without apparent toxicity, and 1 patient with refractory rhabdomyosarcoma (RMS), who only had persistent bone marrow disease, achieved a complete response for greater than 12 months.[22] In addition to HER2-CAR T cells, GD2-CAR T cells are actively being explored (NCT01953900, NCT02107963) for sarcoma. In one study a third generation GD2-CAR with a CD28.OX40.ζ endodomain is expressed in varicella zoster virus (VZV)-specific T cells, and the investigators are evaluating if GD2-CAR VZV-specific T cells can be boosted with an FDA-approved VZV vaccine post infusion. Lastly, one clinical study (NCT02932956) is FDA-approved to evaluate the safety and efficacy of GPC3-CAR T cells[23] in pediatric solid tumor patients including RMS. However, the study is currently not open for accrual.

Brain tumors

Clinical studies with CAR T cells targeting HER2, EGFRvIII, and IL13Rα2 have been conducted in patients with high-grade glioma.[24–27] Two studies only infused adults, whereas 10 out of 17 patients were children on the HER2-CAR T-cell therapy study. T cells were either given intravenously (HER2, EGFRvIII) or directly injected into the tumor and/or ventricle (IL13Rα2). Similar to the clinical results of CAR T-cell therapy studies for solid tumors, the majority of brain tumor patients had progressive disease. However, responses were observed including one partial,[24] one complete,[26] and several patients had stable disease for a prolonged period of time.[24]

Detailed correlative studies performed post infusion of EGFRvIII-CAR T cells revealed that T cells were able to migrate to glioma sites after intravenous infusion.[25] Target antigen expression was reduced in resected gliomas indicative of an 'on target' CAR T-cell effect. In addition, gliomas upregulated the expression of immunosuppressive molecules including indoleamine 2,3 dioxygenase (IDO) and IL-10, highlighting that gliomas have the ability to counteract infiltrating 'pro-inflammatory' CAR T cells. Currently, only one clinical study (NCT02208362) is actively recruiting patients with gliomas including children greater than 12 years of age. This study is evaluating the safety and efficacy of intracranial injections of IL13Rα2-CAR.41BB.ζ T cells.

In conclusion, the initial foray into the clinic with CAR T cells has demonstrated their safety for pediatric solid tumors and brain tumors. However, only subsets of patients have benefited from this approach so far. Potential strategies to increase the efficacy of CAR T cells are reviewed in the next section.

STRATEGIES TO IMPROVE CAR T-CELL THERAPY FOR SOLID TUMORS

Lack of CAR T-cell efficacy is most likely multifactorial. Major roadblocks include the i) availability of targeted antigens and their heterogeneous expression, ii) homing of T cells to tumor cells, and iii) the immunosuppressive tumor microenvironment (Fig 2).

Expanding the repertoire of targetable antigens

The majority of CARs developed so far recognize cell surface proteins, which were originally discovered as targets for MAbs (GD2, HER2, GPC3, and IL13Rα2). Currently, efforts are underway using gene expression array data and proteomics to identify new targetable cell surface antigens.[28] The recent identification of GPC2, which is expressed at high levels on neuroblastoma, highlights the feasibility of this approach.[29] However, it might be difficult to discover antigens that are not expressed at low levels in normal tissues. While private neoantigens are present albeit at low frequency in pediatric tumors,[30] directly targeting these with CAR T cells is not feasible with current technology using viral vectors to generate CAR T cells. Besides the significant regulatory burden and cost, it currently takes more than 6 months to generate a clinical grade viral vector. However, optimizing CAR T cells to efficiently induce immune responses against non-targeted antigen (aka antigen spreading) would be one approach to target private neoantigens, and potentially increase the antitumor activity of CAR T cells similar to cancer vaccines.[31]

CAR T cells are being developed to recognize antigen patterns. Example include designing CARs that recognize two antigens or engineering T cells that express multiple CARs.[32,33] T cells expressing these CARs only get fully activated in the presence of all targeted antigens. In addition, targeting multiple antigens should also offset the risk of selecting antigen loss variants. In addition, so called inhibitory chimeric antigen receptors have been developed that block potential off target T-cell responses.[34] Developing CARs that recognize peptides in the context of major histocompatibility complex (MHC) class I molecules might also increase the potential repertoire of targetable antigens since 2/3 of all expressed proteins reside within the cell. This can be achieved by using a scFv derived from a T-cell receptor (TCR) mimic MAb as a CAR antigen binding domain. Examples include CARs specific for

a HLA-A2-restricted peptide derived from intracellular proteins such as WT1 or Proteinase 3.[35,36]

Lastly, inducible expression system may also provide a potential solution to the 'antigen dilemma'. So called synthetic notch (synNotch) signaling receptors allow the expression of CARs only once a T-cell has migrated to tumor sites, potentially enabling the targeting of antigens that are expressed in normal tissues.[37,38]

Enhancing migration of CAR T-cell to tumor sites and within tumors

Several preclinical studies have highlighted that there is a mismatch between chemokine secreted by solid tumors and chemokine receptors expressed by CAR T cells. Transgenic expression of chemokine receptors has been shown to overcome this roadblock.[39] For example, neuroblastoma secretes high levels of CCL2, however CAR T cells lack expression of the corresponding chemokine receptor (CCR2).[40] Transgenic expression of CCR2b on GD2-CAR T cells resulted in enhanced homing and antitumor activity in preclinical neuroblastoma models.[40] Besides migration to tumor sites, limited migration within tumors might also contribute to the reduced antitumor activity observed in clinical studies. For example, CAR T cells are limited in their ability to degrade the extracellular matrix (ECM), resulting in poor tumor penetration. This can be improved by expressing heparanase.[41] Another approach consists by directly targeting cancer associated fibroblasts (CAFs) with CAR T cells, the main producer of collagen within tumors.[42]

Engineering CAR T cells to resist the immunosuppressive tumor environment

Brain and solid tumors create a hostile tumor environment, which favors T-cell exhaustion and/or dysfunction induced by i) immunosuppressive cytokines (e.g. IL4, IL10, TGFβ), ii) expression of inhibitory molecules (e.g. FAS ligand, PD-L1), iii) the metabolic environment, and/or iv) recruitment of immunosuppressive cells including myeloid derived suppressor cells (MDSCs), cancer associated fibroblasts (CAFs), and/or regulatory T cells (Tregs).[43–45]

While this section is focused on engineering CAR T cells to improve their antitumor activity in the tumor microenvironment, combinatorial therapies are attractive approaches. For example, combining CAR T-cell therapy with checkpoint blockade, oncolytic viruses, chemotherapy, radiation and/or small molecules are actively being explored in preclinical studies with encouraging results.[46–48] Genetic engineering approaches to enhance the antitumor activity of CAR T cells can be divided into two broad categories: i) transgenic expression of immune stimulatory molecules, and ii) silencing negative regulators.

Several preclinical studies have shown that transgenic expression of cytokines (e.g. IL12, IL15, IL18),[49–51] constitutive cytokine receptors,[52] 41BBL,[53] or CD40L[54] enhance the antitumor activity of CAR T cells. One recent study has also demonstrated that transgenic expression of IL7 in combination with the chemokine CCL19 not only enhances the effector function of CAR T cells, but also enables them to induce endogenous T-cell responses against the targeted tumor indicative of antigen spreading.[55]

Directly blocking inhibitory cytokines or converting their signal into a T-cell stimulatory signal are other approaches that are actively being pursued. For example, expressing a

dominant negative TGFβ receptor (DNR) renders T cells resistant to TGFβ in preclinical as well as clinical studies.[56] In addition, DNRs have been developed to provide intrinsic protection from PD1/PD-L1 checkpoint blockade.[57] Chimeric cytokine or switch receptors not only block an inhibitory signal, but convert it into a T-cell stimulatory signal. Examples include receptors that consists of the ectodomain of the IL4 receptor and the transmembrane and intracellular signaling domain of the IL2 or IL7 receptor.[58,59] While siRNA approaches were initially used to silence negative regulators, more recent studies have focused on gene editing technologies such as TALENs and CRIPSR/Cas9. Pertinent examples include the silencing of FAS ligand or the knockout of PD-1 in T cells.[60,61]

As we enhance the effector function of CAR T cells it is advisable to insert safety switches that can be activated if side effects develop. Safety switches that have been tested clinically include the herpes simplex virus thymidine kinase (HSV-*tk*), which enables cell killing in the presence of ganciclovir,[62] or an inducible caspase 9 (iC9),[63] which can be activated by a chemical inducer of dimerization (CID). In addition, expression of cell surface molecules (EGFR, CD20),[64,65] which can be targeted with FDA-approved MAbs are other suicide gene options, which have been successfully evaluated in preclinical models.

CURRENT CHALLENGES

CAR T-cell generation

The majority of clinical studies have used retroviral or lentiviral vectors to generate clinical grade CAR T-cell products. Generating clinical grade viral vectors is time consuming, costly, and their use is associated with a significant regulatory burden. In this regard reducing some of the required testing of CAR T-cell products, as recently advocated,[66,67] would be a step in the right detection. These issues could be overcome with the use of non-viral DNA delivery systems that have been successfully used to generate CAR T cells.[68–70] Lastly, closed cell manufacturing systems[71,72] or the use of ‘off the shelf’ CAR T-cell products[73] hold the promise to streamline CAR T-cell production and/or distribution.

What is the optimal T-cell subset to generate CAR T cells?

Several studies have highlighted that CAR T cells generated from central memory T cells with a defined CD4:CD8 ratio have superior effector function in comparison to CAR T cells generated from bulk T cells in preclinical models.[74,75] Epigenetic profiling of T cells has provided novel insight,[76] and holds the promise to further advance our ability to select the most potent T-cell subset for CAR T-cell generation. Lastly, γδ T cells and invariant natural killer T (iNKT) cells are also actively being explored as T-cell platforms for CAR T-cell therapy.[77,78] In this regard a clinical study with iNKT expressing GD2-CARs and IL15 for neuroblastoma patient is FDA approved (NCT03294954), but not actively accruing patients.

Need for preclinical testing in range of animal models

The majority of preclinical studies have relied on xenograft models, which do not reliably recapitulate the complex tumor microenvironment. Immune competent animal models have been adapted for CAR T-cell therapies and these models will be invaluable as combinatorial therapies are being developed.[79–81] In addition patient-derived xenograft (pdx) models

should enable preclinical testing of CAR T cells against a panel of human tumors that more closely mimic patient tumors than tumor cell lines that have been propagated *in vitro*. Lastly, large animal models hold the promise to evaluate CAR T cells in spontaneous tumor models.[82]

Correlative studies, *in vivo* tracking of infused T cells, and clinical response criteria

There is currently only one published CAR T-cell therapy (see Brain tumor section) that has systematically studied tumor biopsies post infusion.[25] These type of studies will be critical to understand current therapeutic failure and devise evidence-based approaches to overcome them. In addition, our ability to track infused CAR T cells in patients is limited unless they are genetically modified with a reporter gene such HSV-*tk*.[83] Even being able to routinely rack CAR T cells for 48 to 72 hours post infusion would be a major advance, giving us invaluable insight into their initial biodistribution and ability to migrate to tumor sites. While diagnostic imaging immune response criteria have been implemented for the assessment of immunotherapies for solid tumors and brain tumors,[84,85] these were developed in the ‘cancer vaccine era’ and might require further fine tuning for cell-based immunotherapies including CAR T cells.

CONCLUSIONS

The initial foray of CAR T cells into the clinic for pediatric solid tumor and brain tumors demonstrated their safety, but also has highlighted their limited antitumor activity. Additional genetic modification of CAR T cells has greatly enhanced their anti-tumor activity in preclinical studies, and we are hopeful that some of the devised strategies will translate into improved anti-tumor activity in humans. To advance the field there is an urgent need to discover novel antigens that can be targeted with CAR T cells, and to improve our ability to evaluate CAR T-cell therapies in preclinical models. Lastly, being able to track CAR T cells noninvasively and preform detailed studies on patients enrolled on CAR T-cell therapy should enable us to advance the field. We remain hopeful that within the next 5 to 10 years solid tumor patients will benefit from CAR T-cell therapies to the same degree as patients with B-cell derived malignancies today.

ACKNOWLEDGEMENTS

This work was supported by NIH grants 5T32HL092332 and 1R01CA173750, and CRPIT grant RP101335.

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PRACTICAL APPLICATIONS BULLET POINTS

- CAR T cells for pediatric solid tumors are safe, but have limited antitumor activity in early phase clinical studies
- Carefully designed correlative studies will be critical to understand current failures of CAR T-cell therapies and devise strategies to improve them
- Additional genetic modification have improved the antitumor activity of CAR T cells in preclinical models, but ‘improved’ CAR T cells have not been tested in the clinic
- Costs and regulatory requirements associated with clinical testing of CAR T cells have the potential to impede progress

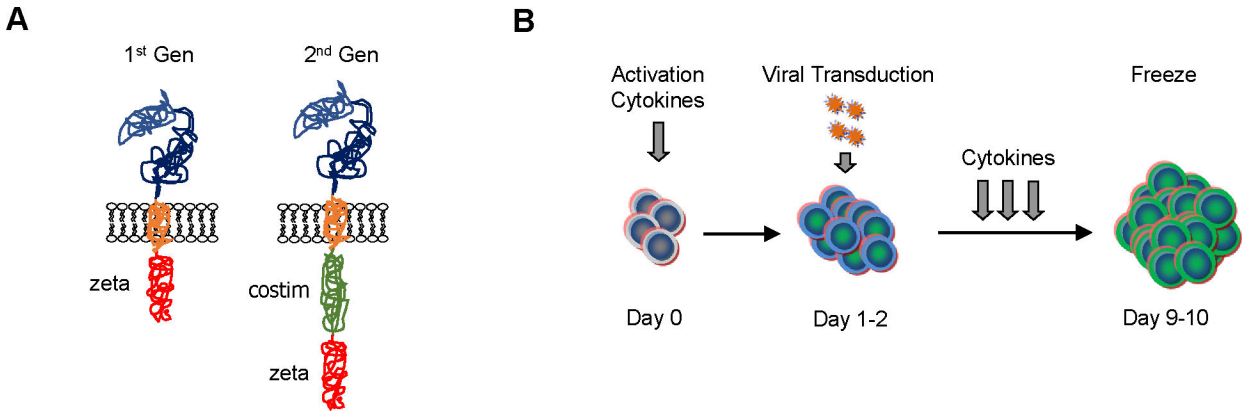

Figure 1: Scheme of CAR T-cell generation

(**A**) CARs consist of an ectodomain, hinge and transmembrane domain, and endodomain. CARs have been designed with or without costimulatory (costim) signaling domains. As examples 1st and 2nd generation (Gen) CARs are shown. (**B**) CAR T cells are produced by activating T cells in the presence of cytokines. Once T-cell proliferate, they are transduced with viral vectors encoding CARs. CAR T cells are subsequently expanded with cytokines, and sufficient CAR T cells for preclinical or clinical studies are generated within 9 to 10 days of culture initiating.

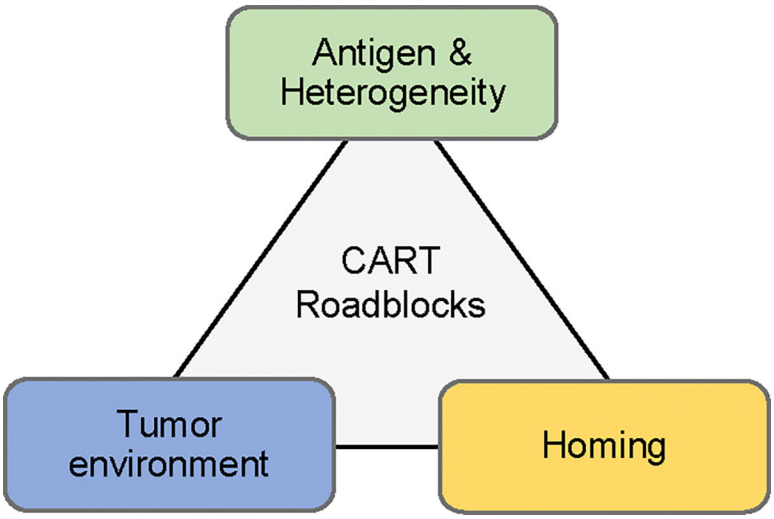

Figure 2: Roadblocks of CAR T cells for solid tumors.
For detail see text.

Table 1:

Selected completed and future CAR T-cell therapy studies for pediatric solid tumors or brain tumors

Disease	Target	CAR signaling domain	Vector	Cell type & delivery	Lymph depl	Comment, NCT #, or ref if published
Neuroblastoma	CD171	ζ	pl	ATC, iv	−	16
	CD171	41BB.ζ or CD28.41BB.ζ	LV	ATC, iv	+	NCT02311621
	GD2	ζ	RV	VST or ATC, iv	−	17,18
	GD2	CD28.Ox40.ζ	RV	ATC, iv	+/−	pembrolizumab, 19
	GD2	ζ, ND	RV	ATC, iv	+	NCT03373097
	GD2	ζ, ND	RV	ATC, iv	+/−	NCT02761915
	GD2	ζ, ND	RV	iNKT, iv	+	2nd transgene: IL15, NCT03294954
Neuroblastoma & sarcoma	GD2	CD28.OX40.ζ	RV	ATC, iv	+	NCT02107963
Sarcoma	HER2	CD28.ζ	RV	ATC, iv	+/−	21,22
	GD2	CD28.OX40.ζ	RV	VST, iv	+/−	VZV Vaccine, NCT02932956
Hepatoblastoma & sarcoma	GPC3	41BB.ζ	RV	ATC, iv	+	NCT02932956
Brain tumors	HER2	CD28.ζ	RV	VST, iv	−	24
	IL13Rα2	41BB.ζ	LV	ATC, ic	−	NCT02208362, 26

pl: plasmid, LV: lentivirus, RV: retrovirus, ATC: activated T cells, VST: virus-specific T cells, iNKT: invariant NK T cells, iv: intravenous, ic: intracranial, ND: not disclosed