

CAR T Cell Therapy: Remedies of Current Challenges in Design, Injection, Infiltration and Working

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Abstract: Chimeric antigen receptor (CAR) T cell therapy, as an innovative immunotherapy, plays a huge role in current cancer therapy. Although CAR T cell therapy has demonstrated therapeutic effects in some subtypes of B cell leukemia or lymphoma, there are many challenges that limit the therapeutic efficacy of CAR T cells in solid tumors. And how to efficiently transport CAR T cells to tumor tissues is a continuing concern for us. In this review, experiments have been extensively studied and compared. We finally compared the influence of different injection methods on therapeutic efficacy. We also carefully explored the difficulties of designing, homing, and working of CAR T cells, and ultimately came up with better solutions for each process to help CAR T cells reach tumor tissue more efficiently and quickly. These results will have significant implications for guiding CAR T cell therapy in cancer treatment.

Keywords: immunotherapy, CAR T cells, metastasis, infiltration, tumor microenvironment

Introduction

Chimeric antigen receptor (CAR) T cell therapy is a landmark immunotherapy in tumor therapy, since it has a very effective and lasting therapeutic effect and broad clinical application prospects. The CAR is a synthetic receptor consisting of an antigen recognition fraction and a T cell signal transduction domain that can redirect lymphocytes to identify and eliminate cells expressing specific target antigens.¹ CAR T cell therapy is currently the most widely used form of this technology. CAR T cell therapy targeting the B cell marker CD19 has shown clear efficacy in a variety of hematologic malignancies such as B cell lymphoma and the lymphoblastic of acute lymphoblastic leukemia according to clinical trials.²⁻⁴ As early as 2017, the US Food and Drug Administration (FDA) launched the CAR T cell preparation for refractory large B lymphoma, and then various CAR T cell products for hematological tumors have been launched.² On June 23, 2021, the CAR T cell therapy product relma-cel (JWCAR029) was approved for marketing in China, becoming the first drug in China for the treatment of adult patients with relapsed or refractory large B-cell lymphoma after two or more lines of systemic therapy.⁵ At the same time, scientists are developing new CAR engineering strategies to extend the clinical success of CAR T cells to patients with other malignancies.⁶ Studies have confirmed that CAR T cell therapy has a significant effect on the treatment of lung cancer and triple-negative breast cancer.^{7,8}

Despite the remarkable success in treating hematologic tumors, CAR T cell therapy still faces many challenges in the treatment of solid tumors due to the difficulty in reaching and infiltrating the tumor.⁹ Theoretically, the modified T cells have a poor homing ability to the tumor sites, and a hostile tumor microenvironment (TME) containing many immunosuppressive cells. The other suppressors may impair the migrating CAR T cells' cytotoxic.¹⁰ Helping CAR T cells homing more efficiently and quickly is a challenge that all current trials must address. Therefore, a comprehensive understanding of the various conditions that CAR T cells can encounter during their migration to the tumor site is

absolutely necessary to advance the application of CAR engineering in cancer immunology. This review briefly describes a series of difficulties in transporting CAR T cells to the tumor site, including the specific mode of recognition / administration / exhaustion of CAR T cells. A comparative analysis of the efficacy of various injection routes is conducted in the article. In addition, we further explored the methods to optimize CAR structure / improve tumor microenvironment and genetic screening, so as to further promote the treatment of CAR T cells for solid tumors.

Recognition of Tumor Cell Surface Antigens by CAR T Cells

CAR T cell therapy is an emerging strategy for treating tumors by isolating and extracting T cells from human blood, modifying them *in vitro* by genetic engineering to increase their anti-tumor activity, at last infusing them back into patients after expansion. The first generation of CARs consisted of the extracellular antigen recognition single chain fragment variable (scFv), merging the transmembrane region and the intracellular signaling domain of the T cell receptor CD3 ζ molecule.¹¹ However, due to the slow expansion and poor persistence of the first generation CAR T cells, the scientists added an ITAM region from the co-stimulatory molecule CD28/CD137 to the intracellular segment of the first generation CAR T cells. After the extracellular antigen recognition region was combined with the target antigen, the T cells obtained both antibody-stimulating and co-stimulatory signals.^{12,13} It exerts direct tumor-killing effect and releases cytokines to recruit more immune cells. Currently, there are widely used bispecific T cell compound such as CD3 / CD19, CD19/CD20, CD5/CD7, etc.^{14–18} This dual recognition mechanism makes CAR T cell therapy more specific, better tumor-killing efficacy, and greatly reduces the risk of off-target toxicity.

The selection of tumor surface-specific antigens plays a pivotal role in the design of CAR T cells. That is, we need to find as much as possible an antigen only expressed on the surface of the tumor. In the early days, the site of this study mostly relied on databases and literature searches for these specific antigens. With the rapid development of gene sequencing technologies, as well as bioinformatics, WES and RNA sequencing of tumor tissues as well as normal tissues to contrasting the tumor-specific mutation sites are diffusely performed. Then, we can select for the predominant sequences with a strong affinity, as determined by HLA typing and the interaction with the specific antigen-TCR. Finally, the gene fragment with the strongest T cell activation was selected *in vitro*.¹⁹ At present, there have been corresponding clinical validation trials, including specific antigen sites for glioma NCT04749641, melanoma NCT03715985, and NCT03558945 in pancreatic cancer, which are widely conducted. Moreover it has been reported that the target PVRL4 of epithelial cancer can be obtained by suppression subtractive hybridization.²⁰ Now, thanks to the in-depth study of representational difference analysis / suppression subtractive hybridization and continuity analysis of gene expression, scientists can isolate differentially expressed genes and thus discover gene segments closely related to malignancy.^{21–23}

The Effects of Different Injection Methods on CAR T Cell Infiltration

It is not difficult to find that scientists are now investigating the effects of different injection methods on the efficacy of CAR T cell immunotherapy. In a study on the treatment of atypical teratoma and rhabdomyoma, the researchers used B7-H3.BB.z-CAR T cells to inject into the tumor (IT), ventricle (ICV) or caudal vein (IV) in order to treat the tumor.²⁴ Finally ICV eradicated all formed tumors, with a very significant effect compared to other controls, whereas intravenous administration of the same dose of B7-H3.BB.z-CAR T cells did not cure any mice.²⁴ Meanwhile, the study found that the delivery of CAR T cells by the IT and ICV pathway did not cause the elevation of systemic inflammatory cytokines. However, systemic inflammatory cytokines increased after IV delivery. Since intravenously injected CAR T cells activated monocytes in the circulatory system, it leads to the massive release of inflammatory cytokines IL-1 and IL-6 from monocytes and cause a cytokine storm (CRS).^{25–27} This will potentially cause severe necrosis of the tissue, so that CAR T cell transport is also closely related to the levels of systemic inflammatory cytokines. Different modes of administration may also therefore have a huge impact on the body's immune function. In order to more clarify the impact of different injection methods on the treatment effect, firstly scientists need to clarify the approximate path of CAR T cells to the tumor under the three different administration methods, as shown in Figure 1.

CAR T cells that are injected into the body via vein travel in the human vascular system. When it recognizes the specific receptors expressed by vascular endothelial cells, it will purposefully migrate out of the circulatory system and

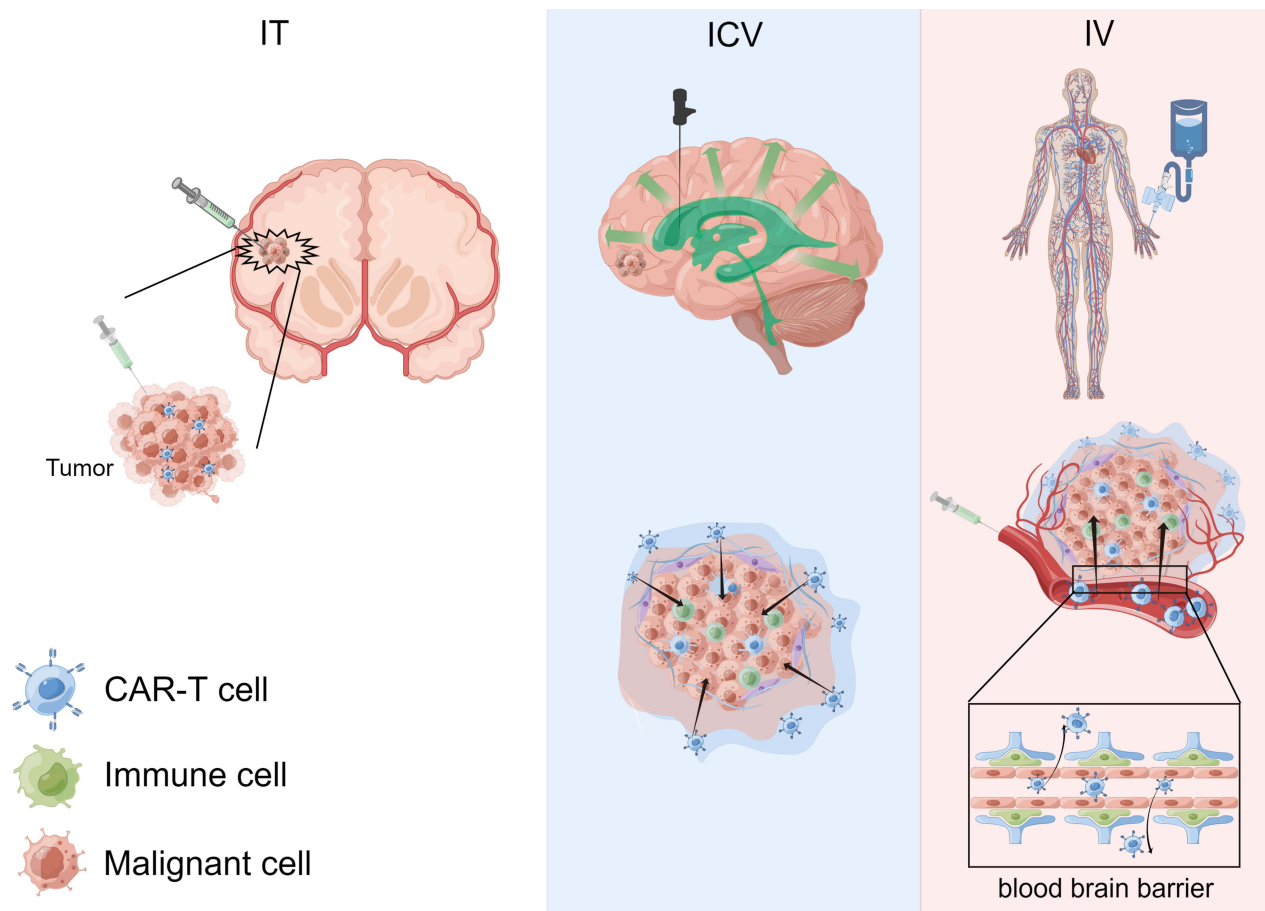


Figure 1 Transport paths of CAR T cells under different injection modes.

Note: By Figdraw.

Abbreviations: IV, intravenous injection; IT, intratumoral injection; ICV, intra-cerebroventricular injection.

into the tissues. In the tissue, CAR T cells can look for traces of tumor everywhere. Once it recognizes the tumor mass, it can further increase in value, activate and finally break through the tumor envelope into the tumor.²⁸ According to Figure 1, compared with the tumor area injection, the blood brain barrier is an additional obstacle for CAR T cells to reach the local tumor tissue of the central nervous system.²⁹ It has been shown that CAR T cells first anchor activated white blood cell adhesion molecules on endothelial cells of the blood-brain barrier. CAR T cells then successfully cross the blood-brain barrier by sensing secondary waves, which are mostly mediated by intercellular adhesion molecule-1 and vascular cell adhesion molecule-1, on the endothelial cells.³⁰ When CAR T cells break through the blood brain barrier, they can reach the tumor site in the cerebrospinal fluid under the guidance of chemokines and try to penetrate the tumor surface capsule into the tumor tissue, and then overcome the role of immunosuppressive factors in the harsh tumor microenvironment, playing a tumor killing effect and trying to recruit more immune cells to achieve the effect of eliminating tumors. In recent years, it has been found that the migration of CAR T cells in the blood system leads to the loss of a large number of CAR T cells.²⁴ The way of intratumoral injection has attracted extensive attention. It not only avoids the loss of CAR T cells caused by long-distance migration, but also helps to reduce the systemic toxicity. Some tumors in other special parts can also be injected through the third space, such as the cerebrospinal fluid of central nervous system tumors and the pleural cavity of lung tumors.^{24,31} In all dosing regimens, CAR T cells may encounter the following common problems in the above path:¹ Because the tumor tissue surface is coated with the extracellular matrix, the first step of CAR T cells infiltration is to penetrate this layer of barrier.³² It has been found that expressing heparanase in CAR T cells can cleave the main component of heparan sulfate chain in the extracellular matrix, thus breaking through the tumor surface envelope, and it does not affect the active of CAR T cells.^{2,33,34} Abnormal vasculature and high

interstitial hydraulic pressure will reduce the ability of CAR T cells to play an anti-tumor effect in view of the adverse environment in the tumor. The experiment with a method of photothermal ablation in tumor tissue can use near infrared light irradiation to produce effective heat to burn tumor cells and can improve the curative effect of CAR T cell tumor killing.³⁵ Another nanoengineering strategy through CAR T cell biohybridization not only improves the internal environment of tumor tissue to retain the original function of CAR T cells, but also has the ability of fluorescent tracing and microenvironment remodeling, which allows CAR T cells to perform within tumor tissue.³⁶

Having defined the pathway of CAR T cells into the tumor, attention should be paid to trying to compare the advantages and disadvantages of various injection modalities in order to find a much more suitable regimen for CAR T cell therapy. Some of the current clinical studies were collated and as shown in Table 1.

According to Table 1, CAR T cells need to travel further away to reach the tumor when systemic i. v. On the one hand, this process will inevitably produce some quantity and time consumption. To achieve the same treatment, intravenous often takes several times or more doses and longer time to work.^{24,31,37} On the other hand, when the

Table 1 Comparison of the Advantages and Disadvantages of Different Injection Methods Within Various Tumors

Tumor	Injection Method	Dosage × 10 ⁶ Cells/kg	Curative Effect	Shortcoming	Merit
Atypical malformation / rhabdomyoma (ATRT) ²⁴	IV	1	No tumor was eliminated.	Causing to cytokine release syndrome	Mediating the antigen-specific immune memory
	ICV	0.5	Curing some tumors	-	Mediating antigen-specific immune memory with rapid onset
	IT(coincedes with xenograft tumor coordinates)	1	Curing all tumors	-	Mediating the antigen-specific immune memory
HER2-positive breast cancer brain metastasis ³⁷	IV	5	Some tumors resolved.	Time of drug arriving to the tumor site delayed.	-
	ICV	0.5	The tumor resolved completely.	-	Rapidly infiltrating tumor tissue and promising multifocal brain metastases (including spinal lesions)
Primary pleural malignancy (malignant pleural mesothelioma MPM) ³¹	IV	0.1	Generating marginal antitumor effect	The triple dose still did not reach the median survival of intrapleural administration.	-
	Intrapleural injection	0.1	The tumor resolved completely.	-	The effect is rapid and sustained, and it can provide systemic tumor protection.
Malignant mesothelioma ³⁸	IV	10	It has an effective antitumor effect.	Tumor regression was delayed as compared to the intratumoral injection route.	-
	IT	10	It has an effective antitumor effect.	-	The antitumor effect is fast and long-lasting.
	Intraperitoneal injection	10	Nearly unavailable	-	

(Continued)

Table I (Continued).

Tumor	Injection Method	Dosage × 10 ⁶ Cells/kg	Curative Effect	Shortcoming	Merit
Medulloblastoma ³⁹	IT	5	The tumor resolved completely.	-	The ability to exert faster antitumor effect can reduce systemic toxicity and cytokine release syndrome outside the target.
	IV	5	The tumor continued to progress.	Higher doses can exert an antitumor effect.	-
Liver metastases of colorectal cancer were ⁴⁰	High pressure area delivery	2	The tumor burden was significantly reduced.	-	Improving the likelihood of durable tumor control and mitigate toxicity
	Low pressure area delivery	2	The tumor burden was reduced.	Tumor progression was increased compared with high pressure regional delivery.	-
	System delivery	2	The tumor continued to progress.	Higher doses are required compared to regional delivery.	-

CAR T cells pass through the normal tissues that coexpress the surface antigen with the tumor, they may also exert a killing effect, leading to systemic toxicity outside of the target.^{24,39} But it also has the advantage that it can be more comprehensive in tumor removal (NCT03146234). So sometimes we need to weigh the pros and cons of choosing the injection method. Furthermore, intravenous administration can also cause a cytokine release syndrome, which may be fatal to the body.^{24,41}

For intracranial tumors, compared to intraventricular injection, intratumor injection limitation is strong, that is to say, intratumor injection may only have killing effect on local tumor and for some distant tumor tissue killing effect is weak, since in intraventricular injection we can also find it for tumor cells in the spine can also have the effect of clearing.³⁷ Both of them are regional injections, which can not only achieve effective treatment at the minimum dose but also avoid systemic toxicity and generate immune memory to maintain the effect.^{24,37}

In addition to comparisons in theoretical efficacy, we should also focus on the practical operational difficulties of these injection pathways. Intravenous administration requires intravenous catheter administration, and intraventricular administration requires ventricular puncture. These operations often require clinicians to perform in places with certain requirements, so the requirements for location and operators are higher.^{24,37} Regional delivery of high pressure and low pressure requires a series of regulation of pressure.⁴⁰ Moreover, the way difficulty of intratumoral in situ injection is also greatly increased due to the complexity of the intratumoral vasculature. In contrast, for common primary tumors, regional administration is a more ideal way of administration for both therapeutic effects and adverse effects (NCT03198546).

Strategies for Optimizing CAR T Cell Homing

Promoting the Expression of Chemokines

Chemokines act as a similar tour guide in directing the CAR T cells to the tumor site, providing a forward direction for the CAR T cells. Different tumor cells may secrete different chemokine.⁴² When it interacts with the chemokine receptors on the CAR T cells, it can recruit the CAR T cells into the tumor. We have now attempted to develop CAR T cells coexpressing chemokine receptors to promote the efficient progression of CAR T cells towards the tumor site.^{43–45} Malignant pleural mesothelioma highly secretes the chemokine CCL2. The transduction of the chemokine receptor

CCR2b into mesoCAR T cells using lentiviral vectors can significantly increase the number of CAR T cells at tumor sites and significantly enhance the anti-tumor efficacy, capable of treating established mesothelin-expressing tumor.⁴⁶ A variety of tumor cells, such as pancreatic and ovarian cancers, can secrete IL-8 and can be detected in the circulation of the mouse models. IL-8 can act on the G protein-coupled receptor CXCR2 to recruit downstream signaling proteins and activate second messenger-mediated signalling. CXCR2 can chemotaxis a range of biological effects such as wandering and degranulation of neutrophils and lymphocytes. This biological effect plays a huge role in multiple inflammatory responses, cell development and the proliferation of tumor cells. Therefore, IL-8 and CXCR2 are very important for the construction of malignant tumor microenvironment.⁴⁷ CAR T cells using targeted $\alpha\beta$ 6 integrins co-expressing CXCR1 and CXCR2 were able to migrate more efficiently toward IL-8 maintaining cytolytic activity, and significantly improved therapeutic efficacy in pancreatic and ovarian xenograft models.⁴⁸

Enhancing the Expression of Vascular Endothelin Receptor

Vascular endothelial selectin receptors play a sentinel-like role in the homing of CAR T cells distributed in circulation in specific paths, directing it to the specific sites by binding to vascular endothelial selectin ligands expressed by CAR T cells.^{49,50} Circulating cells have been found to transport to the bone marrow when the tetra-sugar sialic acid-Lewis X-modified E (sLeX) glycosylated binds to E-selectin receptors expressed in the vascular endothelium.⁵¹ When the CAR T cell-autonomous sLeX expression/E-selectin binding capacity is enhanced, it can be bound to E-selectin receptors in vascular endothelium, thus enabling it to transport to the bone marrow in the circulating blood.

Transforming the Tumor Microenvironment

Although various targets can be artificially created from CAR T cells to the tumor site to guide CAR T cells as far as possible, the inhibitory effect of tumor microenvironment is a great obstacle for CAR T cells.⁵² Specific performance is: The tumor site secretion of transforming growth factor β (TGF β) or IL-10 immune suppressive cytokines, such as regulatory T cells (Tregs) or myeloid suppressive cells and PD-L1 on the cell surface expression and immunosuppressed metabolic environment may be on CAR T cells in the tumor site aggregation and exert a powerful inhibitory effect.⁵³ To overcome this limitation, we reviewed strategies: (1) directly counteracting immunosuppressive factors, such as transgene expression of IL-15 in cytotoxic T cells counteracting Treg-mediated immunosuppression.⁵⁴ (2) improving the metabolic environment of T cells in TME, such as timely supplementation of amino acids critical for T cell function, such as arginine and glutamine.^{55,56} (3) The pretreatment method was used to promote the colonization of CAR T cells.⁵⁷ It has been shown that pretreatment with oxaliplatin (Ox) and cyclophosphamide (Cy) contributes to CAR T cells accumulate within the tumor and enhances the disease response to immune checkpoint suppression. The reason behind this may be that oxaliplatin induced immunogenic tumor cell death, which promotes the local inflammatory process, while oxaliplatin reverses STAT6-mediated immunosuppression, which upregulates the susceptibility of cytotoxic T cells and eventually leads CAR T cells into the tumor site. But it should be noted that the combinatorial approach of pretreatment drugs is a key for the success of this pathway.⁵⁸

However, the tertiary lymphatic structure in the field of immunity has attracted much attention. The tertiary lymphoid structure is composed of T cell regions with endothelial system. It can recruit various immune cells and induce differentiation of mature T cells and B cells, presenting antigen to play antitumor effects. The presence of an additional tertiary lymphatic structure has a clear predictive effect on the prognosis of cancer. According to the current study, it can be found in various solid tumors, such as hepatocellular carcinoma and renal cell carcinoma et al.^{59,60} Studies have confirmed that the tertiary lymphoid structure contributes to the infiltrating of CAR T cells.⁶¹ It promotes the infiltration of CAR T cells in the following aspects: (1) it is rich in immune cells for presenting antigens and a stable endothelial system. This suggests that it can support CAR T cells for survival in the tumor microenvironment. (2) TLS can promote the continuous influx of naive immune cells to maintain immunity. This would greatly expand the effect of CAR T cells.⁶² We can stimulate the maturation of tertiary lymphatic structures to enhance CAR T cell infiltration and cooperate with CAR T cells to clear tumor tissue. Multiple approaches have been developed to stimulate the generation of tertiary lymphatic structures, including promoting vascular normalization in the therapeutic melanoma microenvironment to promote tertiary lymphatic structure formation through treatment with STING agonists.⁶³ It is also noteworthy that the

application of a scaffold of a large number of biomaterials can induce the formation of tertiary lymphatic structures by allowing the infiltration of immune cells, including artificial collagen matrix and hydrogel.^{64,65} In conclusion, the tertiary lymphoid structures are a trusted ally of CAR T cells.

Improving Cytokine Release and Immune Memory

In the process of tumor killing, the ability of locally infiltrating CAR T cells alone may not be enough to fully eliminate, so CAR T cells are required to express more cytokines to recruit more immune cells (such as dendritic cells, T cells, etc.) into the tumor for common cooperation. Currently, the cytokines that can be successfully expressed are IL-7 / CCL19 / CCL21 / IL-12 / CXCL11 and CCR2b.^{66–71}

Temporary drug action can lead to substantial tumor reduction. But the residence time of drugs in the human body is limited. Failure of the CAR T cells is also inevitable. Clearing tumor tissue in the short term is not our ultimate goal. Some scientists are trying to study the methods to give CAR T cells endogenous immunity by analyzing the exhaustion mechanism of CAR T cells.⁷² Currently the immune memory capacity can be achieved by delivering immunostimulants such as RN7SL1, STING, etc.^{73–75} They activate endogenous RNA and promote CAR T cell proliferation and effector memory differentiation. This can enhance the effect of the drug in the body and help maintain the anti-tumor effect.

Conclusion

CAR T cell therapy has been a great success in some clinical trials against hematological malignancies, but the degree of aggregation and activity in solid tumor sites is a huge challenge. In order to improve the efficacy of CAR T cell therapy, feasible solutions were proposed in detail from the aspects of design, injection, infiltration and working of CAR T cells. For the infiltration degree of CAR T cells, we can guide CAR T cells to the target aggregation more often by finding more specific and abundant surface antigens. Secondly, we need to help CAR T cells overcome the immunosuppression of the local tumor microenvironment to facilitate the smooth entry of CAR T cells into the tumor interior. The most advanced approach is to construct tertiary lymphatic structures. In addition, we can better guide CAR T cells into the tumor by promoting the expression of chemokines, etc. In our careful study, we found that the efficacy and side effects of CAR T cell therapy varied with different injection methods in the current clinical studies. However, there are few comparative analyses on the specific efficacy of each injection route. In this context, we also focused on analyzing the impact of various injection methods on CAR T cell transport, which is inspired by the understanding of the CAR T cell transport path. Every step of the research we do is designed to contribute to improving anti-tumor efficacy. Although the challenges remain, we want newer strategies and solutions to create a safer and more effective treatment modality.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

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