BiTE-Secreting HIP Dual CAR-γδT Cells in Non-Small Cell Lung Cancer

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Abstract: Lung cancer is the most frequently diagnosed cancer globally, with non-small cell lung cancer (NSCLC) constituting approximately 85% of cases. Current treatments face challenges such as resistance to targeted therapies and adverse effects associated with immunotherapies. This study aims to enhance NSCLC treatment by utilizing BiTE-secreting hypoimmune (HIP) dual CAR-γδT cells. Building on previous research, we propose to engineer human γδT cells by knocking out B2M and CIITA genes, followed by transfection with CD47 and CAR genes. To evaluate the reduction of allorejection contributed by BiTE-secreting HIP dual CAR-γδT cells and to have a better understanding of their effects in human NSCLC, these modified cells will be injected into humanized NOD SCID IL2RgammaNULL (NSG)-bone marrow liver thymus (BLT) mice bearing A549 tumors. We will assess persistence and anti-tumor activity via bioluminescence imaging and flow cytometry. Results are expected to indicate that HIP dual CAR-γδT cells exhibit superior persistence and reduced immunogenicity, enhancing antitumor efficacy compared to control groups. Additionally, rechallenge experiments are likely to confirm prolonged effectiveness. Our findings suggest that BiTE-secreting HIP dual CAR-γδT cells will offer a promising, targeted treatment for NSCLC, combining reduced immunogenicity with potent antitumor activity.

1 Introduction

Lung cancer has been the most commonly diagnosed cancer globally for several decades. [1] In 2018, approximately 2.1 million new cases of lung cancer were reported, accounting for about 12% of the worldwide cancer burden.[1] The geographical patterns of lung cancer mortality closely mirror the incidence rates due to the poor survival rates and the high lethality of this disease. [2] Lung cancer is the leading cause of cancer-related deaths among men worldwide and ranks second among women. In 2018, it was estimated that 1.8 million deaths occurred (1.2 million in men and 576,100 in women), making up one in five cancer-related deaths globally. [1] These geographical and gender variations are largely linked to historical smoking trends and the stages of the tobacco epidemic. [3] Although lung cancer incidence is on the decline in developed countries, it is increasing in less developed regions, likely due to less stringent smoking regulations. [3]

The most important risk factor for developing NSCLC is tobacco smoking, followed by environmental exposure to radon and air pollution. [4] Most patients are diagnosed with this disease in the middle or advanced stages due to a lack of screening programs and the late onset of symptoms, leading to a poor prognosis. [3] Various diagnostic tools include imaging studies using X-ray, CT scan, and PET imaging. Accurate staging of cancer is essential for providing the most appropriate therapeutic approach, which includes surgery, radio chemotherapy, immunotherapy, and targeted therapies with monoclonal antibodies—all of which improve therapeutic outcomes. [3]

Non-small cell lung cancer constitutes about 85% of new lung cancer cases and includes adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma subtypes, while small-cell lung cancer (SCLC) makes up the remaining 15%. [5,6] The classification of lung cancer is continually advancing, with specific terms and standards used to differentiate adenocarcinoma from squamous cell carcinoma, especially in poorly differentiated tumors. Histological subtyping of lung cancer has gained increasing importance due to the expanding therapeutic possibilities targeting specific subtypes. [3] Tumors that were previously difficult to classify due to unclear features of squamous or

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adenocarcinoma can now be re-evaluated using limited immunohistochemical techniques, which also help preserve tissue for molecular testing. [3,5]

However, despite significant advances in NSCLC therapy, challenges remain. Resistance to targeted drugs often develops, effectively reducing their efficacy over time.[3] Much progress has been made in cancer treatment, particularly through immunotherapeutic procedures that enhance the body's mechanisms in eliminating cancer cells. These procedures come with the major constraint that there is no one-size-fits-all formula for treating cancer patients, as most treatments may not work due to a lack of understanding of the specific type of cancerous cell involved. Currently, palliative care remains the most promising approach, but it is costly and less accessible. As our understanding of NSCLC improves, advancements in patient care and clinical trial design will lead to better outcomes for patients with NSCLC. [3]

2 Approach

2.1 Summary of Primary Research

2.1.1 HIP CAR-T Cells Show Better Persistence and Tumor Suppression

Allogeneic chimeric antigen receptor (CAR) T cells are present in the host for a short time before being eliminated by the immune system, compromising their antitumoral effect. In contrast, hypoimmune (HIP) T cells are designed to avoid immune rejection and at the same time provide a durable antitumor reaction. Researchers used CRISPR-Cas9 to edit specific genes in human HIP T cells to reduce their immunogenicity. For example, CRISPR-Cas9 has been used to target beta-2-microglobulin and Class II Major Histocompatibility Complex Transactivator genes to knock out the expression of HLA class I and II. [7] Such genetic modifications abrogate T cell-mediated allorejection of the edited cells, which still remain susceptible to attack by the innate arm of the immune system, necessitating lentiviral introduction of CD47 to protect from such responses. [8] In addition, the introduction of CD47-targeting fusion proteins may provide a safety switch for killing these cells if necessary. [9] In vitro studies revealed that HIP CAR-T cells had superior persistence and did not lose their specificity and anti-tumor activity with time. Humanized mouse models also confirmed in vivo that the superiority of HIP CAR-T cells persisted compared to traditional allogeneic CAR-T cells with respect to persistence and efficacy in tumor suppression. [9]

2.1.2 Dual CAR-T Cells Enhance the Antitumor Acitivity and Persistence.

CAR-T cells targeting a single antigen have been found to produce significantly better results in patients with refractory hematological cancers and are considered a major therapeutic advance. [1] However, in the context of solid tumors, the variable expression of antigens and the insufficient persistence of CAR-T cells pose significant challenges for achieving effective clinical outcomes. Researchers at the University of North Carolina at Chapel Hill demonstrated that CAR-T cells designed to target two tumor-associated antigens simultaneously, equipped for dual costimulation through CD28 and 4-1BB but sharing the same CD3ζ-chain, were capable of mediating rapid antitumor responses under in vivo stress conditions, preventing tumor recurrence, and inhibiting tumor escape caused by low antigen density. [10] This technique represents a significant breakthrough as it addresses one of the primary challenges in CAR-T cell therapy. Metabolic analyses further showed that dual CAR-T cells enhance glycolysis to enable rapid effector function while maintaining oxidative processes, which are critical for long-term T-cell persistence. [10] In conclusion, dual-targeting CAR-T cells substantially enhance antitumor activity and improve persistence in solid tumors by preventing tumor escape. [10]

2.1.3 BITE-secreting CAR-γδT Cells Enhance the Recruitment and Activation.

CAR-T cell therapy has achieved remarkable success in the clinical treatment of hematological malignancies. However, challenges remain in using CAR-T cells to treat patients with solid tumors. [11] Engineered CAR-γδT cells were developed to address immune escape mechanisms in solid tumors. By targeting both HLA-G and PD-L1 antigens on the cancer surface, the engineered γδT cells can specifically attack and eliminate target cancer cells [12]. Besides, γδT cells reduce the risk of graft-versus-host disease (GvHD) due to their MHC-independent recognition and innate-like function, making them suitable for allogeneic therapies. [13] Furthermore, the CAR-γδT cells were modified to secrete a PD-L1/CD3ε bispecific T-cell engager (BiTE) and then recruit and activate surrounding T cells, thereby enhancing the overall anti-cancer immune response. [11] The literature demonstrated *in vivo* that this approach can effectively reduce tumor growth and extended survival, while maintaining a minimum side effect. [11] This dual-targeting strategy offers a highly effective therapeutic option for treating solid tumors, combining γδT cells and BiTEs to overcome the limitations of current CAR-T therapies. [11]

In light of the foundational studies conducted, our objective aims to innovate a novel therapeutic strategy. This led us to formulate the following hypothesis:

We hypothesize that BiTE-secreting HIP dual CAR-γδT cells augment multiple dimensions of CAR-T cell functionality in NSCLC. The reduction of antigenicity, achieved through HIP with PD-L1 dependent immune occupation and gated dual CAR expression, will amplify the persistence, efficacy, specificity, and safety of CAR-γδT cells in their combat against NSCLC (Figure 1).

2.2 Materials and Method

2.2.1 Working Model.

NSCLC cells will be identified based on their surface expression of PD-L1, HLA-G, and HER2 proteins. γδ T

cells will be engineered to have dual CARs that will target HER2 and HLA-G proteins, thereby increasing the specificity of these γδ T cells towards NSCLC cells in the future. The dual CAR-T cells will be genetically engineered to secrete BiTEs that will target PD-L1 on tumor cells and CD3 on T cells, thus enhancing the targeted elimination of tumor cells in the future. These γδ T cells will lack expression of MHC class I (B2M-/-) and MHC class II (CIITA$^{-/-}$), which will reduce allogeneic responses and extend their persistence without compromising specificity or anti-tumor efficacy in the future.

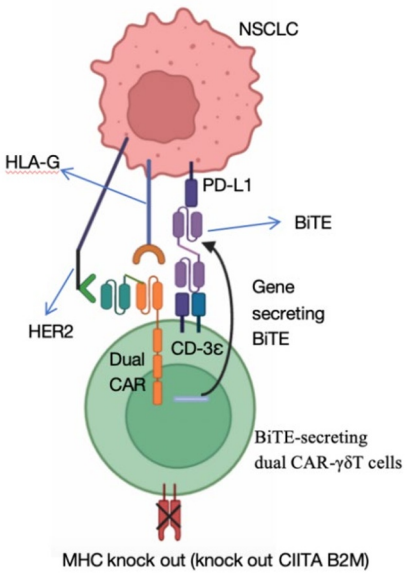

Figure 1. BiTE-secreting HIP dual CAR-γδT cells in non-small cell lung cancer

To enhance the persistence of γδ T cells and minimize their immunogenicity, we will genetically disrupt MHC class I (B2M) and MHC class II (CIITA). Additionally, we propose modifying T cells to express anti-HLA-G and anti-HER2 CARs to circumvent immune evasion. [11]

This statement is intended to stimulate further academic discourse and investigation into this promising area of study.

2.2.2 General Approach.

The initial objective is to create B2M$^{-/-}$ CIITA$^{-/-}$ allogeneic γδT cells (HIP γδT cells) and evaluate their persistence compared to unedited allogeneic γδT cells. To accomplish this, we will utilize the BLT humanized mouse model. γδT cells will be isolated and purified from NSG-BLT humanized mice. [14,15]. These γδT cells will be then subjected to CRISPR-Cas9 editing to knock out the B2M and CIITA genes. Additionally, CD47 and CAR genes are transduced into the B2M$^{-/-}$ CIITA$^{-/-}$ γδT cells via electroporation using edited pUC19 plasmid. The B2M$^{-/-}$ CIITA$^{-/-}$ allogeneic γδT cells would be injected into one group of NSG-BLT humanized mice[1,2], along with another group receiving unedited allogeneic γδT cells derived from the same donor. Besides, the control group would receive autologous γδT cells for comparison. Flow cytometry will be employed to measure the proportion of MHC class I negative and γδ2 positive cells, indicative of the B2M$^{-/-}$ CIITA$^{-/-}$ allogeneic γδT cells. γδ2 marker represents the γδT cells and MHC class I KO indicates the B2M$^{-/-}$ CIITA$^{-/-}$ edition.

Then, we aim to assess the impact of the efficacy of BiTE-secreting HIP dual CAR γδ T cells in non-small cell lung cancer (NSCLC) model. Experiments will be conducted using both control and experimental groups within immunocompetent NSG-BLT mouse models. The control groups consist of 5 mice injected with either A549 human lung adenocarcinoma cells to establish a tumor model or the pUC19 plasmid to serve as a vehicle control. γδ T cells in experimental groups (5 mice each group) will receive various modifications: HIP CAR γδ T cells with reduced immunogenicity, BiTE-secreting HIP CAR γδ T cells designed to enhance T-cell recruitment, dual-targeting CAR γδ T cells aimed at targeting HLA-G and HER2 antigens, and HIP Dual BiTE CAR γδ T cells, which combined both BiTE secretion and dual antigen targeting. This approach allows us to systematically evaluate the impact of these modifications on tumor suppression and overall therapeutic efficacy in NSCLC.

Furthermore, to ensure that the persistence conferred by B2M and CIITA KO also functions effectively and γδT cells' function is not disrupted in more complex models, we rechallenged BiTE-secreting HIP dual CAR γδT cells and BiTE-secreting dual CAR γδT cells (control group) with the same A549 tumor cells, which were transduced with firefly luciferase 60 days after the initial inoculation. Utilizing BLI tracking, we will observe the tumor size in groups of five mouse each, monitoring every four days.

2.2.3 Cell Line.

The cell line utilized in this study is the γδ T cell line harvested from NSG-BLT humanized mice. These mice are engrafted with human fetal liver and thymus tissues, building up human immune system and facilitating the development of human immune cells, including γδ T cells. The human A549 lung adenocarcinoma cell line is also used to establish tumor models in the BLT mice for subsequent therapeutic evaluations. Both of the cell line used in our study are cultivated in 37°C.

2.2.4 Mice Model and Animal Care.

6-weeks-old BLT humanized mice with mixed genders will be utilized in the study. All mice will receive standard food and water supplement. The mice will be treated at certain temperature between 20-26 degree and moisture should be kept at 40-70% along with a daylight cycle switching every 12h. The experiment will be conducted according to the animal care guidelines. All of the following experiments are based on '3R' (Refinement, Reduction, Replacement) animal care principle.

2.2.5 Isolation of γδT Cells. γδ

T cells are isolated from the human using Fluorescence-activated Cell Sorting (FACS). This process involves the extraction of peripheral blood, spleen, or lymphoid

tissues, followed by the enrichment and purification of γδ T cells based on their specific surface markers, such as Vγ9Vδ2 (γδ2). γδ T cells will be separated through FACS in which γδ2-specific antibodies modified with fluorescence combine with γδ T cells and become activated to present fluorescence signals, sorting γδ T cells from other cell types.

2.2.6 B2M and CIITA Knock-out via CRISPR-Cas9.

The isolated γδ T cells will undergo CRISPR-Cas9 gene editing to knock out the B2M and CIITA genes, which inhibit the expression of MHC class I and II molecules, respectively. This genetic modification reduces immune rejection of the allogeneic γδ T cells and enhances their persistence in the host, resulting in a more sustained antitumor response. The CRISPR-Cas9 system will be delivered via ribonucleoprotein complexes or plasmids to achieve efficient gene editing.

BiTE-secreting HIP dual CAR-γδT cells enhance antitumor efficacy through the secretion of bispecific T-cell engagers (BiTEs), which recruit and activate surrounding T cells. This recruitment strengthens the immune response by increasing the concentration of active cytotoxic T cells at the tumor site, thereby improving the overall tumor suppression effect.

2.2.7 Transduction of CD47 and CAR Genes.

The $B2M^{-/-}$ $CIITA^{-/-}$ γδ T cells are further transfected with CD47 and CAR genes by electroporation after editing with CRISPR-Cas9. Because the CIITA and B2M genes are knocked out, the immunogenicity is significantly reduced; however, the loss of MHC class I leads to susceptibility to NK cells. Therefore, CD47 is added to the γδ T cells to protect them from phagocytosis by the host's innate immune cells, while the CAR genes enable the γδ T cells to specifically target tumor antigens. This transfection process is performed under optimized conditions to achieve high transduction efficiency and stable gene expression.

2.2.8 Construction of B2M-/- CIITA-/- Mice.

$B2M^{-/-}$ $CIITA^{-/-}$ γδ T cells will then be infused into NSG-BLT humanized mice to determine their persistence in vivo. This serves as the experimental model to study the impact of the genetic modifications on the survival and function of the allogeneic γδ T cells within a humanized immune system.

2.2.9 Flow Cytometry.

Flow cytometry is used to determine the percentage of MHC class I negative and γδ2 positive cells, which indicate the presence of $B2M^{-/-}$ $CIITA^{-/-}$ γδ T cells in the host. Specific markers, such as γδ TCR and MHC class I molecules, are employed to distinguish these modified cells from unmodified and autologous γδ T cells within the host. This technique enables a quantitative analysis of the persistence and distribution of the genetically modified γδ T cells over time, assessed weekly.

2.2.10 Bioluminescence Imaging (BLI).

To analyze the distribution and persistence of $B2M^{-/-}$ $CIITA^{-/-}$ γδ T cells in vivo, bioluminescence imaging (BLI) will be utilized. The γδ T cells will be labeled with a luciferase reporter gene prior to injection, allowing their activity to be imaged and tracked in real-time within the host. Following the administration of the luciferin substrate, the luminescent signal emitted by the luciferase-tagged cells will be captured using a bioluminescence imaging system. This technique will provide detailed insights into the migration patterns and localization of the γδ T cells within the living organism throughout the experiment.

2.2.11 Measurement of Tumor Size and Weight.

The effectiveness of the γδ T cell treatments will be further evaluated through the measurement of tumor size and weight in the NSG-BLT mice. Tumor dimensions will be regularly recorded using calipers, focusing on the longest and shortest dimensions to calculate the tumor volume. Upon sacrifice, the tumors will be surgically excised, and their weight will be measured. These metrics will provide quantitative data on the anti-tumor activity of the modified γδ T cells, contributing to the assessment of their therapeutic potential in NSCLC models.

2.2.12 Safety Strategy.

A safety strategy is implemented to prevent harmful effects caused by the persistence of BiTE-secreting HIP dual CAR-γδT cells. The first component of this strategy involves providing a reliable off-switch for the entire model. Given that HIP T cells exhibit increased CD47 expression, it is hypothesized that the delivery of CD47-targeting fusion proteins will disrupt CD47 functionality and lead to the depletion of HIP T cells through host innate cell-mediated killing. [9] The second component of the safety strategy leverages the innate advantages of γδT cells. As γδT cells can directly recognize and eliminate a wide variety of tumor cells without requiring typical MHC markers, they are less likely to induce graft-versus-host disease (GvHD). [11]

3 Anticipate Result

3.1 Expression of B2M and CIITA, CD47 and CAR

To study the expression efficiency and antitumoral effects of B2M, CIITA, CD47 and CAR in γδ T cells, flow cytometry will be conducted respectively (Figure 2).

We anticipated that the tumor burden in the control groups would significantly increase over time, leading to decreased survival. In contrast, we expected progressive improvement in the experimental groups, particularly in Group 6. We anticipated that the application of HIP Dual BiTE CAR γδ T cells would yield the most notable results in terms of tumor suppression and survival.

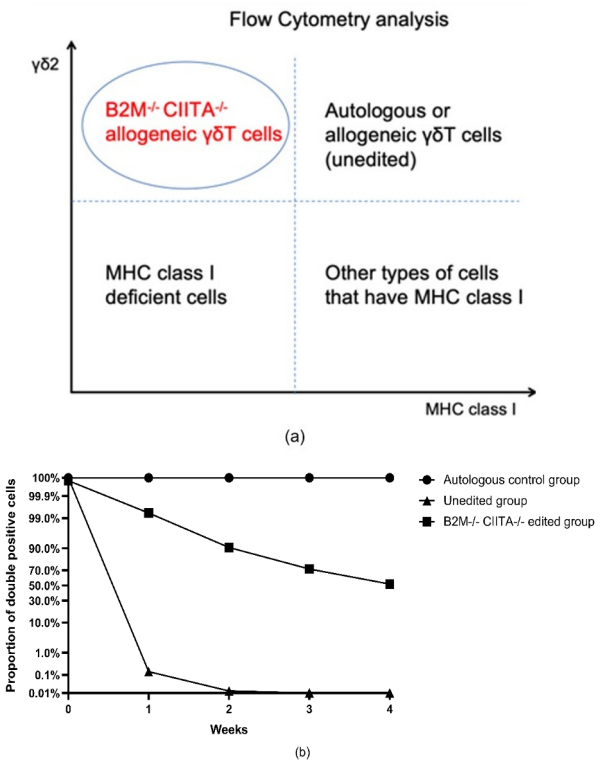

Figure 2. (a) Flow cytometry It is anticipated that the proportion of unedited allogeneic γδT cells will significantly decrease over time, while the proportion of autologous γδT cells (MHC class I+ γδ2+, control group) will remain stable. (b) Furthermore, it is expected that the proportion of B2M$^{-/-}$ CIITA$^{-/-}$ allogeneic γδT cells will gradually decline, suggesting enhanced persistence.

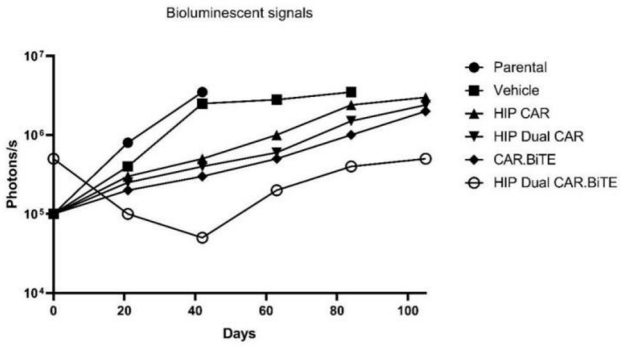

Figure 3. Predicted BLI result

3.2 Bioluminescence Imaging (BLI) Results.

The first control group exhibited increasing tumor growth over time. The second control group served as a baseline to confirm that the effects observed were due to the active treatments and not the vehicle. Group 3, which received HIP CAR γδ T cells, demonstrated some tumor suppression but not as effectively as the other experimental groups. Group 4, which received BiTE-secreting CAR γδ T cells, displayed enhanced tumor suppression due to BiTE secretion. Group 5, treated with dual targeting CAR γδ T cells, had better tumor control compared to single-target CAR groups. Finally, Group 6, which received HIP Dual BiTE CAR γδ T cells, exhibited the most significant reduction in tumor size and delayed tumor growth, supporting our hypothesis that this combination provides enhanced efficacy (Figure 3, 4).

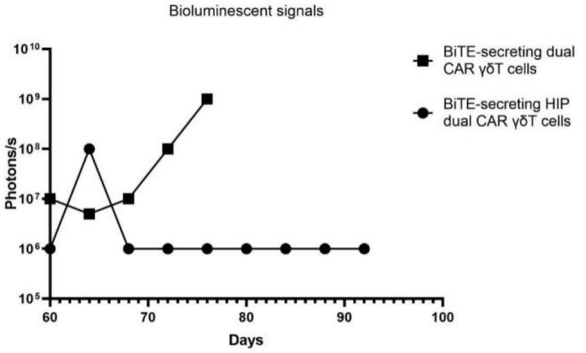

Figure 4. Predicted BLI result

3.3 Ensure HIP Functional Viability

To ensure that the persistence conferred by HIP also functions effectively and γδT cells' function is not disrupted in more complex models, we would rechallenge BiTE-secreting HIP dual CAR-γδT cells and BiTE-secreting dual CAR-γδT cells (control group) with the same A549 tumor cells, which will be transfected with firefly luciferase, 60 days after the initial inoculation. Utilizing BLI tracking, we observe the tumor size in groups of five mice each, monitoring the BLI signals every four days. It is anticipated that BiTE-secreting HIP dual CAR-γδT cells will exhibit superior persistence and anti-tumor efficacy compared to the control group.

4 Discussion

4.1 Addressing Tumor Escape and Enhancing CAR-T Cell Persistence

Overcoming tumor escape due to antigen expression heterogeneity, ensuring optimal T cell costimulation, and enhancing the persistence of allogeneic CAR-T cells within the host's internal environment are crucial factors for achieving effective clinical responses in solid tumors. [10] This project aims to develop CAR-γδT cells that target two antigens simultaneously, HLA-G and HER2, while also secreting bispecific T-cell engagers (BiTEs) that target PD-L1 on tumor cells and CD3 on T cells. This approach is designed to improve the recruitment and activation of surrounding T cells, thereby enhancing tumor cell elimination and ensuring sustained CAR-T cell persistence. To reduce immunogenicity, we propose knocking out MHC-I (B2M) and MHC-II (CIITA) molecules. Additionally, a safety mechanism could be integrated to mitigate toxicity associated with allogeneic CAR-T cells by incorporating a reliable off-switch for the entire model. Since HIP T cells demonstrate elevated CD47 expression, it is postulated that delivering CD47-targeting fusion proteins could disrupt CD47 function, resulting in the depletion of HIP T cells via host innate cell-mediated killing. [9] In NSG-BLT mouse models [15,14], BiTE-secreting HIP dual CAR-γδT cells are expected to exhibit strong and sustained antitumor activity in vivo

while preventing tumor escape.

4.2 Challenges and Toxicities in Traditional CAR-T Cell Therapies

In acute lymphoblastic leukemia/lymphoma (ALL/LBL) patients treated with conventional CAR-T cell therapy, nearly all of them experienced mild toxicity, and 23-46% had severe adverse effects. These severe effects were due to extreme cytokine secretion and significant in vivo T cell expansion. [16] Toxic levels of systemic cytokine release and intense immune cell cross-activation can lead to several life-threatening conditions, including cytokine release syndrome (CRS), macrophage activation syndrome (MAS), [17] and immune effector cell-associated neurotoxicity syndrome (ICANS). [18]

4.3 Strategies to Mitigate Toxicity and Enhance CAR-T Cell Efficacy

To mitigate the toxicity associated with CAR T cell therapy, we propose to employ HIP CAR-T cells by knocking out MHC molecules, which leads to reduced cytokine release and decreased CAR-T cell proliferation. This strategy aims to reduce the immunogenicity of CAR constructs, thereby preventing their recognition by the host immune system. However, such approach only partially alleviates toxicity. Mechanistically, CRS is induced by the extensive activation of administered CAR-T cells, resulting in the release of massive amounts of cytokines. Thus, the toxicity associated with CAR-T cell therapy cannot be entirely avoided.

4.4 Overcoming Experimental and Logistical Challenges

Additional limitations include the intrinsic experimental challenges associated with the substantial workload of combining BiTE, HIP CAR-T cells, and γδT cells, and their efficacy needs to be rigorously tested. Moreover, the extensive experimental processes require significant financial investment, manpower, and material resources. In view of these challenges, several potential strategies can be considered. Improved delivery methods should be developed to localize CAR T cells directly to the tumor site, minimizing systemic exposure and reducing the potential for widespread cytokine release. Combination therapies could be employed by integrating HIP CAR T cell therapy with other immunomodulatory agents or checkpoint inhibitors to enhance anti-tumor activity and mitigate immune-related toxicities. Advanced preclinical models, such as more refined humanized mouse models, should be developed to better mimic the human immune system and tumor microenvironment, allowing for more accurate preclinical testing of CAR T cell therapies. Incorporating suicide genes into CAR T cell constructs could provide a safety mechanism, allowing for the elimination of CAR T cells in the event of severe adverse reactions. Additionally, implementing streamlined and automated manufacturing processes would help reduce the cost and complexity of producing CAR T cells.

5 Conclusions

By using BLI tracking tumor existence and tumor size in NSG BLT mouse models injected with A549 NSCLC tumor cells, it is anticipated that BiTE-secreting HIP dual CAR-γδT cells would perform the best antitumoral efficacy and better persistence by the engineering than any other groups we mentioned before. CD47 off-switch safety strategy is also introduced to prevent toxicity caused by the long duration of BiTE-secreting HIP dual CAR-γδT cells. Thus, we reckon that BiTE-secreting HIP dual CAR-γδT cells would become a reliable gene-editing method for better persistence and antitumor effect which also provides a safety off-switch strategy and possibility of allogeneic products.

By using BLI tracking of tumor presence and size in NSG-BLT mouse models injected with A549 NSCLC tumor cells, it is anticipated that BiTE-secreting HIP dual CAR-γδT cells will demonstrate superior antitumor efficacy and persistence compared to other groups previously mentioned. Traditional CAR-T therapies, while successful in hematological malignancies, often face challenges in solid tumors like NSCLC due to antigen heterogeneity and immune evasion. The dual CAR-γδT cells in this study, targeting both HLA-G and HER2 antigens, coupled with BiTE secretion, are designed to overcome these challenges, enhancing immune recruitment, persistence, and efficacy. Additionally, the CD47 off-switch safety strategy is introduced to mitigate toxicity associated with prolonged BiTE-secreting HIP dual CAR-γδT cell activity. We believe that BiTE-secreting HIP dual CAR-γδT cells represent a promising gene-editing approach for improved persistence and antitumor effects, while also providing a safety mechanism and potential for allogeneic applications.

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Kaiheng Zhang, Mufeng Wang, Biyou Deng, Tianyi Chen and Xuexia Shi contributed equally to this work and should be considered co-first authors

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394-424. doi:10.3322/caac.21492
2. Schabath MB, Cote ML. Cancer Progress and Priorities: Lung Cancer. *Cancer Epidemiol Biomark Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol*. 2019;28(10):1563-1579. doi:10.1158/1055-9965.EPI-19-0221
3. Herbst RS, Morgensztern D, Boshoff C. The biology and management of non-small cell lung cancer. *Nature*. 2018;553(7689):446-454. doi:10.1038/nature25183
4. Darby S, Hill D, Auvinen A, et al. Radon in homes

and risk of lung cancer: collaborative analysis of individual data from 13 European case-control studies. *BMJ*. 2005;330(7485):223. doi:10.1136/bmj.38308.477650.63

5. Travis WD, Brambilla E, Riely GJ. New Pathologic Classification of Lung Cancer: Relevance for Clinical Practice and Clinical Trials. *J Clin Oncol*. 2013;31(8):992-1001. doi:10.1200/JCO.2012.46.9270
6. Govindan R, Page N, Morgensztern D, et al. Changing Epidemiology of Small-Cell Lung Cancer in the United States Over the Last 30 Years: Analysis of the Surveillance, Epidemiologic, and End Results Database. *J Clin Oncol*. 2006;24(28):4539-4544. doi:10.1200/JCO.2005.04.4859
7. Kagoya Y, Guo T, Yeung B, et al. Genetic Ablation of HLA Class I, Class II, and the T-cell Receptor Enables Allogeneic T Cells to Be Used for Adoptive T-cell Therapy. *Cancer Immunol Res*. 2020;8(7):926-936. doi:10.1158/2326-6066.CIR-18-0508
8. Deuse T, Hu X, Agbor-Enoh S, et al. The SIRPα–CD47 immune checkpoint in NK cells. *J Exp Med*. 2021;218(3):e20200839. doi:10.1084/jem.20200839
9. Hu X, Manner K, DeJesus R, et al. Hypoimmune anti-CD19 chimeric antigen receptor T cells provide lasting tumor control in fully immunocompetent allogeneic humanized mice. *Nat Commun*. 2023;14(1):2020. doi:10.1038/s41467-023-37785-2
10. Hirabayashi K, Du H, Xu Y, et al. Dual-targeting CAR-T cells with optimal co-stimulation and metabolic fitness enhance antitumor activity and prevent escape in solid tumors. *Nat Cancer*. 2021;2(9):904-918. doi:10.1038/s43018-021-00244-2
11. Huang S, Pan C, Lin Y, et al. BiTE-Secreting CAR-*γδ* T as a Dual Targeting Strategy for the Treatment of Solid Tumors. *Adv Sci*. 2023;10(17):2206856. doi:10.1002/advs.202206856
12. Li D, English H, Hong J, et al. A novel PD-L1-targeted shark VNAR single-domain-based CAR-T cell strategy for treating breast cancer and liver cancer. *Mol Ther - Oncolytics*. 2022; 24: 849-863. doi: 10.1016/j.omto.2022.02.015
13. Du H, Hirabayashi K, Ahn S, et al. Antitumor Responses in the Absence of Toxicity in Solid Tumors by Targeting B7-H3 via Chimeric Antigen Receptor T Cells. *Cancer Cell*. 2019;35(2):221-237.e8. doi:10.1016/j.ccell.2019.01.002
14. Shultz LD, Lyons BL, Burzenski LM, et al. Human Lymphoid and Myeloid Cell Development in NOD/LtSz- *scid IL2R γ null* Mice Engrafted with Mobilized Human Hemopoietic Stem Cells. *J Immunol*. 2005;174(10):6477-6489. doi:10.4049/jimmunol.174.10.6477
15. Biradar S, Agarwal Y, Lotze MT, Bility MT, Mailliard RB. The BLT Humanized Mouse Model as a Tool for Studying Human Gamma Delta T Cell-HIV Interactions In Vivo. *Front Immunol*. 2022; 13: 881607. doi: 10.3389/fimmu.2022.881607
16. Frey NV, Porter DL. Cytokine release syndrome with novel therapeutics for acute lymphoblastic leukemia. *Hematology*. 2016;2016(1):567-572. doi:10.1182/asheducation-2016.1.567
17. Neelapu SS, Locke FL, Bartlett NL, et al. Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. *N Engl J Med*. 2017;377(26):2531-2544. doi:10.1056/NEJMoa1707447
18. Santomasso BD, Park JH, Salloum D, et al. Clinical and Biological Correlates of Neurotoxicity Associated with CAR T-cell Therapy in Patients with B-cell Acute Lymphoblastic Leukemia. *Cancer Discov*. 2018;8(8):958-971. doi:10.1158/2159-8290.CD-17-1319