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CAR-T cell therapy for lung cancer: a promising but challenging future

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Lung cancer is one of the most commonly diagnosed cancers and the leading cause of cancer death. For many years, the main treatments for lung cancer include surgery, chemotherapy, radiotherapy and targeted therapy. Recently immunotherapy, in particular, the programmed death 1 (PD-1) inhibitors, has become the first-line therapy for lung cancer (1). The emergence of chimeric antigen receptor (CAR)-T cell immunotherapy also provides a new approach and new hope for the treatment of lung cancer. However, the challenges for CAR-T cell therapy in eradicating solid tumors are immense (2). Currently, there are more than 250 clinical trials worldwide evaluating the safety and efficacy of CAR-T cell therapy in the treatment of solid tumors. China and the United States have the largest number of CAR-T clinical trials (3). This paper summarizes some of the recent results for lung cancer treatment and discusses the many challenges and problems we still face in translating these new CAR-T therapies into the clinic to treat lung cancer patients. These challenges include, improvement in the flexibility of the CAR structure, more specificity in tumor antigen targeting, overcoming the complexities of the hostile lung tumor microenvironment (TME), and in many cases the accessibility and penetration of the large tumor volume for effective treatment.

Evolution of the CAR structure

From initial conception the use of CARs in T-cell therapy has undergone four progressive generations generically based on the intracellular signal domains of the CAR (2). The first generation of CARs, containing only the antigen recognition signal, had poor activity and a short survival time *in vivo* (4). The design of the second and third generation CARs included one and two costimulatory molecules within the signal transduction region, respectively. These modifications were designed to enhance T cell proliferation, cytotoxic, and prolonged T cells' survival time. The optimization of the co-stimulatory molecules in the CARs led to enhanced CAR-T cell function. Most commonly used second-generation co-stimulatory domains are 4-1BB or CD28. DNAX-activating protein 10 (DAP10) has also been shown to enhanced cytotoxicity, cytokine secretion and T cell activation. In *in vivo* mouse models of human lung cancer xenotransplantation, delayed growth of primary lung cancer and improved anti-tumor efficacy were observed based on non-small cell lung cancer (NSCLC) cell lines (5). The fourth generation CAR-T design introduced pro-inflammatory cytokines and co-stimulating ligands, to enhance the ability of the T-cells to penetrate and overcome the suppressive nature of the hostile TME (2).

In addition to the intracellular signal transduction modules, the improvement of the extracellular module structure has also been shown to improve the amplification and anti-tumor efficacy of CAR-T cells. Qin *et al.* proposed that the incorporation of a hinge structure improved the flexibility of the single-chain variable fragment (scFv) which binds and promotes the expansion, migration and invasion of cluster of differentiation 4 $(CD4)^+$ CAR-T cells (6).

Table 1 CAR-T cell clinical trials for lung cancer

NCT number	Target(s)	Sponsor/collaborators	Phases
NCT03330834	–	Sun Yat-sen University; Guangzhou Yiyang Biological Technology Co., Ltd.	Phase 1
NCT03525782	MUC1, PD-1	The First Affiliated Hospital of Guangdong Pharmaceutical University; Guangzhou Anjie Biomedical Technology Co., Ltd.; University of Technology, Sydney	Phase 1/2
NCT04153799	EGFR	Sun Yat-sen University; Guangzhou Bio-gene Technology Co., Ltd.	Phase 1
NCT03198052	HER2/MSLN/ Lewis-Y/PSCA/ MUC1/PD-L1/ CD80/86	Second Affiliated Hospital of Guangzhou Medical University; Hunan Zhaotai Yongren Medical Innovation Co. Ltd.; Guangdong Zhaotai In Vivo Biomedicine Co. Ltd.; First Affiliated Hospital, Sun Yat-sen University	Phase 1
NCT02587689	MUC1	PersonGen BioTherapeutics (Suzhou) Co., Ltd.; The First People's Hospital of Hefei; Hefei Binhu Hospital	Phase 1/2
NCT02349724	CEA	Southwest Hospital, China	Phase 1
NCT03198546	GPC3	Second Affiliated Hospital of Guangzhou Medical University; Hunan Zhaotai Yongren Medical Innovation Co. Ltd.; Guangdong Zhaotai InVivo Biomedicine Co. Ltd.; First Affiliated Hospital, Sun Yat-sen University	Phase 1
NCT04025216	TnMUC1	Tmunity Therapeutics	Phase 1
NCT03356808	–	Shenzhen Geno-Immune Medical Institute	Phase 1/2
NCT02713984	HER2	Zhi Yang; Southwest Hospital, China	Phase 1/2
NCT03638206	–	Shenzhen BinDeBio Ltd.; The First Affiliated Hospital of Zhengzhou University	Phase 1/2
NCT01583686	MSLN	National Cancer Institute (NCI); National Institutes of Health Clinical Center (CC)	Phase 1/2
NCT02414269	MSLN	Memorial Sloan Kettering Cancer Center	Phase 1
NCT02706392	ROR1	Fred Hutchinson Cancer Research Center; National Cancer Institute (NCI)	Phase 1
NCT03054298	MSLN	University of Pennsylvania	Phase 1
NCT03740256	HER2	Baylor College of Medicine; The Methodist Hospital System; Texas Children's Hospital	Phase 1
NCT02862028	PD-1, EGFR	Shanghai International Medical Center	Phase 1/2

The structural design of the CARs is continuously being optimized and key in the efficacy of CAR-T, although the second-generation CAR-T cells still remains the mainstream approach for therapeutic application.

Antigenic heterogeneity and specific targeting of NSCLC

The ideal target for CAR-T cell therapy is when the target-antigen is only expressed on cancer cells or overexpressed on all or most lung cancer cells compared to normal cells. Although many tumor-associated antigens (TAA) have been detected in NSCLCs (7), CAR-T cells have been designed to target only a small number of these antigens (8). At the same time, some of these target-antigens are also expressed in low amounts in normal tissues, thus some CAR-T cells have the potential to attack normal cells.

Targets currently under evaluation for CAR-T cell therapy for lung cancer include: epidermal growth factor receptor (EGFR); human epidermal growth factor receptor 2 (HER2); mesothelin (MSLN); prostate stem cell antigen (PSCA); mucin 1 (MUC1); carcinoembryonic antigen (CEA); tyrosine kinase-like orphan receptor 1 (ROR1); programmed death ligand 1 (PD-L1) and CD80/CD86. *Table 1* lists the current clinical research targets and clinical trials of CAR-T cell therapy for lung cancer.

EGFR is expressed in both epithelial cells and many epithelium-derived malignancies. Compared to normal lung tissues, the significant elevation of affinity of binding sites in lung carcinomas makes EGFR a promising therapeutic target. The second-generation lentivirus-transduced EGFR-CAR-T cells proved to be safe and a feasible option

for patients with EGFR-positive (>50% expression), relapsed/refractory NSCLCs in a phase I clinical study (NCT01869166) (9).

HER2 is also a potential CAR-target antigen in lung cancer. Generally, HER2-targeted CAR-T cells have demonstrated good therapeutic benefits in patients with recurrent/refractory HER2-positive sarcomas with no observed respiratory distress after treatment. However, in one case study, a patient, with metastatic colon cancer migrating to the lungs and liver, experienced respiratory distress within 15 minutes after 1×10^{10} HER2-targeted CAR-T cells infusion. Morgan *et al.* speculated that it was related to low levels of HER2 expression on the normal lung epithelial cells, which may have caused an auto-immune response (10). Thus, the safety and efficacy of HER2-targeted CAR-T may be compromised in the treatment of some lung cancer patients depending on the HER2 expression. Therefore, although HER2 is generally considered a strong candidate-target, the cause of respiratory distress caused by HER2-targeted CAR-T, albeit not common exemplifies the need to understand tumor characteristics and design of alternative specific-antigen targets.

In a different study, the lung cancer target, MSLN, was shown to be expressed in 69% of lung adenocarcinoma. One in five adenocarcinoma patients strongly expressing MSLN, with no MSLN expression detected in normal lung tissue (11). MSLN CAR-T cell therapy reduced the tumor burden in pre-clinical mouse models (12).

The expression of MUC1, a transmembrane glycoprotein, is aberrantly upregulated in NSCLC. PSCA is a glycosylphosphatidylinositol (GPI)-anchored cell surface antigen that is also frequently overexpressed in NSCLC. The design of combinational CAR-PSCA and CAR-MUC1-T cells, as proposed by Wei *et al.*, showed excellent anti-NSCLC efficacy compared with the treatment of CAR-T cells targeting a single antigen (13). The study demonstrates that PSCA and MUC1 are both promising CAR-T cell targets in NSCLC. CEA is overexpressed in nearly 70% of NSCLCs (14). However, some patients who received CAR-T cell therapy targeting CEA, had transient, acute respiratory toxicity. Expression of CEACAM5 on lung epithelium cells has been proposed as a mechanism that may have contributed to this transient toxicity (15). It suggests that methods to control CAR-T 'on-target, off-tissue' toxicity are required to enable a clinical impact of this approach in solid malignancies. ROR1 exhibits high and homogeneous cell surface expression in many epithelial tumors and some B cell malignancies. However, ROR1 was expressed in some normal tissues, raising concerns that targeting ROR1 in patients may cause toxicity. To improve selectivity, Srivastava *et al.* creatively engineered T cells with synthetic Notch (synNotch) receptors specific for EpCAM or B7-H3, which are expressed on ROR1$^+$ tumor cells but not ROR1$^+$ stromal cells. SynNotch receptors induced ROR1 CAR expression selectively within the tumor, resulting in tumor regression without toxicity (16). CD80/86 are costimulatory molecules of the immune cells. Binding of CD80/CD86 to CTLA-4 can lead to downregulation of T cell function through a variety of mechanisms. The central role of the CTLA4-CD80/CD86 pathway in co-stimulation makes it a preferred target for immune intervention (17). CD80/CD86 mRNA expression has been detected in a large number of NSCLC cell lines (18). As CD80/CD86 is also expressed in normal immune cells, there is a risk of developing autoimmunity. New strategies are expected to be developed to enable CD80/CD86 CAR-T cells to differentiate between normal cells and tumor cells.

In summary, EGFR, MSLN and multi-targeted combinations may be more suitable targets in the treatment of lung cancer in the light of HER2, CEA and ROR1 CAR-T cells causing serious adverse reactions in some patients and CD80/CD86 CAR-T may induce autoimmunity.

Immune microenvironment and checkpoint inhibitors

To evade attack from the immune system tumor cells have developed an evasion strategy. The immune system is in constant surveillance. When T cells are activated they express immune checkpoint proteins, such as the PD-1 on the cell surface which binds to its ligand (PD-L1) expressed on the surface of host cells to prevent a host autoimmune reaction. Tumor cells express the PD-1 ligand (PD-L1 or PD-L2) and by binding to PD-1 on the T cell they evade immune cell recognition and attack from the immune system (2). Blocking the interaction between PD-1 and PD-L1 to allow the T cells to recognise cancer cells and to enhance immune function is now being utilized as an anti-tumor therapy and a promising strategy for the treatment of lung cancer.

The use of PD-1 and PD-L1 monoclonal antibodies (mAbs) to block the PD-1-PD-L1 interaction as a cancer therapy has FDA approval and have been in clinical use

for a number of years (1). Another effective approach to block the PD-1/PD-L1 interaction is through the design of CAR-T cells engineered to secrete the checkpoint PD-1 inhibitor. Rafiq *et al.* demonstrated that CAR-T cells with scFv secreting PD-1 enhanced the survival rate of PD-L1 (+) tumor-bearing mice in both homogenous and xenograft mouse models, acting through autocrine and paracrine mechanisms (19). This strategic approach enhanced the efficacy of CAR-T cells in cancers within the immunosuppressive microenvironment. Our group, Chen *et al.*, successfully applied the combination of CAR-T cells and PD-1 knockout in the clinical treatment of lung cancer. The clinical trial (NCT03525782) indicated that the treatment was safe, but the therapeutic effect varied greatly depending on the individual patient. Factors influencing the variation in clinical outcomes are currently under investigation (20).

Problems with CAR-T cells infiltration into solid tumor tissue

Infiltration of CAR-T cells into solid tumor tissues is a prerequisite for their anti-tumor function, which relies on their efficient and specific trafficking capabilities. Mismatching of chemokine-chemokine receptor pairs, down-regulation of adhesion molecules, aberrant vasculature, the immunosuppressive TME and anatomical location of immune effector cells, may all contribute to the poor homing of these cells (21). To overcome the problems associated with the CAR-T cells entering into the solid tumor environment or penetrating the extracellular matrix (ECM) of the tumor, Caruana *et al.* modified CAR-T cells to express heparinase (HPSE), an enzyme that aids in the degradation of the tumor ECM components, and hence promote T-cell invasion and anti-tumor activity (22). Another approach to successfully infiltrate large solid tumors in the lung was developed by Hu *et al.*, where they co-administered interleukin 12 (IL-12) DNA and the chemotherapy drug doxorubicin before CAR-T cell infusion (23). The combination of IL-12 plus doxorubicin not only promoted NKG2D (+) CD8(+) T cell infiltration into large solid tumors in the mouse lung cancer model, but also co-up-regulated the production of chemokines CXCL9 and CXCL10 that attracted T cells. Thus, the accumulation of T cells in the tumor microenvironment was promoted, and the effector function of infiltrating T cells was enhanced by increasing the ratio of the stimulator and regulator. Intrapleural administration of CAR-T cells enabled more effective infiltration of T cells into the tumor microenvironment, requiring 30 times fewer CAR-T cells than systemic intravenous administration. These CAR-T cells rapidly expanded and differentiated, and induced long-term remission of tumors, and regional T cell administration also promoted effective elimination of tumors outside the thoracic cavity (24).

T cell exhaustion

T cells infiltrating into lung tumors is also affected by a phenomenon known as T cell exhaustion. A recent study by Chen *et al.* found that a transcription factor family called NR4A played an important role in T cell exhaustion, and these transcription factors were shown to limit CAR-T cell function in solid tumors (25). Using mouse models, they demonstrated that CAR-T cells function more effectively when NR4A transcription factors were lacking, reducing tumor size and increasing the survival rate of mice with cancer.

Although these findings have not been directly applied to clinical studies of CAR-T therapy for lung cancer, analyzing the role of NFAT and NR4A transcription factors solves an immunological mystery and provides scientists with new clues for designing better anti-tumor strategies. NR4A enriched in CD8^{+} PD-1^{hi} TILs in NSCLC (25), so blocking NR4A, may also be a promising treatment for NSCLC.

In summary, the clinical application of CAR-T in lung cancer treatment is still undergoing extensive research. However, the continuous improvement of CAR-T technology for lung cancer is providing much promise but many challenges. Although the toxicology results are favorable, we still face many generic challenges before using CAR-T based therapy as a viable alternative, or as an adjunct treatment for lung cancers. Future efforts are being made to find more specific target antigens for lung cancer cells to reduce adverse side effects, as well as continuously optimization of CAR-T cells through improvement in genetic engineering, enabling an increase in the number of CAR-T cells that migrate to tumor sites and enhance the anti-lung cancer ability.

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