Modified Enhanced Immunocyte Targeting HPV and Pharmaceutical Use Thereof

Technical Field

The present application relates to the technical field of immunotherapy, in particular to a modified immune cell and medical uses thereof. Meanwhile, the present application also provides a T cell receptor or a fragment thereof targeting HPV E7 antigen and its related applications.

Background Art

1.1 Molecular Biological Characteristics and Subtypes of HPV

Human papillomavirus (HPV) is a DNA virus with a genomic DNA of approximately 8 kb, consisting of three parts: an early protein coding region, a late protein coding region, and an upstream regulatory region (URR). The early coding region comprises 4,500 base pairs and encodes six early regulatory proteins (E1/E2/E4/E5/E6/E7), which are involved in DNA replication, transcription, translation regulation, and cell transformation. The late protein coding region comprises 2,500 base pairs and encodes the major capsid protein L1 and the minor capsid protein L2. The upstream regulatory region comprises 1,000 base pairs and contains the origin of replication and gene expression regulatory elements of the HPV genome, regulating viral transcription and replication. Additionally, the HPV genome does not encode polymerases or other enzymes essential for viral replication; therefore, HPV must rely on host cell replication proteins to mediate the synthesis of viral DNA. HPV is genotyped based on the homology of the E6, E7, and L1 genes sequence, with 90% homology as the standard, and more than 200 subtypes have been identified currently. Depending on the pathogenicity, HPVs can be further classified into high-risk types (hrHPVs) and low-risk types. There are approximately 14 high-risk HPV types, including HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66, and HPV68. High-risk HPVs cause various cancers, including almost all kinds of cervical cancer, the majority of anal cancer and varieties of oropharyngeal cancer, vaginal cancer, vulvar cancer, and penile cancer. Thereof, the high-risk HPV16 and HPV18 have been found to be associated with the majority of HPV-related cancers. Persistent infection with HPV16 and HPV18 accounts for 70% of cervical cancer cases, and these two types are also primarily responsible for 84.1% of invasive cervical cancers.

The Human Leukocyte Antigen (HLA) is the most polymorphic gene complex in the human genome, which includes subtypes of HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQB1, and others. Studies have found that, HLA-A alleles play a more critical role in the Chinese Han population compared with other HLA genes. The most common HLA-A alleles in China are HLA-A*24:02，HLA-A*11:01, and HLA-A*02:01.

1.2 Oncogenic Pathway of HPV

Taking cervical cancer as an example, 80% of sexually active women experience HPV infection, of which approximately 90% can regress spontaneously within 1–2 years through the immune system, a small proportion turn into persistent infection, and about 1% of patients develop into cervical cancer Eventually. Typically, 5–10 years of high-risk HPV infection causes cytological abnormalities and induces cervical cancer. Precancerous lesions of cervical cancer/cervical epithelial dysplasia are known as Cervical Intraepithelial Neoplasia (CIN), which can be classified into three grades depending on severity:

CIN1: Lesions are limited to the lower 1/3 of the epithelial layer, i.e., mild cervical dysplasia;

CIN2: Lesions are limited to 1/2–2/3 of the epithelial layer, i.e., moderate cervical dysplasia;

CIN3: Lesions involve almost the entire epithelial layer, leaving only 1–2 layers of normal squamous epithelium, i.e., severe cervical dysplasia and carcinoma in situ.

All grades of precancerous lesions have the potential to develop into invasive cancer, with higher grades carrying greater risk. Studies have shown that HPV-DNA integration is found in 83% of cervical cancers, and HPV genes play an important role in the occurrence and development of HPV-related tumors.

HPV infects the host through epithelial tissue damage, and its life cycle is closely associated with the differentiation process of keratinocytes. During the development of keratinocytes to the spinous layer, viral DNA replication and gene expression occur simultaneously. The virus is internalized into the cell via endocytosis and penetrates to the nucleus to initiate the replication process. Specifically, the E1 and E2 proteins play critical roles in the initiation of HPV replication and transcription, while the E4 and E5 proteins regulate functions in late-stage life cycle of the viral. They downregulate the P21 gene, leading to disrupted regulation of cell cycle and causing HPV-infected cells to become malignant. Additionally, HPV-DNA integration occurs within the coding region of the E2 gene. The inactivation of E2 expression leads to the upregulation of E6 and E7 gene transcription. E6 and E7 are the only two viral genes consistently found in cervical cancer tissues, suggesting their direct involvement in the development of HPV-related tumors. Studies have shown that, upon binding to the cellular ubiquitin ligase E6AP, the E6 protein undergoes a conformational change, enabling it to specifically bind to the tumor suppressor protein p53 and form an E6/E6AP/p53 complex which induces the ubiquitination and subsequent degradation of p53, thereby impeding apoptosis of cells. Similarly, the E7 protein binds to and inactivates pRb, promoting the transcriptional activation of genes by the E2F transcription factor family and leading to cell cycle dysregulation. The inactivation of the tumor suppressor genes p53 and pRb results in uncontrolled cell cycle

progression and impaired apoptosis, causing excessive cellular proliferation and subsequent carcinogenesis. Furthermore, E7 can bind to the DREAM domain of RB-like, E2F4, and MuvB proteins, leading to their degradation via the proteasome pathway, activating downstream p53 signaling pathways, and causing cell cycle abnormalities. In other words, the E6 and E7 proteins are key factors driving the malignant transformation and pathological lesions in HPV-infected cells.

In summary, HPV infection induces changes in host cell gene expression, involving the activation of numerous signaling pathways which regulates apoptosis and the cell cycle, thereby conferring tumor characteristics to the cells. Persistent HPV infection promotes chronic inflammation, leading to the generation and release of reactive oxygen species (ROS). This subsequently damages DNA and drives the malignant transformation of infected cells. The E5, E6, and E7 proteins promote the expression of cyclooxygenase-2 (COX-2), triggering the massive release of prostaglandins which participate in cell proliferation, angiogenesis, and apoptosis inhibition, exerting adverse effects on cervical tissues. Moreover, the E6 and E7 proteins inhibit the NF-κB signaling pathway, facilitating immune evasion by HPV. The E7 protein also suppresses the expression of the apoptosis-related genes Bcl-xL, Fas, and Bad, promoting the malignant proliferation of infected cells and supporting HPV replication, which contributes to viral persistence. This chronic and persistent immune response manifests pathologically as cervical hyperplasia and ultimately leads to cervical cancer.

Since the initial formation and subsequent progression of cervical cancer are entirely dependent on the constitutive expression of the two major HPV oncogenes, E6 and E7, the oncoproteins E6 and E7 serve as biomarkers for cervical cancer cells. If the expression and/or function of E6 and/or E7 are inhibited, the proliferation of tumor cells will be arrested, and apoptosis will be induced. When target antigens such as E6 and E7 are delivered into the body in various forms, MHC class I and class II molecules present intracellular and extracellular antigens, respectively, activating CD8+ T cells and CD4+ T cells to induce an HPV-specific cellular immune response. Following the presentation of intracellular antigens, and with the synergistic action of cytokines, effector CD8+ T cells eliminate HPV-infected cells or tumor cells expressing the target antigen genes. Therefore, the E6 and E7 proteins are ideal targets for treating HPV infection-related inflammation and tumors, possessing broad prospects for application and development.

1.3 Global Prevalence of HPV

HPV (Human Papillomavirus) is a non-enveloped double-stranded circular DNA virus with high specificity and affinity to its host, and mainly spreads via sexual transmission, mother-to-child transmission, or skin-mucosa contact. 80% of sexually active women have HPV infection. A molecular epidemiological survey found that HPV infection rates are highest in Africa and

South America, reaching 22.9% and 18.6%, respectively. Globally, HPV16 is the most prevalent high-risk subtype, followed by HPV18, HPV58, HPV52, HPV31, HPV33, and HPV45. High-risk HPV DNA is present in 9 malignant tumors including oropharyngeal, cervical, vulvar, vaginal, penile, and anal cancers.

According to Global Cancer Statistics 2020, the annual new cases of HPV-related cancers worldwide reach 852,620, accounting for 4.40% of all cancers; annual deaths reach 447,900, accounting for 4.50% of all cancers. In 2022, the estimated new HPV-related cancer cases in China reached 127,804, with 68,370 deaths. HPV infection has become a serious public health problem threatening women's health.

1.4 Current Treatment Status of HPV-Related Cancers

HPV-related oropharyngeal, cervical, vulvar, vaginal, penile, and anal cancers are incurable and difficult to relieve once they develop into advanced stages, with no effective treatments are available currently. Although chemotherapy provides some relief for these tumors, the duration of response is usually short.

Cervical cancer accounts for approximately 70% of HPV-related cancers. Due to the popularization of cervical cancer screening and HPV vaccines, cervical cancer has become largely preventable. However, the 5-year survival rate of cervical cancer patients remains only about 60%. For early-stage non-metastatic cervical cancer, surgery and chemoradiotherapy are the main treatments; patients with FIGO stage IB–IIA without lymph node metastasis have a 5-year survival rate of 88%–95%. However, traditional treatments have not achieved satisfactory efficacy for metastatic or recurrent cervical cancer.

With the use of targeted therapies such as anti-angiogenic agents and immune checkpoint inhibitors, the survival time of these patients has been significantly prolonged, but a cure has not been achieved. There is an urgent need for in-depth molecular research to reveal new therapeutic targets and methods to meet clinical needs.

Early-stage cervical cancer is treated mainly with radical surgery, while advanced-stage cervical cancer is treated mainly with radiotherapy. With the emergence of new chemotherapeutic agents and routes, concurrent chemoradiotherapy has become the standard regimen for intermediate-stage and advanced-stage cervical cancer, but local recurrence remains high. Approximately 29%–38% of cervical cancer patients relapse after treatment, making recurrent cervical cancer a clinical focus. Chemotherapy options for recurrent cervical cancer are limited, and most cases are incurable. According to the Gynecologic Oncology Group (GOG) evaluation, the optimal regimen for recurrent or metastatic cervical cancer is the combination of cisplatin, paclitaxel, and bevacizumab, with an overall response rate (RR) of

48% and median survival of 17 months. However, due to the poor prognosis of these patients, the benefit of second-line chemotherapy for recurrent cervical cancer has not been agreed upon.

Immunotherapy is a novel cancer treatment relying on the immune system's ability to directly recognize and kill tumor cells. In recent years, several immunotherapeutic strategies for cervical cancer have achieved major breakthroughs.

Therapeutic Vaccines

Prophylactic HPV vaccines are approved and highly effective in preventing HPV infection but appear ineffective in clearing established HPV infection. Research on novel therapeutic vaccines for cervical dysplasia and cervical cancer is ongoing. A preclinical trial using Pseudomonas exotoxin-HPV16 E7ΔKDEL3 fusion protein or TVGV-1 combined with two different adjuvants showed preliminary efficacy in mouse models, associated with prolonged survival and proliferation of HPV16 E7-specific CD8+ T cells.

In addition, preliminary results have been obtained for the therapeutic vaccine pNGVL4a-CRT/E7 targeting patients with CIN2/3 secondary to HPV16. The vaccine consists of a DNA plasmid (pNGVL4a-A) encoding calreticulin linked to a detoxified form of HPV16 E7 antigen. The vaccine is designed to increase cytotoxic T cells to clear established HPV16 infection. In this first-in-human clinical trial, 27 women were vaccinated, with a 30% regression rate to CIN1 or lower, and cytotoxic T cells are increased compared with the control group.

Immune Checkpoint Therapy

Expression of PD-L1 enables tumors to evade CD8+ cytotoxic T cell-mediated destruction. Multiple studies have shown that PD-L1 expression is associated with poor prognosis, independent of other prognostic factors including tumor stage, tumor size, invasion depth, lymph vascular space invasion, or lymph node metastasis.

In addition, checkpoint inhibitors such as CTLA-4, LAG-3, VISTA, and TIGIT are being tested as single agents or in combination with other checkpoint inhibitors for potential efficacy in cervical cancer.

Adoptive T Cell Therapy

Adoptive T-Cell Therapy (ACT) involves vitro modification, culture, and expansion of autologous T cells to specifically recognize tumor cells. Reinfusion of large numbers of anti-tumor reactive cells into cancer patients can mediate durable, complete regression of some advanced malignancies.

E7 is the major oncogenic protein of HPV, and its overexpression in host cells is a key factor in development, progression, and metastasis of cervical cancer. As a foreign viral antigen not

expressed in normal tissues, TCR-T targeting HPV E7 is an ideal target for treating HPV-related advanced malignant tumors, with broad application prospects.

Solid tumors employ extensive active immune evasion strategies; repeated antigen stimulation and the suppressive tumor microenvironment cause T cell exhaustion and impair immunotherapeutic efficacy. Anti-exhaustion TCR-T cells targeting HPV are a self-developed next-generation therapy, transducing T cells with HPV E7-targeting TCR and anti-exhaustion fusion receptors to kill tumor cells and clear viral infection by targeting viral-specific antigen expression and DNA integration mechanisms. The anti-exhaustion fusion receptor serves as a component to counteract the tumor microenvironment and reduce immune suppression, enabling sustained tumor killing and proliferation of TCR-T cells, and thereby inducing durable anti-tumor immune responses. It is applicable to various HPV-induced malignant tumors including cervical, oropharyngeal, head and neck, vaginal, vulvar, and penile cancers.

LN-145 is an ongoing clinical trial (NCT03108495) using a product extracted and expanded from TILs. These TILs are reinfused after myeloablative chemotherapy, complete and durable responses have been observed with this treatment. Among 18 evaluable cervical cancer patients, 5 showed a response, including 2 complete responses. Another phase I clinical trial (NCT02280811) which uses T cells engineered with TCRs recognizing HLA-A*02:01-restricted HPV-16 oncoproteins showed responses in HPV16-positive anal canal cancer patients, preliminarily demonstrating efficacy.

In summary, due to the limitations and risks of currently available treatments and drugs, new strategies are needed to provide more treatment options for patients with HPV-related cervical cancer.

1.5 Role of T Cells in HPV-Related Tumor Immunotherapy and Basic Principles of TCR-T Cell Application

T cells are immune cells originating from the bone marrow and lymphoid system that differentiate and mature in the thymus. They express T cell antigen receptors (TCRs) on their surface and play a crucial role in clearing infections and cancer cells in cell-mediated immunity. The TCR can specifically recognize and bind to target antigenic epitopes presented by major histocompatibility complex (MHC) molecules. Once a T cell recognizes its target, it can eliminate target cells through massive proliferation, cytokine release, and cytotoxicity.

Regarding the scope of application for TCR-T therapy, adoptive T cell immunotherapy is currently widely explored for treating human malignancies (e.g., leukemia), viral diseases (e.g., hepatitis B virus (HBV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV)). However, isolating and expanding virus- or tumor-specific T cells from patient blood is highly difficult

and time-consuming. Therefore, researchers have adopted novel therapeutic strategies by introducing T cell receptors (TCRs) or TCRα/β heterodimers that target specific antigens. This approach enables these genetically engineered T lymphocytes to act against specific viral or tumor antigens, thereby confers defined antigen specificity to the T cells. The application of TCR-T in solid tumors, particularly in HPV-associated cervical cancer, holds significant clinical value and implications.

TCR-T cell therapy relies on the presentation by histocompatibility complex (MHC) molecules to recognize targets and activate T cell functions. It can recognize intracellular antigenic fragments presented by MHC molecules. This characteristic determines that the target scope of TCR-T cell therapy is broader, encompassing intracellular antigens, cell surface antigens, and neoantigens generated from tumor cell mutations. It can overcome the differences in antigen expression of malignant tumor cells caused by tumor heterogeneity, demonstrating superior therapeutic value.

Studies have shown that T cells genetically edited to express HPV-specific T cell receptors (TCRs) can specifically recognize HPV16 E7-associated cervical cancer cells, and subsequently activated and expanded, thereby effectively eliminate HPV16 E7-positive tumor cells. Therefore, the HPV16 E7 protein serves as a therapeutic target for HPV-related cancers.

1.6 T Cell Exhaustion and Countermeasures

T cell exhaustion is a persistent issue and a major cause of the suboptimal efficacy of cell therapies in solid tumors. T cell exhaustion is commonly observed in malignancies and represents a state of T cell dysfunction resulting from prolonged exposure to persistent antigen stimulation. Specifically, T cells will ultimately lose their functions being an effector after continuous effector activity, characterized by defective production of tumor necrosis factor (TNF) and interferon-γ (IFN-γ). This is one of the mechanisms by which malignant tumor cells evade immune responses.

Studies have shown that the binding of programmed death-ligand 1 (PD-L1) expressed on tumor cells to programmed cell death protein 1 (PD-1) on lymphocytes is a critical signaling pathway leading to lymphocyte exhaustion. The exhausted T cell phenotype can be restored by antagonizing the PD-1/PD-L1 interaction. This finding provides significant implications for addressing TCR-T exhaustion and improving therapeutic efficacy in solid tumors.

During tumor progression, tumor cells, mesenchymal fibroblasts, and other cells in the tumor microenvironment (TME) secrete massive amounts of transforming growth factor β (TGF-β). On the one hand, tumor cells establish tolerance by secreting large quantities of TGF-β, thereby creating a high-concentration TGF-β microenvironment for themselves. On the other hand,

TGF-β suppresses the anti-tumor activity of immune cells, facilitating tumor immune evasion and further promoting tumor progression. Most malignancies employ broad active immune evasion strategies by producing TGF-β to inhibit the cytotoxic effects of immune cells against tumor cells. Antagonizing the downstream TGF-β signaling pathway holds the potential to counteract the immunosuppressive effects caused by TGF-β in the TME, preventing the attenuation and exhaustion of T cell cytotoxicity. This can further improve therapeutic efficacy, prolong the survival of TCR-T cells in vivo, and promote T cell infiltration. Ultimately, this addresses the pain points of insufficient persistence, suboptimal therapeutic outcomes, and tumor recurrence in TCR-T cell therapy, thereby better meeting patient needs.

Summary of the Invention

In view of existing technical problems, the present application provides a modified immune cell targeting specific HPV E7 antigen, capable of overcoming the issue of immune cell exhaustion caused by tumor microenvironment immunosuppression. The modified immune cell can specifically kill cancer cells induced by HPV infection, including cells of cervical cancer, oropharyngeal cancer, head and neck cancer, vaginal cancer, vulvar cancer, penile cancer, and anal cancer, thereby treating these abovementioned HPV-associated malignancies.

The first aspect of the present application provides a modified immune cell, which comprises a T cell receptor targeting the HPV E7 antigen (HPV TCR) and a chimeric switch receptor.

In some embodiments, the HPV TCR comprises a TCRα chain variable domain and a TCRβ chain variable domain; wherein the αCDR3 of the TCRα chain variable domain has an amino acid sequence shown as SEQ ID NO: 3, or a variant thereof with one or two amino acids substituted; and the βCDR3 of the TCRβ chain variable domain has an amino acid sequence shown as SEQ ID NO: 6, or a variant thereof with one or two amino acids substituted.

In some embodiments, the TCRα chain variable domain comprises complementarity-determining regions αCDR1, αCDR2, and αCDR3, and the TCRβ chain variable domain comprises complementarity-determining regions βCDR1, βCDR2, and βCDR3, wherein:αCDR1, αCDR2, and αCDR3 are shown as SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3, respectively; βCDR1, βCDR2, and βCDR3 are shown as SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6, respectively; or variants thereof with one or two amino acids substituted in one or more CDRs.

In some embodiments, the TCRα chain variable domain comprises an amino acid sequence at least 90% identical to SEQ ID NO: 7; in some embodiments, the TCRβ chain variable domain comprises an amino acid sequence at least 90% identical to SEQ ID NO: 8.

In some embodiments, the HPV TCR comprises an amino acid sequence shown as SEQ ID NO:

13. In some embodiments, the chimeric switch receptor comprises an extracellular domain (ECD) of an immunosuppressive protein, wherein the ECD is fused to an intracellular domain (ICD) of a co-stimulatory molecule mediating immune cell activation signals; wherein binding of the ECD of the immunosuppressive protein to its ligand generates an activation signal rather than an inactivation signal in the modified immune cell.

In some embodiments, the immunosuppressive protein is selected from PD-1, CTLA4, BTLA, TIM3, TIGIT, TGFβ receptor, and any other proteins with immunosuppressive functions or related to immunosuppressive signaling pathways, or a combination thereof, and the ECD sequence of the immunosuppressive protein may have at least one amino acid mutation.

In some embodiments, the ECD is TGFβ receptor II ECD; in some embodiments, the TGFβ receptor II ECD sequence may have at least one amino acid mutation; in some embodiments, the ECD amino acid sequence of TGFβ receptor is shown as SEQ ID NO: 12.

In some embodiments, the ECD is PD-1 ECD; in some embodiments, the PD-1 ECD sequence may have at least one amino acid mutation; in some embodiments, the PD-1 ECD has a single amino acid mutation (alanine to leucine at position 132) increasing affinity for PDL1, with the ECD amino acid sequence shown as SEQ ID NO: 9.

In some embodiments, the co-stimulatory molecule is selected from any one of CD28, 4-1BB, ICOS, CD27, IL-12R, CD3, OX40, or a combination thereof, and the ICD sequence of the co-stimulatory molecule may have at least one amino acid mutation.

In some embodiments, the ICD is 4-1BB ICD; in some embodiments, the 4-1BB ICD sequence may have at least one amino acid mutation; in some embodiments, the amino acid sequence of 4-1BB ICD is shown as SEQ ID NO: 11.

In some embodiments, the TCR comprises a TCRα chain and a TCRβ chain, wherein the TCRα chain variable domain comprises an amino acid sequence at least 90% identical to SEQ ID NO: 7; and/or the TCRβ chain variable domain comprises an amino acid sequence at least 90% identical to SEQ ID NO: 8.

In some embodiments, the ECD and ICD are connected via a transmembrane (TM) sequence; in some embodiments, the transmembrane region comprises a transmembrane domain of the protein selected from TCR α, β, or ζ chain, CD28, CD3ε, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154, or a combination thereof, and the transmembrane domain may have at least one amino acid mutation.

In some embodiments, the transmembrane sequence is CD8 transmembrane sequence; in some embodiments, the CD8 transmembrane sequence is shown as SEQ ID NO: 10.

In some embodiments, the modified immune cell expresses the following exogenous polypeptides:

A. A T cell receptor (TCR) specifically targeting HPV E7 antigen; and

B. A chimeric switch receptor comprising an extracellular domain (ECD) and an intracellular domain (ICD), wherein the ECD is selected from ECDs of immunosuppressive molecules, and the ICD is selected from ICDs of co-stimulatory molecules.

In one embodiment, the TCR is capable of binding to an HPV E7 antigen peptide presented by HLA-A*02, and more preferably, the TCR is capable of binding to an HPV E7 antigen peptide presented by HLA-A*02:01; the antigen peptide comprises the amino acid sequences at positions 11-19 of HPV16 E7 and HPV52 E7, which correspond to YMLDLQPET or YILDLQPET, respectively.

In some embodiments, the chimeric switch receptor is PD1(ECD)-CD8(TM)-4-1BB(ICD) (PD1-BB); in some embodiments, the amino acid sequence of PD1-BB is shown as SEQ ID NO: 14. In some embodiments, the chimeric switch receptor is TGFβ receptor(ECD)-CD8(TM)-4-1BB(ICD) (TGF-BB); in some embodiments, the amino acid sequence of TGF-BB is shown as SEQ ID NO: 16.

In some embodiments, the modified immune cell comprises an HPV TCR and a chimeric switch receptor with the structure of PD1(ECD)-CD8(TM)-4-1BB(ICD)(PD1-BB). Preferably, the aforementioned modified immune cell comprises the amino acid sequence shown as SEQ ID NO: 15. Preferably, the modified immune cell comprises an HPV TCR and a chimeric switch receptor with the structure of TGFβ receptor(ECD)-CD8(TM)-4-1BB(ICD) (TGF-BB). In some embodiments, the aforementioned modified immune cell comprises the amino acid sequence shown as SEQ ID NO: 17.

In some embodiments, the immune cell is selected from lymphocytes, dendritic cells, monocytes/macrophages, granulocytes and mast cells. In some embodiments, the immune cell is a T cell.

The second aspect of the present application provides a nucleic acid molecule comprising a nucleic acid sequence encoding the TCR molecule of the first aspect; and/or a nucleic acid sequence encoding the chimeric switch receptor of the first aspect.

In some embodiments, the HPV TCR is encoded by a nucleic acid sequence shown as SEQ ID NO: 18.

In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence encoding HPV TCR and a nucleic acid sequence encoding PD1-BB chimeric switch receptor; in some

embodiments, the nucleic acid molecule comprises a sequence shown as SEQ ID NO: 19.

In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence encoding HPV TCR and a nucleic acid sequence encoding TGF-BB chimeric switch receptor; in some embodiments, the nucleic acid molecule comprises a sequence shown as SEQ ID NO: 20.

The third aspect of the present application provides a vector, wherein the vector comprises the nucleic acid molecule of the second aspect of the present application.

The fourth aspect of the present application provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and the modified immune cell of the first aspect, the nucleic acid molecule of the second aspect, or the vector of the third aspect of the present application.

The fifth aspect of the present application provides use of the modified immune cell of the first aspect, the nucleic acid molecule of the second aspect, the vector of the third aspect, or the pharmaceutical composition of the fourth aspect in the manufacture of a medicament for preventing or treating diseases associated with HPV infection; the HPV infection-associated diseases comprise one or more of various malignancies caused by HPV, including cervical cancer, oropharyngeal cancer, head and neck cancer, vaginal cancer, vulvar cancer, penile cancer, and anal cancer.

The sixth aspect of the present application provides a T cell receptor targeting HPV E7 antigen or a fragment thereof, wherein said T cell receptor comprises a TCRα chain variable domain and a TCRβ chain variable domain selected from the group consisting of:

(1) The TCRα chain variable domain comprises three CDRs: αCDR1 shown as SEQ ID NO: 1, αCDR2 shown as SEQ ID NO: 2, and αCDR3 shown as SEQ ID NO: 3, or variants thereof with one or two amino acids substituted; and

(2) The TCRβ chain variable domain comprises three CDRs: βCDR1 shown as SEQ ID NO: 4, βCDR2 shown as SEQ ID NO: 5, and βCDR3 shown as SEQ ID NO: 6, or variants thereof with one or two amino acids substituted.

In some embodiments, the TCRα chain variable domain comprises three CDRs: αCDR1 shown as SEQ ID NO: 1, αCDR2 shown as SEQ ID NO: 2, and αCDR3 shown as SEQ ID NO: 3; and the TCRβ chain variable domain comprises three CDRs: βCDR1 shown as SEQ ID NO: 4, βCDR2 shown as SEQ ID NO: 5, and βCDR3 shown as SEQ ID NO: 6.

In some embodiments, the TCRα chain variable domain comprises an amino acid sequence at least 90% identical to SEQ ID NO: 7; and/or the TCRβ chain variable domain comprises an amino acid sequence at least 90% identical to SEQ ID NO: 8.

In some embodiments, the T cell receptor or fragment is capable of binding to HPV E7 antigen peptide presented by HLA-A*02; more preferably, the TCR is capable of binding to HPV E7 antigen peptide presented by HLA-A*02:01; In some embodiments, the peptide comprises the amino acid sequence of YMLDLQPET or YILDLQPET.

The seventh aspect of the present application provides a nucleic acid molecule, which comprises a nucleic acid sequence encoding the T cell receptor targeting HPV E7 antigen of the sixth aspect or a fragment thereof, or a complementary sequence thereof.

The eighth aspect of the present application provides a vector, wherein the vector comprises the nucleic acid molecule of the seventh aspect, wherein the vector is selected from the group consisting of a plasmid, a binary vector, a DNA vector, an mRNA vector, a retroviral vector, a lentiviral vector, a transposon-based vector, and an artificial chromosome.

The ninth aspect of the present application provides an isolated polypeptide encoded by the nucleic acid molecule of the seventh aspect or the vector of the eighth aspect.

The tenth aspect of the present application provides isolated cells, wherein the cells comprise the T cell receptor or fragment thereof of the sixth aspect, the nucleic acid molecule of the seventh aspect, the vector of the eighth aspect, or the polypeptide of the ninth aspect; preferably, the cells comprise HPV E7_SCG61 TCR, and the amino acid sequence of the HPV E7_SCG61 TCR is shown as SEQ ID NO: 13.

The eleventh aspect of the present application provides a pharmaceutical composition, wherein the composition comprises a pharmaceutically acceptable carrier and the T cell receptor or fragment thereof of the sixth aspect, the nucleic acid molecule of the seventh aspect, the vector of the eighth aspect, the polypeptide of the ninth aspect, or the cell of the tenth aspect.

Use of the T cell receptor or fragment thereof of the sixth aspect, the nucleic acid molecule of the seventh aspect, the vector of the eighth aspect, the polypeptide of the ninth aspect, the cell of the tenth aspect, or the pharmaceutical composition of the eleventh aspect in the manufacture of a medicament for preventing or treating diseases associated with HPV infection. In some embodiments, the HPV infection-associated diseases comprise one or more of various malignancies caused by human papillomavirus (HPV), including cervical cancer, oropharyngeal cancer, head and neck cancer, vaginal cancer, vulvar cancer, penile cancer, and anal cancer.

The beneficial effects of the present application are as follows:

The present application provides an exhaustion-resistant TCR-T product targeting the E7 antigen of HPV-16- and HPV-52-associated tumor cells. Structurally, the enhanced TCR-T cells targeting HPV can simultaneously express a specific TCR and a PD-1 or TGFβRII co-

stimulatory fusion receptor. Wherein, the TCR is MHC-restricted (specifically for HLA-A*02:01) and can specifically recognize the HPV-16/52 E7 viral peptide presented by HLA-A*02:01, thereby the TCR-T cells produces cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), and by which exerting a cytolysis effect on tumor cells. The enhanced TCR-T cells contains the PD-1 or TGFβRII co-stimulatory domain fusion receptor, which leads to a replacement of the original intracellular inhibitory signaling domain of the inhibitory receptor to an intracellular activation signaling domain. The purpose of this replacement is to convert the inhibitory signal of T cells into an activation signal, thereby enhancing cell proliferation capacity, cytokine release, and the exhaustion resistance of the TCR-T cells.

The use of the enhanced TCR-T cells targeting HPV-16 E7 of the present invention for treating HPV-associated solid tumors has the following main advantages:

(a) The anti-tumor treatment targeting HPV E7 can eliminate the protection effect of the Tumor Barrier on solid malignant tumor tissues. The Tumor Barrier is a self-protection mechanism of tumors that can restrict the entry of immune effector cells into tumor tissues. Unlike TAA targets, the precancerous lesions caused by chronic HPV infection form a region with high expression of HPV E7 targets between normal tissues and tumor tissues. These targets can guide the corresponding innate immune cells to infiltrate into the tumor to exert a cytolytic effect, thereby overcoming the Tumor Barrier.

(b) HPV E7 is a foreign antigen and is not expressed in normal tissues. The cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) induced by TCR-T drugs are mild and well-tolerated; the main adverse reactions are mostly caused by the drug attacking normal tissue cells that express the corresponding targets outside the tumor. Therefore, the enhanced TCR-T cells targeting HPV possess superior safety profiles.

(c) The enhanced TCR-T cells targeting HPV can recognize the corresponding HPV E7 peptides that match HLA-A*02:01, regardless of whether the complete E7 antigen is expressed. Studies have shown that the enhanced TCR-T cells targeting HPV exhibit a significant dose-dependent inhibitory effect on the growth of CDX cervical cancer and head and neck squamous cell carcinoma xenografts expressing HPV-16 E7 in immunodeficient mice.

(d) The inventors successfully screened and obtained a fully natural, ultra-high-affinity HPV-specific TCR, and engineered the constant regions of the TCR to reduce the risk of mispairing with endogenous TCR chains.

(e) The enhanced TCR-T cells targeting HPV can simultaneously recognize various HPV virus-associated tumors related to the HPV-16 and HPV-52 genotypes, such as cervical cancer and

head and neck cancer, thereby significantly increasing the coverage of the patient population.

(f) The co-stimulatory molecule fusion receptor carried by the enhanced TCR-T cells targeting HPV can also reverse T cell exhaustion mediated by signaling pathways, enabling the TCR-T cells to continuously exert a tumor-killing effect. Antagonizing downstream signaling pathways of immunosuppression aims to prolong the survival time of TCR-T cells in vivo and promote T cell infiltration, thereby solving the pain points of insufficient durability, suboptimal therapeutic efficacy, and the tumor recurrence in TCR-T cell therapy, and better meeting patient needs.

(g) Compared with enhanced CAR-T cells equipped with chimeric switch receptors, the enhanced TCR-T cells can recognize intracellular antigen fragments presented to the cell surface by MHC molecules, overcoming the differences in antigen expression of malignant tumor cells caused by tumor heterogeneity. Furthermore, TCR-T cells can recognize low-abundance tumor antigens, when combined with a chimeric switch receptor, it further enhances the proliferation and tumor-killing effects of CD8+ and CD4+ TCR-T cells while overcomes the inhibitory tumor microenvironment simultaneously, and promotes the long-term persistence of memory T cells, to ensure durable and significant therapeutic efficacy of the immune cell therapy, which is crucial for achieving breakthrough efficacy in solid tumors. Additionally, the risk of cytokine release syndrome (CRS) associated with enhanced TCR-T cell therapy may be lower compared to enhanced CAR-T cells.

Brief Description of the Drawings

In order to more clearly illustrate the technical solutions in the embodiments of the present application, the accompanying drawings required to be used in the description of the embodiments will be briefly introduced below. It is obvious that the drawings in the following description are merely some embodiments of the present application. For those of ordinary skill in the art, other drawings can also be obtained based on these drawings without involving any inventive effort.

Figure 1: Schematic diagram of SCG61 TCR structure;

Figure 2: TCR-T production flow chart;

Figure 3: Expression detection of SCG61 TCR-T cells infected at different MOIs;

Figure 4: SCG61 TCR binding activity assay;

Figure 5: In vitro cytotoxicity and cytokine secretion of SCG61 TCR-T against target cells;

Figure 6: Proliferation of SCG61 TCR-T after multiple rounds of stimulation;

Figure 7: Recognition of SCG61 TCR-T for similar target epitopes;

Figure 8: Recognition of SCG61 TCR-T for peptides of different HPV subtypes;

Figure 9: In vivo efficacy of SCG61 TCR-T in CaSki xenograft model;

Figure 10: Structure and sequence of SCG61-PD1-BB expressing HPV TCR and chimeric switch receptor;

Figure 11: Expression and phenotype detection of SCG61-PD1-BB TCR-T;

Figure 12: Proliferation of SCG61-PD1-BB TCR-T;

Figure 13: In vitro cytotoxicity of SCG61-PD1-BB against target cells;

Figure 14: In vitro cytotoxicity of SCG61-PD1-BB against target cells with different HLA subtypes and HPV/PDL1 status;

Figure 15: In vitro cytotoxicity of SCG61-PD1-BB after multiple tumor stimulations;

Figure 16: Proliferation of SCG61-PD1-BB after multiple tumor stimulations;

Figure 17: In vivo efficacy of SCG61-PD1-BB in CaSki xenograft model;

Figure 18: In vivo efficacy of SCG61-PD1-BB in SCC-090 xenograft model;

Figure 19: Mismatch rate of SCG61-PD1-BB with endogenous TCR;

Figure 20: Identification of key amino acids recognized by SCG61-PD1-BB using alanine scanning library;

Figure 21: Functional affinity of SCG61-PD1-BB for T2-loaded E7 peptide;

Figure 22: In vivo function of SCG61-PD1-BB in CaSki xenograft model;

Figure 23: Distribution of SCG61-PD1-BB in mice cancer, lungs, spleen, etc;

Figure 24: Structure and sequence of SCG61-TGF-BB expressing HPV TCR and chimeric switch receptor;

Figure 25: Expression and phenotype detection of SCG61-TGF-BB TCR-T;

Figure 26: Mismatch rate and memory/exhaustion phenotype of SCG61-TGF-BB TCR-T;

Figure 27: Recognition of SCG61-TGF-BB TCR-T for peptides of different HPV subtypes;

Figure 28: Cytotoxicity of SCG61-TGF-BB TCR-T against target cells of different HPV subtypes;

Figure 29: Cytokine secretion of SCG61-TGF-BB TCR-T against target cells of different HPV subtypes;

Figure 30: Mechanism validation of SCG61-TGF-BB TCR-T to inhibit pSMAD2/3 pathway;

Figure 31: Function validation of affinity of SCG61-TGF-BB TCR-T for TGFβ1;

Figure 32: Cytotoxicity of SCG61-TGF-BB against target cells expressing TGFβ1;

Figure 33: In vitro cytotoxicity of SCG61-TGF-BB after multiple tumor stimulations;

Figure 34: Proliferation of SCG61-TGF-BB after multiple tumor stimulations;

Figure 35: Cross-reactivity of SCG61-TGF-BB with human peptide epitope;

Figure 36: In vitro cytotoxicity of SCG61-TGF-BB (animal batch);

Figure 37: In vivo cytotoxicity of SCG61-TGF-BB in CaSki-E7 xenograft model;

Figure 38: In vivo expansion and persistence of SCG61-TGF-BB in CaSki-E7 xenograft model;

Figure 39: In vivo cytotoxicity of SCG61-TGF-BB in HepG2-E7 xenograft model;

Figure 40: In vivo expansion and persistence of SCG61-TGF-BB in HepG2-E7 xenograft model.

Detailed Description

To facilitate an understanding of the present invention, certain technical and scientific terms are described below prior to the description of the embodiments. Unless otherwise explicitly defined elsewhere in the application documents of the present invention, all other technical and scientific terms used in the present invention have the meanings commonly understood by those of ordinary skill in the art to which the present invention pertains.

The term 'HPV E7 antigen T cell receptor (TCR)' is defined herein as a TCR that binds to an HPV E7 surface antigen in the context of a major histocompatibility complex (MHC) molecule, thereby inducing a helper or cytotoxic response in a cell expressing the recombinant TCR. The TCR or a fragment thereof of the present application can recognize a corresponding peptide of HPV-16 E7 that matches HLA-A*02:01, regardless of whether the complete E7 antigen is expressed.

The term 'MHC molecule' refers to a protein of the immunoglobulin superfamily, which may be a class I or class II MHC molecule. Therefore, it is specific for antigen presentation. Different individuals have different MHCs, which can present different short peptides of a protein antigen to the surface of their respective antigen-presenting cells (APCs). The human MHC is commonly referred to as the HLA gene or HLA complex.

TCR is a glycoprotein on the cell surface existing as a heterodimer composed of an α chain/β chain or a γ chain/δ chain. In 95% of T cells, the TCR heterodimer consists of α and β chains, whereas in 5% of T cells, the TCR consists of γ and δ chains. A natural αβ heterodimeric TCR comprises an α chain and a β chain, which constitute the subunits of the αβ heterodimeric TCR. Each of the α and β chains comprises a variable region and a constant region. Each variable region comprises three complementarity-determining regions (CDRs), i.e., CDR1, CDR2, and CDR3, embedded in framework regions (FRs). The CDR regions of the α and β chains of the TCR of the present application are defined according to the IMGT numbering scheme. The CDR regions determine the binding of the TCR to the pMHC complex. The sequences of the TCR constant domains can be found in the public database of the International Immunogenetics Information System (IMGT).

In the present application, the terms "T cell receptor", "TCR" and "TCR molecule" are used interchangeably. TGFβRII and TGFβ receptor II have the same meaning and are used interchangeably.

TCR Molecules

The TCR or a fragment thereof of the present application recognizes a corresponding peptide of HPV-16 E7 that matches HLA-A02:01. Approximately 50% of the general population expresses the MHC class I molecule HLA-A*02; therefore, HLA-A*02-restricted TCRs can have universal therapeutic utility. Specifically, the TCR of the present application can recognize the products of many HLA-A02 alleles, including HLA-A*0201, HLA-A*0202, HLA-A*0203, HLA-A*0206, and HLA-A*0207. Although there may be significant differences in HLA gene subtypes between Caucasians and Asians, more than 95% of HLA-A2-positive Caucasians are HLA-A*0201. In contrast, the HLA-A2 subtypes in HLA-A2-positive Chinese individuals consist of: 23% HLA-A*0201; 45% HLA-A0*207; 8% HLA-A*0206; and 23% HLA-A*0203.

In some embodiments, the TCR comprises a TCRα chain variable domain and a TCRβ chain variable domain, each with three CDRs.

In some embodiments, the αCDR3 of the TCRα chain variable domain is shown as SEQ ID NO: 3 or a variant with 1~2 substitutions; the βCDR3 of the TCRβ chain variable domain is shown as SEQ ID NO: 6 or a variant with 1~2 substitutions.

In some embodiments, the TCRα chain variable domain comprises complementarity-determining regions αCDR1, αCDR2, and αCDR3, and the TCRβ chain variable domain comprises complementarity-determining regions βCDR1, βCDR2, and βCDR3, wherein:
αCDR1, αCDR2, and αCDR3 are shown as SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3, respectively, and βCDR1, βCDR2, and βCDR3 are shown as SEQ ID NO: 4, SEQ ID NO: 5,

and SEQ ID NO: 6, respectively; or a CDR variant, wherein one or two amino acids in one or more of the CDRs are substituted with other amino acids.

In some embodiments, αCDR1, αCDR2, and αCDR3 are shown as SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3, respectively; and/or βCDR1, βCDR2, and βCDR3 are shown as SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6, respectively.

In some embodiments, the TCR comprises the amino acid sequence shown as SEQ ID NO: 13.

The CDR amino acid sequences of the present application described above can be grafted into any suitable framework regions to prepare chimeric TCRs. As long as the framework regions are compatible with the CDR regions of the TCR of the present application, those of ordinary skill in the art can design or synthesize TCR molecules with corresponding functions based on the CDR regions disclosed in the present application. Therefore, the TCR molecules of the present application refer to TCR molecules comprising the aforementioned α and/or β chain CDR sequences and any suitable framework regions. The TCRα chain variable domain of the present application is an amino acid sequence having at least 90%, preferably 95%, and more preferably 98% sequence identity to SEQ ID NO: 7; and/or the TCRβ chain variable domain of the present application is an amino acid sequence having at least 90%, preferably 95%, and more preferably 98% sequence identity to SEQ ID NO: 8.

In some embodiments, the TCR is an αβ heterodimer comprising a TCRα chain constant domain and a TCRβ chain constant domain. In some embodiments, the constant domains of the TCR molecules of the present application are human constant domains. Those of ordinary skill in the art know, or can obtain, the amino acid sequences of human constant domains by consulting relevant literature or the public database of the International Immunogenetics Information System (IMGT). For example, the constant domain sequence of the α chain of the TCR molecule of the present invention may be "TRAC*01", and the constant domain sequence of the β chain may be "TRBC1*01" or "TRBC2*01". In some embodiments, an additional disulfide bond is introduced into the constant domains to improve stability and reduce mispairing between the exogenously introduced TCR molecules and endogenous TCR molecules. The constant domains of the TCR molecules of the present application may also be murine constant domains. Replacing both TRAC and TRBC with murine-derived constant domains simultaneously can avoid mispairing between the exogenously introduced TCR molecules and endogenous TCR molecules, thereby preventing TCR mis-targeting. This effect is similar to the purpose of artificially introducing disulfide bonds.

Chimeric switch receptors

As used herein, “chimeric switch receptors” and “chimeric co-stimulatory molecule” are interchangeable.

The chimeric switch receptor comprises an ECD of an immunosuppressive protein fused to an ICD of a co-stimulatory molecule mediating activation signals; binding of the ECD to its ligand or immunosuppressive factor generates an activation signal rather than an inactivation signal.

Proteins contained in immune cells that trigger inhibitory signaling upon binding to their ligands include immunosuppressive proteins such as PD-1, CTLA4, BTLA, TIM3, TIGIT and TGFβ receptors, as well as any other proteins with immunosuppressive functions or associated with immunosuppressive signaling pathways, either alone or in any combination. The extracellular domains (ECDs) of the aforementioned immunosuppressive proteins may contain at least one amino acid mutation.

In some embodiments, the ECD herein is the PD-1 ECD. In certain embodiments, the PD-1 ECD sequence has at least one amino acid mutation. In one embodiment, the PD-1 ECD carries a single amino acid mutation, in which alanine (A) at position 132 is substituted with leucine (L). This mutation enhances the binding affinity between the PD-1 ECD and PDL1, and the amino acid sequence of the ECD is shown as SEQ ID NO: 9.

In some embodiments, the ECD of the present application is the TGFβ receptor ECD. In certain embodiments, the TGFβ receptor ECD sequence contains at least one amino acid mutation. The amino acid sequence of the TGFβ receptor ECD is shown as SEQ ID NO: 12.

The co-stimulatory molecules of the present application include any one or a combination of CD28, 4-1BB, ICOS, CD27, IL-12R, CD3 and OX40 proteins. The intracellular domain (ICD) sequences of the above co-stimulatory molecules may have at least one amino acid mutation.

In some embodiments, the ICD of the present application is a 4-1BB ICD. In some embodiments, the 4-1BB ICD sequence contains at least one amino acid mutation. In some embodiments, the amino acid sequence of the 4-1BB ICD is shown as SEQ ID NO: 11.

In some embodiments, the TCR comprises a TCR α chain and a TCR β chain. The variable domain of the TCR α chain comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 7; and/or the variable domain of the TCR β chain comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 8.

In some embodiments, the extracellular domain (ECD) and the ICD are linked by a transmembrane (TM) sequence. The transmembrane domain is selected from the transmembrane domains of the following proteins: TCR α chain, TCR β chain, TCR ζ chain, CD28, CD3ε, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154, or any combination thereof. The transmembrane domains of the

aforementioned proteins may contain at least one amino acid mutation.

In some embodiments, the transmembrane sequence is a CD8 transmembrane sequence or a CD28 transmembrane sequence. In some embodiments, the CD8 transmembrane sequence is shown as SEQ ID NO: 10.

In some embodiments, the chimeric co-stimulatory molecule is PD1(ECD)-CD8(TM)-4-1BB(ICD) (PD1-BB). In some embodiments, the amino acid sequence of PD1-BB is shown as SEQ ID NO: 14. In some embodiments, the chimeric co-stimulatory molecule is TGFβ(ECD)-CD8(TM)-4-1BB(ICD) (TGF-BB). In some embodiments, the amino acid sequence of TGF-BB is shown as SEQ ID NO: 16.

Nucleic Acid Molecules

The nucleic acid molecule of the present application comprises a nucleic acid sequence encoding an HPV TCR molecule; and/or a nucleic acid sequence encoding a chimeric switch receptor.

The present application provides nucleic acid molecules encoding the aforementioned TCR molecules or fragments thereof. Such fragments may be one or more CDRs, variable domains of the α chain and/or β chain, as well as the full-length α chain and/or β chain.

In some embodiments, the nucleic acid encodes one or more structural features that enhance and/or stabilize the association between the expressed TCR α chains and β chains. In certain embodiments, the features are specific amino acids or amino acid sequences. In some embodiments, the nucleic acid may encode one or more non-natural cysteine residues to form one or more disulfide bonds between the TCR α chain and β chain. In further embodiments, the nucleic acid encodes one or more non-natural cysteine residues located in the constant domains of the TCR α chain and β chain.

The nucleotide sequence of the nucleic acid molecule herein may be single-stranded or double-stranded. The nucleic acid molecule can be RNA or DNA, and may or may not contain introns. Preferably, the nucleotide sequence of the nucleic acid molecule contains no introns and is capable of encoding the TCR and/or the chimeric switch receptors of the present application.

The nucleotide sequence may be codon-optimized. Different cell types exhibit distinct codon usage preferences. Codons within the sequence can be modified according to cell types to increase expression levels. Codon usage tables for mammalian cells and many other organisms are well known to those skilled in the art.

In some embodiments, the coding sequence is provided as a single strand. The coding sequence of the TCR β chain is linked to that of the TCR α chain via a P2A coding sequence, which is

further linked to the coding sequence of the co-stimulatory molecule via a T2A coding sequence. All coding nucleotides on this single strand are arranged in the same open reading frame.

It is understood that appropriate restriction sites are commonly designed during gene cloning, which inevitably introduce one or more irrelevant residues at the termini of the expressed amino acid sequences. Such residues do not affect the activity of the target sequence. To construct fusion proteins, boost the expression of recombinant proteins, achieve autonomous secretion of recombinant proteins out of host cells, or facilitate protein purification, additional amino acids are often added to the N-terminus, C-terminus or other suitable regions of a recombinant protein. Examples include, but are not limited to, suitable linker peptides, signal peptides, leader peptides and terminal extensions. Accordingly, the N-terminus or C-terminus of the fusion protein disclosed herein may further contain one or more polypeptide fragments serving as protein tags. Any suitable tags may be adopted herein, such as FLAG, HA, HA1, c-Myc, Poly-His, Poly-Arg, Strep-Tag II, AU1, EE, T7, 4A6, ε, B, gE and Ty1. These tags can be used for protein purification.

In some embodiments, the HPV TCR is encoded by the nucleotide sequence shown as SEQ ID NO: 18.

In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence encoding the HPV TCR molecule and a nucleic acid sequence encoding the chimeric co-stimulatory molecule PD1-BB. In certain embodiments, said nucleic acid molecule comprises the nucleotide sequence shown as SEQ ID NO: 19.

In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence encoding the HPV TCR molecule and a nucleic acid sequence encoding the chimeric co-stimulatory molecule TGF-BB. In certain embodiments, said nucleic acid molecule comprises the nucleotide sequence shown as SEQ ID NO: 20.

Vectors

The present invention also relates to vectors comprising the nucleic acid molecule sequences described herein, as well as one or more regulatory sequences operably linked to these sequences. The nucleic acid molecules of the present invention can be modified in various ways to ensure the expression of the fusion proteins. Techniques for altering polynucleotide sequences using recombinant DNA methods are known in the art.

The regulatory sequence may be a suitable promoter sequence. A promoter sequence is typically operably linked to the coding sequence of the protein to be expressed. A promoter may be any nucleotide sequence that exhibits transcriptional activity in the selected host cell, including mutated, truncated, and chimeric promoters, and may be derived from a gene encoding an

extracellular or intracellular polypeptide that is homologous or heterologous to the host cell. The regulatory sequence may also be a suitable transcription terminator sequence, which is recognized by the host cell to terminate transcription. The terminator sequence is operably linked to the 3' end of the nucleotide sequence encoding the polypeptide. Any terminator that is functional in the selected host cell may be used in the present invention. The regulatory sequence may also be a suitable leader sequence, which is an untranslated region of the mRNA that is important for translation in the host cell. The leader sequence is operably linked to the 5' end of the nucleotide sequence encoding the polypeptide. Any leader sequence that is functional in the selected host cell may be used in the present application.

The nucleic acid molecules of the present application can be cloned into a variety of vectors. For example, they can be cloned into plasmids, phagemids, phage derivatives, animal viruses, and cosmids. Furthermore, the vector is an expression vector. The expression vector can be delivered to a cell in the form of a viral vector. Viral vector techniques are well known in the art and are described, for example, in Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York) and other virology and molecular biology manuals. Viruses that can be used as vectors include, but are not limited to, retroviruses, adenoviruses, adeno-associated viruses (AAVs), herpesviruses, and lentiviruses.

In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter sequence, convenient restriction enzyme sites, and one or more selectable markers (see WO 01/96584 and U.S. Patent No. 6,326,193).

For example, in certain embodiments, a lentiviral vector is adopted in the present invention. The lentiviral vector comprises an origin of replication, a 3' LTR, a 5' LTR, the polynucleotide sequence described herein, and an optional selectable marker.

One example of a suitable promoter is the immediate early cytomegalovirus (CMV) promoter, a strong constitutive promoter capable of driving high-level expression of any polynucleotide sequence operably linked thereto. Another suitable promoter is elongation factor 1α (EF-1α). Other constitutive promoters are also available, including but not limited to the early promoter of simian virus 40 (SV40), mouse mammary tumor virus (MMTV) promoter, human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, avian leukemia virus promoter, Epstein-Barr virus immediate early promoter, Rous sarcoma virus promoter, as well as human gene promoters such as actin promoter, myosin promoter, heme promoter and creatine kinase promoter.

In addition, inducible promoters may also be used. An inducible promoter acts as a molecular switch, which enables the expression of operably linked polynucleotide sequences to be turned on when expression is needed and turned off when expression is undesired. Examples of

inducible promoters include, but are not limited to, metallothionein promoter, glucocorticoid promoter, progesterone promoter and tetracycline promoter.

To assess the expression of target genes, expression vectors introduced into cells may contain either or both selectable marker genes and reporter genes, facilitating the identification and selection of expressing cells from cell populations transfected or infected with viral vectors. Alternatively, the selectable marker may be carried on a separate DNA fragment for co-transfection procedures. Both selectable markers and reporter genes may be flanked by appropriate regulatory sequences to allow their expression in host cells. Useful selectable markers include antibiotic resistance genes such as the neo gene.

Reporter genes are used to identify potential transfected cells and evaluate the functionality of regulatory sequences. After DNA is introduced into recipient cells, the expression of reporter genes is detected at an appropriate time point. Suitable reporter genes include those encoding luciferase, β-galactosidase, chloramphenicol acetyltransferase, secreted alkaline phosphatase or green fluorescent protein. Suitable expression systems are well known in the art, and can be constructed using established techniques or obtained commercially.

Methods for introducing and expressing genes in cells are known in the art. Vectors can be readily introduced into host cells including mammalian, bacterial, yeast or insect cells via conventional approaches. For instance, expression vectors can be delivered into host cells by physical, chemical or biological means.

Physical methods for introducing polynucleotides into host cells include calcium phosphate precipitation, lipofection, particle bombardment, microinjection and electroporation. Biological methods utilize DNA and RNA vectors. Chemical approaches include colloidal dispersion systems such as macromolecular complexes, nanocapsules and microspheres, as well as lipid-based systems including oil-in-water emulsions, micelles, mixed micelles and liposomes.

Viral vectors, particularly lentiviral vectors, are the most widely used biological method for gene delivery into mammalian cells such as human cells. Other viral vectors can be derived from poxviruses, herpes simplex virus type 1, adenoviruses, adeno-associated viruses and the like. A variety of viral-based systems have been developed for gene transfer into mammalian cells. For example, retroviruses serve as a convenient platform for gene delivery systems. Selected genes can be inserted into vectors and packaged into retroviral particles using art-recognized techniques. The recombinant viruses can then be isolated and delivered to target cells *in vivo* or *ex vivo*. Multiple retroviral systems are well documented in the art.

In some embodiments, adenoviral vectors are used, and a wide range of adenoviral vectors are known in the field. In one embodiment, lentiviral vectors are employed.

Immune Cells

The immune cells of the present application are selected from lymphocytes, dendritic cells, monocytes/macrophages, granulocytes and mast cells. In some embodiments, the immune cells are lymphocytes. In some embodiments, the immune cells are NK cells. In some embodiments, the immune cells are B cells. In some embodiments, the immune cells are TILs. In some embodiments, the immune cells are T cells. The T cells may be derived from T cells isolated from a subject, or part of a mixed cell population isolated from a subject, such as peripheral blood lymphocytes (PBLs). For example, the cells can be isolated from peripheral blood mononuclear cells (PBMCs), and can be $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cells. The cells may also exist in a mixed population of $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells. Generally, the cells can be activated with antibodies (e.g., anti-CD3 antibodies) to improve their susceptibility to transfection.

The modified immune cells of the present application refer to immune cells comprising the aforementioned TCR molecules and immune co-stimulatory molecules. In some embodiments, the modified immune cells are prepared by introducing coding sequences encoding the TCR molecules and immune co-stimulatory molecules, or vectors carrying such coding sequences, into isolated immune cells. In some embodiments, the modified immune cells are generated by delivering the aforesaid coding sequences or corresponding vectors into immune cells *in vivo*. In some embodiments, the coding sequences of the TCR molecules and immune co-stimulatory molecules are expressed in tandem within the same open reading frame.

In some embodiments, the modified immune cells comprise HPV TCR and the chimeric co-stimulatory molecule PD1(ECD)-CD8(TM)-4-1BB(ICD) (PD1-BB). In some embodiments, the modified immune cells comprise the amino acid sequence shown as SEQ ID NO: 15.

In some embodiments, the modified immune cells comprise HPV TCR and the chimeric co-stimulatory molecule TGFβ receptor (ECD)-CD8(TM)-4-1BB(ICD) (TGF-BB). In some embodiments, the modified immune cells comprise the amino acid sequence shown as SEQ ID NO: 17.

Compositions

The present application further provides compositions comprising the modified immune cells, nucleic acids or vectors disclosed herein. In some embodiments, the composition is a pharmaceutical composition. In some embodiments, the composition is suitable for research, treatment, prevention and/or diagnosis.

In preferred embodiments, the modified immune cells, nucleic acids or vectors of the present application are formulated into medicaments together with one or more pharmaceutically

acceptable ingredients well known to those skilled in the art. Such ingredients include, but are not limited to, pharmaceutically acceptable carriers, adjuvants, excipients, diluents, fillers, buffers, preservatives, antioxidants, lubricants, stabilizers, solubilizers, surfactants, masking agents, colorants, flavoring agents and sweeteners. As used herein, the term "pharmaceutically acceptable" refers to compounds, ingredients, materials, compositions and dosage forms that, within sound medical judgment, are suitable for contact with the tissues of a subject (e.g., human beings) without excessive toxicity, irritation, allergic reactions or other adverse complications, and present a reasonable benefit-risk profile. Each carrier, adjuvant, excipient and the like must also be compatible with other components of the formulation. Suitable carriers, adjuvants and excipients can be found in standard pharmaceutical textbooks, such as *Remington's Pharmaceutical Sciences* and *Handbook of Pharmaceutical Excipients*.

The pharmaceutical compositions of the present invention may be administered in a manner appropriate for the disease to be treated or prevented. The dosage and administration frequency are determined by factors including the patient's physical condition, as well as the type and severity of the disease.

When referring to an "immunologically effective amount", "anti-tumor effective amount", "tumor-inhibiting effective amount" or "therapeutically effective amount", the exact dosage of the composition to be administered shall be determined by a physician in consideration of the subject's age, body weight, tumor size, extent of infection or metastasis, and individual physical differences. Generally, the pharmaceutical composition comprising the T cells described herein may be administered at a dose of 10^4 to 10^9 cells per kilogram of body weight, preferably 10^5 to 10^7 cells per kilogram of body weight. The T cell composition may also be administered multiple times at the aforementioned dosages. The cells can be administered via standard infusion techniques commonly used in immunotherapy (see, e.g., Rosenberg et al., *New Eng. J. of Med.* 319:1676, 1988). The optimal dosage and treatment regimen for a specific patient can be readily determined by medical practitioners by monitoring disease manifestations and adjusting treatment accordingly.

The composition may be administered to a subject via any convenient route, including spraying, injection, oral administration, infusion, implantation or transplantation. The compositions herein can be administered to patients via subcutaneous, intradermal, intratumoral, intranodal, intraspinal, intramuscular, intravenous or intraperitoneal injection.

Medical Uses

In another aspect, the present application provides the use of the modified immune cells, nucleic acids, vectors or pharmaceutical compositions described herein in the manufacture of a medicament for treating or preventing diseases or disorders.

In some embodiments, the modified immune cells, nucleic acids, vectors or pharmaceutical compositions of the present application are used for the prevention or treatment of diseases associated with HPV infection. Such diseases associated with HPV infection include one or more of cervical cancer, oropharyngeal cancer, head and neck cancer, vaginal cancer, vulvar cancer and penile cancer.

Methods of Treatment and Prevention

Treatment can be performed by isolating T cells from patients suffering from HPV-associated diseases or healthy volunteers, introducing the nucleic acid molecules or vectors of the present application into the above T cells, and subsequently infusing these genetically modified cells back into the patients. Accordingly, the present application provides a method for treating HPV-associated diseases, which comprises administering isolated T cells expressing the TCR of the present application into a patient. Preferably, the T cells are derived from the patient.

In general, the method includes the following steps: (1) isolating T cells from the patient; (2) transducing the T cells with the nucleic acid molecules or vectors of the present application *in vitro*; (3) infusing the genetically modified T cells into the patient. The quantity of cells for isolation, transfection and reinfusion shall be determined by a physician.

In some embodiments of the present invention, the modified immune cells, nucleic acids, vectors or pharmaceutical compositions herein may be used in combination with other therapies known in the art. Such therapies include, but are not limited to, chemotherapy, radiotherapy, immunosuppressants and viral inhibitors. For instance, the treatment can be combined with nucleotide analogs or interferons conventionally used for diseases induced by HPV.

The terms "patient", "subject" and "individual" are used interchangeably herein, referring to a living organism capable of mounting an immune response, such as a mammal. Examples include, but are not limited to, humans, dogs, cats, mice, rats and transgenic species thereof.

The technical solutions of the present application will be described clearly and completely in conjunction with the following specific examples. Apparently, the described examples are merely a part rather than all of the examples of the present application. All other examples obtained by those skilled in the art without creative efforts shall fall within the protection scope of the present application. It should be noted that the order of description of the following examples does not constitute any limitation on the preferred sequence thereof.

Unless otherwise specified, the experimental methods in the following examples are carried out under conventional conditions, such as those described in *Molecular Cloning: A Laboratory Manual* (3rd Edition, 2001) by Sambrook et al., or in accordance with the conditions recommended by the manufacturers. Percentages and parts are calculated by weight unless

otherwise stated.

Unless otherwise specified, all reagents and materials mentioned herein are commercially available or can be prepared by those skilled in the art based on common general knowledge. Any methods and materials similar or equivalent to those described herein can be applied to the present application. The preferred methods and materials presented in the examples are for illustrative purposes only and shall not limit the scope of the present application.

Example 1: Construction of Viral Vector Expressing SCG61 TCR

Through large-scale screening, the present inventors unexpectedly identified a TCR that recognizes the HPV-16 E7 peptide presented in the context of HLA-A*02:01. This TCR is designated SCG61 TCR, which comprises the variable domains of the TCR α chain and TCR β chain. Each of the TCR α chain and β chain contains three complementarity-determining regions (CDRs). Specifically, αCDR1, αCDR2 and αCDR3 are shown as SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3, respectively; and βCDR1, βCDR2 and βCDR3 are shown as SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6, respectively.

A preferred structure of SCG61 TCR is schematically illustrated in Figure 1. The coding sequence of the full-length HPV TCR fragment was synthesized. The synthesized gene was digested with two restriction enzymes and inserted into a lentiviral vector to construct a recombinant plasmid, which was subsequently used for lentivirus packaging.

SCG61 TCR consists of the variable region of HPV E7 TCR β chain (E7 TCR Vβ), the constant region of TCR β chain (TCR Cβ), a 2A self-cleaving peptide, the variable region of HPV E7 TCR α chain (E7 TCR Vα), and the constant region of TCR α chain (TCR Cα)，wherein the sequences of the TCR constant regions were modified to reduce mispairing of the TCR with endogenous TCRs.

Example 2: Preparation of SCG61 TCR-T

Preparation of T Cell Complete Medium: mix CTS™ OpTmizer™ + Supplement (Gibco, A379040-01), 5% CTS™ Immune Cell SR (Gibco, A25961-01), 2% Glutamax (Gibco, A12860-01), and 400 IU/mL recombinant IL-2 for injection (Shandong Quangang Pharmaceutical Co., Ltd., Batch No. 08-102) thoroughly, followed by gentle inversion and stored at 4 ℃ for later use.

Preparation of T Cell Cryopreservation Solution: prepare the solution with 75% CS10 (Thermo Fisher Scientific, A2596101) and 25% HSA (FLEXBUMIN, Batch No. S20181007).

The overall production process is shown in Figure 2.

Day 0: Isolate $CD3^+$ T cells from apheresis products and highly purified. Adjust the cell

concentration to 1 × 10^6 cells/mL using T cell complete medium. Add Transact (CD3/CD28 Microbeads, MACS, Cat. No. 6201000014) into the cell suspension at a ratio of 1:30, and mix gently and thoroughly. After 24 hours of stimulation and culture, perform viral transduction at different MOIs.

Day 1: Count the cells and adjust the T cell density to 5 × 10^5 cells/mL, then add viral supernatant.

Day 2 to Day 11: After viral transduction, monitor cell status daily. Supplement with T cell complete medium in a timely manner to maintain the T cell density at 5 × 10^5 cells/mL to support cell expansion.

Day 12: Harvest cells by centrifugation at 300 g for 5 minutes. Wash the cell pellet with normal saline containing 5% human serum albumin. Resuspend the cells in T cell cryopreservation solution at an appropriate cell density for cryopreservation. Cool the cells using a programmable freezer and store the final product in liquid nitrogen.

Example 3: Detection of SCG61 TCR-T cell expression following transduction at different MOIs

Preparation of Flow Cytometry Buffer: Mix DPBS (Gibco, 14190250) with 2% FBS (Gibco, 10099141), and store the prepared buffer at 4 ℃ until use.

. Wash the SCG61 TCR-T cells and UT cells (untransduced cells, control group) once with flow cytometry buffer and discard the supernatant. Add PE-conjugated HLA-A*02:01 HPV E7 Tetramer (MBL, TB-0031-1) into the forementioned cells, incubate for 60 minutes at 4 ℃ in the dark, then wash and resuspend the cells with flow cytometry buffer. Finally, perform detection on the cells using a Beckman CytoFLEX flow cytometer.

The results showed that when the MOI ranged from 0.3 to 8, the positive rate of SCG61 TCR was between 17% and 96%. No significant upregulation of PD1 expression was observed (Figure 3). It indicates that the expression level of SCG61 TCR is positively correlated with MOI, and the exhaustion phenotype marker PD1 is not upregulated.

Example 4: Detection of Binding Activity of SCG61 TCR

TCR affinity reflects the binding strength between TCR and pMHC, while the effects of other molecules (e.g., TCR co-receptors) during the interaction are taking into account. TCR-positive cells were labeled with mTCR to gate the TCR-positive population. The sorted positive cells were then stained with serially diluted E7 11-19 pMHC tetramer. The binding capacity between SCG61 TCR-positive cells and E7 11-19 peptide-MHC tetramer was detected at different concentrations. The results revealed a distinct positive correlation between SCG61 TCR

binding signal and tetramer concentration (Figure 4).

Example 5: In Vitro Cytotoxicity and Cytokine Secretion of SCG61 TCR Against Target Cells

Real-Time Cell Analysis (RTCA) was applied to evaluate the cytotoxic effect of SCG61 TCR on target cells. Human cervical cancer CaSki cells (HPV-16 E7 positive) were cultured in 96-well RTCA plates as target cells. After approximately 16 hours of incubation, SCG61 TCR-T cells and untransduced (UT) cells were added into the target cells respectively for co-culture. The cells were continuously monitored for 36 hours. The survival curves of target cells were plotted for comparison, and the co-culture supernatants were collected for cytokine measurement.

Preparation of M10 Cell Complete Medium: Mix DMEM (Gibco, 11965-092), 10% FBS (Gibco, 10099141), 1% Sodium Pyruvate (Gibco, 11360070), 1% HEPES (Gibco, 15630080) and 1% NEAA (Gibco, 11140-050) thoroughly by gentle inversion and stored at 4 ℃ for subsequent use.

Preparation of R10 Cell Complete Medium: Mix RPMI 1640 (Gibco, 22400-089) and 10% FBS (Gibco, 10099141) thoroughly by gentle inversion and stored at 4 ℃ for subsequent use.

Preparation of T Cell Culture Medium: Mix CTSTM OpTmizerTM + Supplement (Gibco, A379040-01), 5% CTSTM Immune Cell SR (Gibco, A25961-01) and 2% Glutamax (Gibco, A12860-01) thoroughly by gentle inversion and stored at 4 ℃ for subsequent use.

Cytotoxicity of SCG61 TCR-T Cells Against Tumor Cells (RTCA Assay)

Day 0: Harvest well-grown CaSki cells and resuspend them in R10 complete medium. After trypsinization, adjust the cell density to 4×10^5 cells/mL for use. 50μL of R10 complete medium per well in a collagen-coated 96-well RTCA plate was used to measure the baseline, then add 50 μL of target cell suspension (4×10^5 cells/mL) to each well. Allow the plate to stand for around 5 minutes before placing it on the RTCA system, and continuously record the cell growth curves for approximately 16 hours.

Day 1: Collect SCG61 TCR-T cells and UT cells with confirmed positive rate and cell viability. Calculate the number of effector cells based on the positive rate. Add 50μL of effector cells to each well at effector-to-target (E:T) ratios of 1:1 and 1:3. Place the plate on the RTCA system to continuously monitor cytotoxicity curves. After 24 hours of co-culture, collect the supernatants for cytokine detection.

Cytokine Detection: Equilibrate the Human Th1/Th2 Cytokine Cytometric Bead Array Kit II (BD, 551809) to room temperature.

Redissolve the lyophilized reference standard of cytokine with 2 mL Assay Diluent to obtain a stock standard solution at 5000 pg/mL, and equilibrate the solution at room temperature for 30 minutes.

Preparation of reference standard: Label the stock standard solution as S1. S1 diluted by 2-fold serially to generate standards S2 to S9, and set S10 as the blank control.

Preparation of Human Th1/Th2 Cytokine Capture Beads mixture: Vortex bead suspensions A1 to A6 thoroughly, then mix them in equal volumes.

Add 50 μL of the Capture Beads mixture to each well of a 96-well U-bottom plate.

Add 50 μL of test samples and standard samples (S1–S10) to respective wells.

Add 50 μL of Human Th1/Th2 PE Detection Reagent to each well.

Incubate the plate for 180 minutes at room temperature in the dark.

Add 100 μL of Wash buffer to each well, centrifuge at 300 g for 5 minutes, and discard the supernatant.

Resuspend the pellets in 100 μL of Wash buffer, then perform flow cytometric detection. Analyze the raw data using FCAP Array v3 software.

The results demonstrated that SCG61 TCR-T cells exerted potent cytotoxicity against HPV-16 E7-positive CaSki cells, and the killing rate was positively correlated with the effector-to-target ratio (Figure 5A). After 36 hours of co-culture, nearly 100% of target cells were killed in both the E:T = 1:1 and E:T = 1:3 groups. Meanwhile, the secretion level of IFN-γ was markedly higher than that in the Mock T cell group (Figure 5B).

Example 6: Proliferative Capacity of SCG61 TCR-T Cells upon Multiple Rounds of Stimulation

The proliferative capacity of SCG61 TCR-T cells was evaluated via repeated tumor cell stimulation assays.

CaSki cells (HPV-16 E7 positive) were added as target cells at an effector-to-target ratio of 1:1 into culture dishes containing SCG61 TCR-T cells and UT cells respectively. Cell counting was performed every 2 to 3 days. After 5 to 7 days of stimulation, the cell density of SCG61 TCR-T cells and UT cells was adjusted, followed by the addition of an equal number of tumor cells. The above procedures were repeated for a total of three rounds of stimulation.

Experimental data showed that the proliferation fold of SCG61 TCR-T cells was significantly higher than that of UT cells after each round of tumor cell stimulation (Figure 6). SCG61 TCR-

T cells retained robust and sustained proliferative activity after multiple rounds of tumor stimulation.

Example 7: Recognition Capacity of SCG61 TCR-T Cells Against Similar Target Epitopes

T2 cells were loaded with nonapeptides (HPV E7 11-19, HPV E7 12-20) and decapeptide (HPV E7 11-20), then co-incubated with SCG61 TCR-T cells. The IFN-γ concentration in the supernatant was measured to identify the epitope recognized by SCG61 TCR-T cells, providing evidence and support for non-clinical safety studies.

Preparation of Target Cells: Harvest well-grown T2 cells after several passages. Centrifuge the cell suspension at 500 g for 5 minutes and discard the supernatant. Resuspend the cells with R10 complete medium and perform cell counting. Add 100 μL of T2 cell suspension into each well of a 96-well U-bottom plate at a density of 2×10^4 cells per well. Add 11 μL of the corresponding peptide solution to achieve final concentrations of 1 μM and 100 nM respectively. Place the plate in a 37 °C incubator for 2 hours, then add effector cells.

Preparation of Effector Cells: Gently resuspend SCG61 TCR-T cells and count the cells. Adjust the cell density to 4.0×10^5 cells/mL according to the positive rate. Add 50 μL of the effector cell suspension to each well, mix gently, and incubate the plate at 37 °C. After approximately 24 hours of co-incubation, collect samples for flow cytometric detection of cytokine levels.

The results showed that at two peptide concentrations (1μM and 100nM), SCG61 TCR-T cells secreted high levels of IFN-γ when co-cultured with T2 cells loaded with HPV E7 11-19 nonapeptide, its recognition capacity for HPV E7 11-20 decapeptide was relatively weak, and no cytokine secretion was detected in groups treated with HPV E6 29-38 peptide (negative control) or HPV E7 12-20 peptide (Figure 7). These results demonstrate that the epitope specifically recognized by SCG61 TCR-T cells is the HPV E7 11-19 nonapeptide.

Example 8: Recognition of Polypeptides from Different HPV Subtypes by SCG61 TCR-T Cells

Human papillomavirus (HPV) is a non-enveloped double-stranded circular DNA virus with high host specificity and affinity. This experiment focuses on globally prevalent high-risk HPV subtypes, namely HPV16, HPV31 and HPV52. T2 cells were loaded with HPV16-E7, HPV31-E7 and HPV52-E7 polypeptides at varying concentrations. Cytokine secretion in the co-culture system was detected to explore whether SCG61 TCR-T cells are capable of recognizing different HPV subtypes.

Co-incubation Assay of SCG61 TCR-T Cells and T2 Cells Loaded with HPV-E7 Polypeptides of Different Subtypes

Preparation of R10 Complete Medium: Mix RPMI 1640 (Gibco, 22400-089) with 10% FBS (Gibco, 10099141). Invert the container to mix thoroughly and store the medium at 4 ℃ until use.

Preparation of T Cell Culture Medium: mix CTS™ OpTmizer™ + Supplement (Gibco, A379040-01), 5% CTS™ Immune Cell SR (Gibco, A25961-01) and 2% Glutamax (Gibco, A12860-01) thoroughly by inversion and stored at 4 ℃ for later use.

Preparation of Target Cells: Harvest well-grown T2 cells after several passages. Centrifuge the cell suspension at 500 g for 5 minutes and discard the supernatant. Resuspend the cells with R10 complete medium and perform cell counting. Add 100 μL of T2 cell suspension to each well of a 96-well U-bottom plate at a density of 2×10^4 cells per well. For the negative control group, add 100μL of TCM medium to each well. Add serially diluted solutions of HPV16-E7, HPV31-E7, HPV52-E7 and HPV16-E6 polypeptides to reach final concentrations ranging from 10^{-5} M to 10^{-12} M.

Preparation of Effector Cells: Gently resuspend SCG61 TCR-T cells, then centrifuge at 500 g for 5 minutes and remove the supernatant. Resuspend the cell pellet with T cell culture medium and count the cells. Adjust the density of SCG61 TCR-T positive cells to 4.0×10^5 cells/mL and add 50μL of the cell suspension to each well. Mix gently and place the plate in a 37 ℃ incubator. After 24 hours of co-incubation, collect cell culture supernatants and measure cytokine production via flow cytometry.

The results showed that for T2 cells loaded with HPV16-E7, HPV31-E7 and HPV52-E7 polypeptides at concentrations ranging from 10μM to 1pM, the EC_{50} values of SCG61 TCR-T cells were 2.4×10^{-9} M, 1.8×10^{-8} M and 6.8×10^{-9} M, respectively (Figure 8). It demonstrates that SCG61 TCR-T cells have comparable recognition ability against high-risk HPV16 and HPV52, and exhibit stronger recognition activity towards these two subtypes than HPV31.

Example 9: In Vivo Efficacy of SCG61 TCR-T Cells in Immunodeficient Mice Bearing CaSki Xenograft Tumors

To evaluate the anti-tumor activity of SCG61 TCR-T cells *in vivo*, a total of 25 female NPG immunodeficient mice were used. Each mouse was inoculated with 5×10^6 CaSki cells. Ten days after tumor inoculation, the mice were randomly divided into five groups, wherein three groups received low, medium and high doses of SCG61 TCR-T cells via a single injection in tail vein at doses of 2×10^6, 6×10^6 and 2×10^7 positive T cells per mouse, respectively. The negative control group was administered with 2×10^7 UT cells per mouse via a single injection in tail vein. Meanwhile, mice in the model control group received an equal volume of vehicle via a single injection in tail vein. The animals were observed for 24 days after administration.

The long and short diameters of tumors were measured twice a week to calculate tumor volume. On Day 24, the tumor volumes in the low-, medium- and high-dose SCG61 TCR-T groups were significantly reduced compared with those in the model control group and negative control group. The tumor inhibition rate was positively correlated with the administered dose (Figure 9).

The above results indicate that SCG61 TCR-T cells can markedly inhibit the growth of subcutaneous CaSki xenografts derived from human cervical cancer cells. The high dose of 2×10^7 SCG61 TCR-T cells per mouse exerted the most potent anti-tumor effect.

Example 10: Construction of Viral Vector Expressing SCG61-PD1-BB

The structure of SCG61-PD1-BB is shown in Figure 10. The coding sequence of the full-length HPV TCR-PD1-BB fragment was synthesized . The synthesized gene was digested with two restriction enzymes and inserted into a lentiviral vector to generate a recombinant plasmid, which was subsequently used for lentivirus packaging.

SCG61-PD1-BB consists of the variable region of HPV E7 TCR β chain (E7 TCR Vβ), the constant region of TCR β chain (TCR Cβ), a 2A self-cleaving peptide, the variable region of HPV E7 TCR α chain (E7 TCR Vα), the constant region of TCR α chain (TCR Cα), another 2A self-cleaving peptide, the extracellular domain of PD-1, the transmembrane domain of CD8 and the intracellular domain of 4-1BB.

Example 11: Detection of Specific Expression of SCG61-PD1-BB TCR-T and PD-1 by Flow Cytometry

Preparation of Flow Cytometry Buffer: Prepare the buffer with DPBS (Gibco, 14190250) supplemented with 2% FBS (Gibco, 10099141), and store it at 4 ℃ for later use.

Wash the SCG61-PD1-BB TCR-T cells and Mock T cells (control group) once with flow cytometry buffer and discard the supernatant, then added with PE-labeled HLA-A*02:01 HPV E7 Tetramer (MBL, TB-0031-1), PE-Cy7-conjugated anti-human CD4 antibody (BioLegend, 300512) and PerCP/Cy5.5-conjugated anti-human CD8a antibody (BioLegend, 301032). Incubate the mixture for 60 minutes at 4 ℃ in the dark. After incubation, wash and resuspend the mixture with flow cytometry buffer, and perform detection using a Beckman CytoFLEX flow cytometer.

The results demonstrated that the positive rate of SCG61-PD1-BB TCR exceeded 60%. The proportions of $CD4^+$ and $CD8^+$ T cells were both approximately 50%, with no significant difference compared with the Mock T group (Figure 11). It indicates that SCG61-PD1-BB TCR can be stably expressed, and the newly introduced TCR and PD-1 sequences exert no obvious impact on the percentages of $CD4^+$ and $CD8^+$ T cells.

In addition, the *in vitro* expansion assay showed that SCG61-PD1-BB TCR-T cells achieved more than 100-fold expansion around day 10 (Figure 12). This confirms that transduction with TCR and PD-1 constructs does not impair the proliferation ability or viability of T cells.

Example 12: In Vitro Tumor Cell Killing Activity of SCG61-PD1-BB

Real-Time Cell Analysis (RTCA) was used to evaluate the cytotoxic effect of SCG61-PD1-BB on target cells. CaSki cells (HPV-16 $E7^+$/$PD\text{-}L1^+$), human head and neck squamous cell carcinoma SCC-090 cells (HPV-16 $E7^+$/$PD\text{-}L1^-$) and human hepatocellular carcinoma HepG2 cells (HPV-16 $E7^-$/$PD\text{-}L1^-$) were seeded in 96-well RTCA plates as target cells. After approximately 16 hours of incubation, SCG61-PD1-BB TCR-T cells and Mock T cells were separately added for co-culture. The cells were continuously monitored for 60 hours. The survival curves of target cells were plotted for comparison. Upon completion of the cytotoxicity assay, co-culture supernatants were collected for cytokine detection.

Cytotoxicity of SCG61-PD1-BB against Tumor Cells (RTCA Assay)

Day 0: Harvest well-grown human cervical cancer CaSki-Luci-GFP cells (cultured in R10 complete medium), human head and neck squamous cell carcinoma SCC-090 cells and human hepatocellular carcinoma HepG2 cells (both cultured in M10 complete medium). Digest the cells and adjust the cell density to 4×10^5 cells/mL for later use. 50μL of the corresponding complete medium per well in a collagen-coated 96-well RTCA plate was used to measure the baseline, then add 50μL of target cell suspension at a density of 4×10^5 cells/mL to each well. Allow the plate to stand for about 5 minutes before placing it on the RTCA system, and continuously record cell growth curves for around 16 hours.

Day 1: Collect SCG61-PD1-BB TCR-T cells and Mock T cells with confirmed positive rate and cell viability. Calculate the number of effector cells according to the positive rate, and add 50μL of effector cell suspension to each well at effector-to-target (E:T) ratios of 5:1 and 1:1. Place the plate on the RTCA system for continuous monitoring of cytotoxicity curves.

Day 3: Export the growth and cytotoxicity curves from the RTCA system, then stop the device and collect co-culture supernatants for cytokine detection.

The results showed that SCG61-PD1-BB TCR-T cells exerted potent and specific cytotoxicity against CaSki-Luci-GFP cells (HPV-16 $E7^+$/$PD\text{-}L1^+$) and SCC-090 cells (HPV-16 $E7^+$/$PD\text{-}L1^-$). The killing rate was positively correlated with the effector-to-target ratio (Figure 13A). The half-killing time at an E:T ratio of 5:1 was obviously shorter than that at an E:T ratio of 1:1. After 40 hours of co-culture, nearly 100% of the positive target cells were killed in both the 5:1 and 1:1 E:T groups (Figure 13B). The secretion levels of IFN-γ and TNF were significantly higher than those in the Mock T group (Figure 13C, D).

At the same effector-to-target ratio, the half-killing time for CaSki cells with high PD-L1 expression was markedly shorter than that for PD-L1-negative SCC-090 cells. In addition, the concentration of secreted cytokines was positively correlated with the killing rate. These results indicate that SCG61-PD1-BB TCR-T cells possess stronger anti-tumor activity against HPV-16 $E7^+$ tumor cells with high PD-L1 expression.

Example 13: The Anti-Tumor Effect of SCG61-PD1-BB is Specific to HLA-A*02:01 and HPV

To verify whether SCG61-PD1-BB exhibits different cytotoxicity against tumor cells with distinct expression profiles of HLA-A*02:01 and HPV-16 E7, four target cell lines were prepared for function assays (Figure 14A): HLA-A*02:01^+/HPV-16 $E7^+$/PD-$L1^+$ (CaSki cells), HLA-A*02:01^+/HPV-16 $E7^+$/PD-$L1^-$ (SCC-090 cells), HLA-A*02:01^+/HPV-16 $E7^-$/PD-$L1^-$ (human cervical cancer C-33A cells), and HLA-A*02:01^-/HPV-16 $E7^+$/PD-$L1^-$ (human cervical cancer SiHa cells). SCG61-PD1-BB TCR-T cells and Mock T cells were co-cultured with the four types of target cells. Real-Time Cell Analysis (RTCA) was applied to assess cell cytotoxicity. Upon completion of the cytotoxicity assay, co-culture supernatants were collected for cytokine detection.

Cytotoxicity of SCG61-PD1-BB against Tumor Cells (RTCA Assay)

Day 0: Harvest well-grown CaSki cells (cultured in R10 complete medium), SCC-090 cells, C-33A cells and SiHa cells (the latter three cultured in M10 complete medium). Digest the cells and adjust the cell density to 4×10^5 cells/mL for subsequent use. 50μL of the corresponding complete medium per well in a 96-well RTCA plate was used to measure the baseline, then add 50μL of target cell suspension at a density of 4×10^5 cells/mL to each well. Allow the plate to stand for approximately 5 minutes before placing it on the RTCA system, and continuously record cell growth curves for around 16 hours.

Day 1: Collect SCG61-PD1-BB TCR-T cells and Mock T cells with confirmed positive rate and cell viability. Calculate the number of effector cells based on the positive rate, and add 50 μL of effector cell suspension to each well at an effector-to-target (E:T) ratio of 1:2. Place the plate on the RTCA system to continuously monitor cytotoxicity curves.

Day 3: Export the growth and cytotoxicity curves from the RTCA system, stop the device and collect co-culture supernatants for cytokine detection.

The results showed that at an E:T ratio of 1:2, SCG61-PD1-BB specifically recognized target cells positive for both HLA-A*02:01 and HPV-16 E7, with a cytotoxicity rate of over 70% within 24 hours. Moreover, SCG61-PD1-BB exerted stronger killing activity against HPV-16 $E7^+$/PD-$L1^+$ cells than HPV-16 $E7^+$/PD-$L1^-$ cells (Figure 14B). No obvious cytotoxicity was

observed against target cells with HLA-A*02:01^{+}/HPV-16 E7^{-} or HLA-A*02:01^{-}/HPV-16 E7^{+} phenotypes (Figure 14B). Cytokine detection results were highly consistent with the cytotoxicity data (Figure 14C). It is demonstrated that the anti-tumor activity of SCG61-PD1-BB is strictly dependent on the expression of HLA-A*02:01 and HPV-16 E7.

SCG61-PD1-BB can specifically target and kill tumor cells co-expressing HLA-A*02:01 and HPV-16 E7, accompanied by the secretion of effector cytokines, while it does not recognize or eliminate tumor cells expressing only HLA-A*02:01 or HPV-16 E7 alone. In addition, SCG61-PD1-BB converts the inhibitory signal mediated by PD-1 into an activating stimulatory signal, thereby producing more potent anti-tumor effects against HPV-16 E7-positive cancer cells with high PD-L1 expression.

Example 14: SCG61-PD1-BB Retains Cytotoxicity and Proliferative Capacity after Multiple Rounds of Target Cell Stimulation

Repeated tumor cell stimulation assays were performed to evaluate the resistance of SCG61-PD1-BB to T cell exhaustion during tumor cell killing. Target cells including HPV-16 E7^{+}/PD-L1^{+} CaSki cells and HPV-16 E7^{+}/PD-L1^{-} SCC-090 cells were seeded in RTCA plates. After 24 hours of incubation, SCG61-PD1-BB cells and Mock T cells were separately added at an effector-to-target (E:T) ratio of 2:1. Four days after the initiation of cytotoxicity assays, T cells were harvested and transferred to new RTCA plates pre-seeded with target cells for another round of killing. The above procedures were repeated for a total of four consecutive killing cycles.

Experimental data revealed that SCG61-PD1-BB still exerted potent cytotoxicity against CaSki and SCC-090 tumor cells after four rounds of repeated tumor cell stimulation (Figure 15).

For the proliferation assay, CaSki cells (HPV-16 E7^{+}/PD-L1^{+}) were added to culture dishes containing SCG61-PD1-BB cells and Mock T cells at an E:T ratio of 1:1. Cell counting was conducted every 2 to 3 days. Following 5 to 7 days of stimulation, the cell density of SCG61-PD1-BB and Mock T cells was adjusted, and an equal number of tumor cells were supplemented. The operation was repeated for three rounds of stimulation in total.

The results demonstrated that SCG61-PD1-BB exhibited a significantly higher proliferation fold than Mock T cells after each round of tumor cell stimulation (Figure 16).

SCG61-PD1-BB was engineered to express a PD-1 co-stimulatory fusion receptor (PD-1 auxiliary sequence). This design converts the inhibitory signal triggered by tumor cell surface PD-L1 into an activating signal, so as to facilitate cell proliferation and prevent premature T cell exhaustion. In conclusion, these results confirm that SCG61-PD1-BB can maintain sustained anti-tumor activity and proliferative potential even after repeated tumor stimulation,

which mimics the harsh microenvironment that induces T cell exhaustion.

Example 15: In Vivo Efficacy of SCG61-PD1-BB against Subcutaneous Xenografts of Human Cervical Cancer CaSki Cells in NPG Mice

To evaluate the *in vivo* anti-tumor activity of SCG61-PD1-BB, a total of 30 female NPG immunodeficient mice were used. On Day 7, all mice were inoculated with CaSki cells. When the tumor volume reached 99.9–133.9 mm^3, the mice were randomly divided into five groups, wherein three groups were designated as the low-, medium- and high-dose SCG61-PD1-BB groups, which received a single injection in tail vein of SCG61-PD1-BB at doses of 2×10^6, 6×10^6 and 2×10^7 positive T cells per mouse, respectively. The negative control group was administered with 2×10^7 Mock T cells per mouse via a single injection in tail vein. Meanwhile, mice in the model control group received an equal volume of vehicle via a single injection in tail vein. All animals were observed for 4 weeks after administration.

The long and short diameters of tumors were measured twice a week to calculate tumor volume. On Day 29, the mean tumor volumes of the model control group, negative control group, low-dose, medium-dose and high-dose SCG61-PD1-BB groups were 761±127 mm^3, 674±101 mm^3, 444±80 mm^3, 466±69 mm^3 and 206±119 mm^3, respectively (Figure 17).

From Day 8 to Day 29, the tumor volume in the negative control group was slightly lower than that in the model control group, with no statistically significant difference ($P>0.05$). Compared with the model control group, the low-dose SCG61-PD1-BB group exhibited significantly reduced tumor volume from Day 15 to Day 25 ($P<0.05$). The medium-dose group showed a significant decrease in tumor volume on Day 22 and Day 25 ($P<0.05$). Notably, the high-dose SCG61-PD1-BB group presented markedly smaller tumor volumes throughout Day 8 to Day 29 ($P<0.01$).

The above results demonstrate that SCG61-PD1-BB can effectively inhibit the growth of subcutaneous CaSki xenografts. The high dose of 2×10^7 SCG61-PD1-BB cells per mouse achieved the most prominent anti-tumor effect.

Grouping for Efficacy Study of SCG61-PD1-BB against Subcutaneous CaSki Xenografts in NPG Mice

No.	Group	Test Article	Dosage (Positive T cells/mouse)	Number of Animals
1	Model Control Group	Vehicle	NA	6
2	Negative Control Group	Mock T cells	2×10^7	6
3	SCG61-PD1-BB Low-Dose Group	SCG61-PD1-BB	2×10^6	6
4	SCG61-PD1-BB Medium-Dose Group	SCG61-PD1-BB	6×10^6	6
5	SCG61-PD1-BB High-Dose Group	SCG61-PD1-BB	2×10^7	6

Note: NA stands for Not Applicable.

Example 16: In Vivo Efficacy of SCG61-PD1-BB against Subcutaneous Xenografts of Human Head and Neck Squamous Cell Carcinoma SCC-090 Cells in NCG Mice

To investigate the *in vivo* anti-tumor activity of SCG61-PD1-BB, a total of 30 female NCG immunodeficient mice were used. On Day 14, each mouse was subcutaneously inoculated with 5×10^6 human head and neck squamous cell carcinoma SCC-090 cells on the right flank. When the average tumor volume reached approximately 120 mm^3, the mice were randomly divided into five groups, wherein three groups received intravenous injection of SCG61-PD1-BB at different doses: 2×10^6 cells per mouse, 6×10^6 cells per mouse and 1.5×10^7 cells per mouse. The remaining two groups served as control groups, which were administered with vehicle and Mock T cells respectively. During the experiment, tumor volume and body weight were measured twice a week.

On Day 32 after administration, the mean tumor volumes of the model control group, negative control group, low-dose, medium-dose and high-dose SCG61-PD1-BB groups were 1585±72 mm^3, 555±72 mm^3, 98±34 mm^3, 9±6 mm^3 and 9±8 mm^3, respectively (Figure 18). The mean tumor volumes of the negative control group and all three SCG61-PD1-BB dose groups were significantly lower than those of the vehicle control group ($P<0.01$). In addition, the mean tumor volumes of the low-, medium- and high-dose SCG61-PD1-BB groups were markedly reduced compared with the negative control group ($P<0.01$).

In conclusion, a single intravenous infusion of SCG61-PD1-BB at doses ranging from 0.2×10^7 to 1.5×10^7 cells per mouse exerted prominent tumor growth inhibition against SCC-090 xenografts in tumor-bearing NCG mice.

Grouping for Efficacy Study of SCG61-PD1-BB against Subcutaneous SCC-090 Xenografts in NCG Mice

No.	Group	Test Article	Dosage (Positive T cells/mouse)	Number of Animals
1	Model Control Group	Vehicle	NA	6
2	Negative Control Group	Mock T cells	2×10^7	6
3	SCG61-PD1-BB Low-Dose Group	SCG61-PD1-BB	2×10^6	6
4	SCG61-PD1-BB Medium-Dose Group	SCG61-PD1-BB	6×10^6	6
5	SCG61-PD1-BB High-Dose Group	SCG61-PD1-BB	1.5×10^7	6

Note: NA means Not Applicable.

Example 17: Mismatch Rate between Exogenous TCR and Endogenous TCR in SCG61-PD1-BB

The antibody APC Hamster Anti-mTCRβ (BD, 553174) is used to detect the expression of all TCR chains in the SCG61-PD1-BB cell product, while PE HLA-A*02:01 HPV E7 Tetramer (MBL, TB-0031-1) detects the target-specific TCR in this product. These two reagents were applied for combined detection, and the TCR mismatch rate was calculated accordingly.

Calculation formula for the SCG61-PD1-BB TCR mismatch rate: SCG61-PD1-BB mismatch rate = $[1 - (\text{E7 tetramer}^{+}/\text{mTCR}\beta^{+})] \times 100\%$

The results indicated that TCR expression was positively correlated with the Multiplicity of Infection (MOI), which refers to the ratio of lentiviral vectors to target T cells. Specifically, the TCR positive rate of SCG61-PD1-BB was only 22.1% at an MOI of 0.2, and increased to 79.2% when the MOI was raised to 5 (Figure 19). Meanwhile, the TCR mismatch rate remained below 10% across the MOI range from 0.2 to 5.

In conclusion, the introduced TCR and PD-1 sequences hardly induce TCR mismatches, thus posing no potential safety risks.

Example 18: Identification of Key Amino Acids and Binding Affinity of SCG61-PD1-BB for HPV-16 E7 Epitope Peptide

In this study, alanine scanning mutagenesis was performed to individually mutate each amino acid residue of the HPV-16 E7 epitope peptide to alanine (A). This approach was used to clarify the effects of specific amino acid sites on TCR affinity and bioactivity, and to define the key recognition sites of SCG61-PD1-BB for the target antigen peptide. T2 cells are hybridomas of T and B lymphocytes that are HLA-A*02:01 positive. T2 cells were loaded with mutated peptides and incubated in a 37 °C incubator, followed by the addition of SCG61-PD1-BB cells. After 24 hours, the secretion of IFN-γ in the culture supernatant was measured.

The capacity of SCG61-PD1-BB to secrete cytokines upon specific recognition of T2 cells loaded with HPV-16 E7 epitope peptides at serially diluted concentrations was evaluated to characterize the *in vitro* binding activity of SCG61-PD1-BB against the HPV-16 E7 peptide-MHC complex.

Preparation of R10 Complete Medium: Mix RPMI 1640 (Gibco, 22400-089) with 10% FBS (Gibco, 10099141). Invert the container to mix thoroughly and store the medium at 4 °C for later use.

Preparation of T Cell Culture Medium: Mix CTS™ OpTmizer™ + Supplement (Gibco, A379040-01), 5% CTS™ Immune Cell SR (Gibco, A25961-01) and 2% Glutamax (Gibco, A12860-01) thoroughly by inversion and stored at 4 °C until use.

Peptide Preparation: 2 mg of each peptide in vial was dissolved with 190 μL DMSO to obtain

a stock solution at a final concentration of 10 mM. Pipette 3 μL of the 10 mM peptide solution and dilute it with 297 μL TCM medium to a concentration of 100 μM. Subsequently, perform serial 10-fold dilutions down to 1 nM. Detailed information of all peptides is listed in the table below.

No.	Peptide Name	Peptide Sequence	Purity
1	E7	YMLDLQPET (SEQ ID NO:21)	>98%
2	E7-Y1A	AMLDLQPET (SEQ ID NO:22)	>98%
3	E7-M2A	YALDLQPET (SEQ ID NO:23)	>98%
4	E7-L3A	YMADLQPET (SEQ ID NO:24)	>98%
5	E7-D4A	YMLALQPET (SEQ ID NO:25)	>98%
6	E7-L5A	YMLDAQPET (SEQ ID NO:26)	>98%
7	E7-Q6A	YMLDLAPET (SEQ ID NO:27)	>98%
8	E7-P7A	YMLDLQAET (SEQ ID NO:28)	>98%
9	E7-E8A	YMLDLQPAT (SEQ ID NO:29)	>98%
10	E7-T9A	YMLDLQPEA (SEQ ID NO:30)	>98%

Preparation of Target Cells: Harvest well-grown T2 cells after several passages. Centrifuge the cell suspension at 500 g for 5 minutes and discard the supernatant. Resuspend the cell pellet with R10 complete medium. After cell counting, resuspend 2.4×10^6 T2 cells in 12 mL TCM medium. Add 100 μL of the cell suspension to each well of a 96-well U-bottom plate at a density of 2×10^4 cells per well. Add serially diluted peptide solutions to reach final concentrations ranging from 10^{-5} M to 10^{-12} M. Place the plate in a 37 °C incubator for 2 hours before adding effector cells.

Preparation of Effector Cells: Gently resuspend SCG61-PD1-BB cells. After counting, resuspend 3.6×10^6 cells in 6 mL T cell culture medium to adjust the density of SCG61-PD1-BB positive cells to 4×10^5 cells/mL. Add 50 μL of the effector cell suspension to each well, mix gently, and incubate the plate at 37 °C. Following 24 hours of co-incubation, collect the co-culture supernatants for flow cytometric detection of cytokine secretion.

The results showed that cytokine secretion decreased significantly when SCG61-PD1-BB was incubated with HPV-16 E7 peptides carrying mutations at position 2, 4, 5 and 6 (Figure 20), confirming that the amino acids at these four positions are critical recognition sites. These four key amino acid residues are considered to play an essential role in the direct binding between TCR and HPV-16 E7 epitope peptide, or in maintaining the spatial conformation of the peptide.

T2 cells were loaded with HPV-16 E7 epitope peptides at concentrations from 10^{-5} M to 10^{-12} M and co-cultured with SCG61-PD1-BB. The level of IFN-γ secretion was positively correlated with the concentration of loaded HPV-16 E7 epitope peptide. In contrast, Mock T cells failed to recognize T2 cells loaded with the target peptide. The concentration for 50% of maximal effect (EC_{50}) of SCG61-PD1-BB against HPV-16 E7 epitope peptide was 6.76×10^{-10} M (Figure

21). In conclusion, SCG61-PD1-BB can specifically recognize the key binding sites of HPV-16 E7 epitope peptide and exhibits strong binding activity.

Example 19: In Vivo Expansion of SCG61-PD1-BB Cells in NPG Mice Bearing CaSki Tumors

To characterize the sustained expansion of SCG61-PD1-BB *in vivo*, an NPG immunodeficient mouse model bearing human cervical cancer CaSki xenografts was established. This model was used to simulate the *in vivo* expansion of SCG61-PD1-BB cells following cell infusion after lymphodepleting chemotherapy.

On Day 0, a total of 18 mice were randomly divided into three groups and administered with SCG61-PD1-BB via tail vein injection at doses of 2×10^6, 6×10^6 and 2×10^7 positive T cells per mouse, respectively. Peripheral blood samples were collected from the mice every 7 days thereafter. Viral vector copy number (VCN) was quantified by real-time fluorescent quantitative PCR. The obtained data were used to plot the dynamic changes of SCG61-PD1-BB levels in peripheral blood.

The results revealed obvious expansion of SCG61-PD1-BB cells in mice across all dose groups. On Day 21, the VCN values in peripheral blood of the three groups were 40.35±37.88, 44.53±44.10 and 397.50±65.78 copies/μg gDNA, respectively (Figure 22). Mice receiving the highest dose of 2×10^7 SCG61-PD1-BB cells exhibited a markedly higher VCN than the other two groups.

The above data demonstrate that SCG61-PD1-BB can reconstitute immunity in mice, achieve long-term persistence and continuous expansion *in vivo* which displays a certain degree of dose dependence.

No.	Group	Test Article	Dosage (Positive T cells/mouse)	VCN on Day 21 (copies/μg gDNA)	Number of Animals
1	SCG61-PD1-BB Low-Dose Group	SCG61-PD1-BB	2×10^6	40.35±37.88	6
2	SCG61-PD1-BB Medium-Dose Group	SCG61-PD1-BB	6×10^6	44.53±44.10	6
3	SCG61-PD1-BB High-Dose Group	SCG61-PD1-BB	2×10^7	397.50±65.78	6

Example 20: Tissue Distribution of SCG61-PD1-BB Cells in NPG Mice Bearing CaSki Tumors

Female NPG immunodeficient mice bearing CaSki tumor xenografts were used in this study.

On Day 0, each mouse received a tail vein injection of 2×10^7 SCG61-PD1-BB cells. Whole blood and tissue samples were collected prior to administration, as well as on Day 1, Day 7, Day 14, Day 21 and Day 28 post-infusion. The collected tissues included the heart, liver, spleen, lung, kidney, tumor mass, brain, eyes and optic nerves, ovaries, uterus, urinary bladder, stomach, skeletal muscle and the tail vein injection site. Real-time fluorescent quantitative PCR was performed to investigate the tissue distribution of SCG61-PD1-BB cells.

The results demonstrated that the viral vector copy number (VCN) in all tissues except tumor tissue gradually decreased over time after cell infusion. A relatively high VCN was maintained in tumor tissue, which peaked at 8.22×10^4 copies/μg gDNA on Day 7 and then declined moderately. The VCN in tumor tissue rose again from Day 28 and reached 2.25×10^4 copies/μg gDNA on that day.

SCG61-PD1-BB cells exhibited high exposure levels in tumor tissue, lungs, spleen, whole blood, injection site and liver. The exposure levels ranked from high to low were as follows: tumor tissue, lungs, spleen, tail vein injection site, whole blood, liver, heart, left eye and optic nerve, and uterus, with the corresponding VCN values were 6.98×10^5, 5.04×10^5, 2.87×10^5, 9.84×10^4, 6.78×10^4, 2.26×10^4, 9.32×10^3, 2.81×10^3 and 2.12×10^3 days*(copies/μg gDNA), respectively (Figure 23). The lungs showed high exposure because they are the first organ reached by cells after tail vein injection. As a hematopoietic organ, the spleen supports the engraftment and *in vivo* expansion of infused SCG61-PD1-BB cells, leading to considerable cell accumulation in this tissue. No distribution of SCG61-PD1-BB cells was detected in the urinary bladder, stomach, skeletal muscle, kidneys, brain or ovaries.

In conclusion, after intravenous infusion, SCG61-PD1-BB cells can specifically accumulate in tumor tissue. The lungs and spleen present high cell exposure due to abundant blood perfusion. Other organs show low and transient exposure to SCG61-PD1-BB cells.

Example 21: Construction of Viral Vector Expressing SCG61-TGF-BB

The structural design of SCG61-TGF-BB is illustrated in Figure 24. The coding sequence of the full-length HPV TCR-TGF-BB fragment was chemically synthesized. The obtained gene fragment was digested with two restriction enzymes and inserted into a lentiviral vector to generate a recombinant plasmid, which was subsequently used for lentivirus packaging.

SCG61-TGF-BB consists of the variable region of HPV E7 T cell receptor β chain (E7 TCR Vβ), the constant region of T cell receptor β chain (TCR Cβ), a 2A self-cleaving peptide, the variable region of HPV E7 T cell receptor α chain (E7 TCR Vα), the constant region of T cell receptor α chain (TCR Cα), another 2A self-cleaving peptide, the extracellular domain of TGFβRII, the transmembrane domain of CD8 and the intracellular domain of 4-1BB. Notably,

the sequences of TCR constant regions were engineered to reduce endogenous TCR mismatches.

Example 22: Detection of Specific Expression and Phenotype of SCG61-TGF-BB TCR-T Cells via Flow Cytometry

Preparation of Flow Cytometry Buffer： Flow buffer was prepared with DPBS (Gibco, Cat. No. 14190250) supplemented with 2% FBS (Gibco, Cat. No. 10099141), and stored at 4 °C for later use.

Wash SCG61-TGF-BB TCR-T cells, SCG61 TCR-T cells and Mock T cells (control group) once with flow buffer and discard the supernatant. The following antibodies were added: PE HLA-A*02:01 HPV E7 Tetramer (MBL, Cat. No. TB-0031-1), APC mTCRβ (BD, Cat. No. 553174), APC Anti-human TGF-βRII (Biolegend, Cat. No. 399706), and BV421 Mouse Anti-human CD8a (BD, Cat. No. 743064). Samples were incubated at 4 °C in the dark for 60 min, washed with flow buffer, resuspended, and analyzed using a Beckman CytoFLEX flow cytometer.

The results demonstrated that the E7 Tetramer positive rates of SCG61 TCR-T and SCG61-TGF-BB TCR-T cells both exceeded 90%, and TGF-βRII was specifically expressed in SCG61-TGF-BB TCR-T cells. No significant difference was observed in the proportion of CD8+ T cells between the two groups (Figure 25). These data indicate that both SCG61 and SCG61-TGF-BB can be stably expressed, and the newly introduced TCR sequence and 41-BB sequence exert no obvious impact on the percentage of CD8+ T cells.

Washed SCG61-TGF-BB TCR-T cells and Mock T cells (control group) once with flow buffer, and removed the supernatant. Then added antibodies listed in the table below. After incubation at 4 °C in the dark for 60 min, cells were washed and resuspended with flow buffer, followed by detection on a Beckman CytoFLEX flow cytometer.

Reagent Name	**Manufacturer**	**Catalog Number**
PE HLA-A*02:01 HPV E7 Tetramer	MBL	TB-0031-1
APC mTCR β	BD	553174
APC Anti-human TGF- β RII	Biolegend	399706
BV421 Mouse Anti-human CD8a	BD	743064
BV510 Mouse Anti-human CD45	BD	563204
BB700 Rat Anti-human CCR7	BD	566437
PE-Cy7 Anti-human CD223 (LAG-3)	Biolegend	369310
BV421 Mouse Anti-human CD279 (PD-1)	BD	562516
FITC Mouse Anti-human CD4	BD	566911
APC Anti-human CD25	Biolegend	302610

BV421 Mouse Anti-human CD127	BD	562436

The results showed that SCG61-TGF-BB TCR-T cells retained a high proportion of CD8+ T cells, abundant memory T cell subsets (stem cell memory T cells (Tscm) and central memory T cells (Tcm)), as well as low expression of PD-1 and a low percentage of Treg cells (Figure 26).

Example 23: Function Evaluation of SCG61-TGF-BB Against Different HPV Subtypes

Human papillomavirus (HPV) is a group of non-enveloped double-stranded circular DNA viruses with high host specificity and tropism. This study focuses on two high-risk HPV subtypes with the highest global infection prevalence, HPV16 and HPV52. Their corresponding HPV-E7 proteins serve as the major oncogenic proteins, and their sequences are shown in Figure 27.

In this experiment, T2 cells were loaded with different concentrations of HPV16-E7 and HPV52-E7 peptides to detect cytokine secretion upon co-culture. In addition, HepG2 cell lines stably expressing HPV16-E7 or HPV52-E7 were constructed to investigate whether SCG61-TGF-BB can recognize HPV16-E7 and HPV52-E7 epitopes endogenously processed and presented by target cells, so as to evaluate its function activity against distinct HPV subtypes.

Co-incubation Assay of SCG61-TGF-BB and T2 Cells Loaded with Different HPV-E7 Subtype Peptides

Preparation of R10 Complete Medium：RPMI 1640 (Gibco, Cat. No. 22400-089) supplemented with 10% FBS (Gibco, Cat. No. 10099141). The medium was mixed by gentle inversion and stored at 4 °C until use.

Preparation of T Cell Culture Medium： CTS™ OpTmizer™ Base Medium + Supplement (Gibco, Cat. No. A379040-01) supplemented with 5% CTS™ Immune Cell SR (Gibco, Cat. No. A25961-01) and 2% GlutaMAX (Gibco, Cat. No. A12860-01). The medium was mixed by gentle inversion and stored at 4 °C until use.

Preparation of Target Cells： Well-grown T2 cells after multiple passages were harvested and centrifuged at 500 g for 5 min, followed by supernatant removal. Cells were resuspended in R10 complete medium and counted. T2 cells were seeded into U-bottom 96-well plates at 100 μL per well with a density of 2×10^4 cells per well. Wells assigned to the negative control group received 100 μL T cell medium (TCM). Serial dilutions of HPV16-E7 or HPV52-E7 peptide solutions were added to achieve final concentrations ranging from 10^{-5} M to 10^{-12} M.

Preparation of Effector Cells： SCG61-TGF-BB and Mock T cells were gently resuspended and centrifuged at 500 g for 5 min, after which the supernatant was discarded. Cells were resuspended in T cell culture medium and adjust the density of SCG61-TGF-BB positive cells to 4.0×10^5 cells/mL after counting. Effector cells were added at 50 μL per well, mixed

thoroughly, and the plates were incubated at 37 °C. After 24 h of co-culture, cell supernatants were collected to quantify cytokine production via flow cytometry.

Real-Time Cell Analysis (RTCA) of SCG61-TGF-BB-Mediated Cytotoxicity Against HepG2 Cells Expressing Different HPV-E7 Subtypes

Preparation of M10 Complete Medium：DMEM (Gibco, Cat. No. 11965-092) supplemented with 10% FBS (Gibco, Cat. No. 10099141), 1% Sodium Pyruvate (Gibco, Cat. No. 11360070), 1% HEPES (Gibco, Cat. No. 15630080), and 1% NEAA (Gibco, Cat. No. 11140-050). The medium was mixed by gentle inversion and stored at 4 °C until use.

Preparation of T Cell Culture Medium：CTS™ OpTmizer™ Base Medium + Supplement (Gibco, Cat. No. A379040-01) supplemented with 5% CTS™ Immune Cell SR (Gibco, Cat. No. A25961-01) and 2% GlutaMAX (Gibco, Cat. No. A12860-01). The medium was mixed by gentle inversion and stored at 4 °C until use.

Target Cell Seeding (Day 0)：Pre-coated RTCA 96-well plates were filled with 50μL M10 complete medium and incubated at 37 °C for baseline signal measurement. Target cells including wild-type HepG2, HepG2-HPV16-E7-LG and HepG2-HPV52-E7-LG were digested, centrifuged to remove supernatant, and resuspended in M10 complete medium. After counting, target cells were seeded into RTCA plates at 4×10^4 cells per well with a volume of 50 μL per well. Blank control wells received 50μL T cell medium. Plates were shaken gently, incubated at 37 °C, and loaded onto the RTCA instrument for recording.

Addition of Effector Cells：SCG61-TGF-BB and Mock T cells were thawed on Day 0, mixed evenly, and cultured at 37 °C. On Day 1, the cells were resuspended, counted, and added with target cells at effector-to-target (E:T) ratios (according to the positive cell proportion) of 2:1, 1:2 and 1:8 in a volume of 50 μL per well. Plates were incubated at 37 °C and loaded onto the RTCA platform to initiate the assay. On Day 2, RTCA plates were taken out, and 20μL culture supernatants were carefully harvested from each well after 24 h incubation for cytokine detection. The plates were returned to the 37 °C incubator to continue monitoring. The experiment was terminated between Day 3 and Day 4 according to the degree of target cell lysis, and the RTCA plates were collected.

The results demonstrated that SCG61-TGF-BB exhibited comparable affinity for T2 cells loaded with serially diluted HPV16-E7 and HPV52-E7 peptides ranging from 10μM to 1pM, with respective EC_{50} values of 8.39×10^{-9} and 8.82×10^{-9} (Figure 27). Furthermore, SCG61-TGF-BB completely eliminated HPV16-E7- and HPV52-E7-positive target cells at effector-to-target (E:T) ratios of 2:1 and 1:2, achieving over 90% target cell lysis within 72 hours. At an E:T ratio of 1:8, the lysis rate of HPV16-E7-positive target cells remained above 60%, whereas

SCG61-TGF-BB exerted no cytotoxicity against negative control target cells (Figure 28). Measurements of the cytokines IFN-γ and TNF were highly consistent with the cytotoxicity data (Figure 29). Mock T cells showed no function activity against target cells.

In summary, SCG61-TGF-BB exerted potent anti-tumor activity against high-risk HPV16 and HPV52 epitopes, whether presented as exogenous peptides loaded onto T2 cells or endogenously expressed epitopes in cell lines. Accordingly, incorporating patients carrying HPV genotypes 16 and 52 into the enrollment criteria may benefit a broader patient population.

Example 24: Mechanism Verification of SCG61-TGF-BB for Inhibiting the pSMAD2/3 Signaling Pathway

The tumor microenvironment harbors multiple immunosuppressive mechanisms that can impair the therapeutic efficacy of adoptively transferred T cells. One prominent mechanism is mediated by transforming growth factor-β (TGF-β), a cytokine secreted by tumor cells and infiltrating immunosuppressive immune cells that directly suppresses the activity of effector T cells. Effector T cells express TGF-β receptor I (TGFBRI) and TGF-β receptor II (TGFBRII). Upon exposure to TGF-β, heterodimerization of these receptors is triggered, followed by phosphorylation of SMAD2 and SMAD3—the primary downstream mediators of TGF-β signaling. Phosphorylated SMAD proteins initiate an inhibitory transcriptional program, ultimately resulting in diminished cytokine production, attenuated cytotoxicity, and defective proliferation upon antigen stimulation. In this experiment, intracellular phosphorylation levels of SMAD2/3 were measured after TGF-β incubation to verify the inhibitory effect of SCG61-TGF-BB on the TGF-β signaling cascade.

Preparation of T Cell Culture Medium：CTS™ OpTmizer™ Base Medium with Supplement (Gibco, Cat. No. A379040-01) supplemented with 5% CTS™ Immune Cell Serum Replacement (SR, Gibco, Cat. No. A25961-01) and 2% GlutaMAX Supplement (Gibco, Cat. No. A12860-01). Mix thoroughly by gentle inversion and stored at 4 °C until use.

Preparation of Flow Cytometry Buffer：Dulbecco's Phosphate-Buffered Saline (DPBS, Gibco, Cat. No. 14190250) supplemented with 2% fetal bovine serum (FBS, Gibco, Cat. No. 10099141). Stored at 4 °C for standby application.

Preparation of TCR-T Cells：Suspend SCG61-TGF-BB and Mock T cells by gentle pipetting, then centrifuge at 500 g for 5 min and discard the supernatant. Resuspend cell pellets in T cell culture medium and adjust the cell density to 1.0×10^6 cells/mL after counting. Two replicate groups were set up, with 1 mL cell suspension added per well in a 24-well plate, Wherein one group was treated with 10 ng/mL TGF-β, and the other served as an untreated control. After thorough mixing, plates were placed in a 37 °C incubator. Cells were harvested after 1 h of co-

incubation for subsequent flow cytometric analysis.

Flow Cytometry Staining Protocol：Wash SCG61 TCR-T and SCG61-TGF-BB TCR-T cells once with flow cytometry buffer and discard the supernatant, then added with PE Hamster Anti-mTCRβ (BD, Cat. No. 553172), BV421 Mouse Anti-hCD8 (BD, Cat. No. 743064), and Ghost Dye Red 710 (TONBO, Cat. No. 13-0871-T100). Incubate samples at 4 °C in the dark for 60 min, followed by washing and centrifugation with flow cytometry buffer. Pre-warmed BD PhosFlow Fix Buffer I (BD, Cat. No. 557870) to 37 °C was added to cell pellets, fix cells at 37 °C under light protection for 15 min, then wash the cells with flow cytometry buffer and centrifuge again.

Add pre-chilled BD PhosFlow Perm Buffer III (BD, Cat. No. 558050) into the cells of previous step and permeabilized at 4 °C in the dark for 60 min, then wash and centrifuge.

Add AF647 Mouse Anti-pSmad2/3 antibody (BD, Cat. No. 562696) into the cells of previous step and incubated at 4 °C protected from light for 60 min. After final washing and resuspension in flow cytometry buffer, samples were analyzed on a Beckman CytoFLEX flow cytometer.

The results demonstrated that compared with SCG61 TCR-T cells, nearly no upregulation of SMAD2/3 phosphorylation was detected in SCG61-TGF-BB positive cells. Moreover, the proportions of cells with activated versus non-activated pSMAD2/3 signaling were comparable across $CD4^+$ and $CD8^+$ T cell subsets (Figure 30). These data demonstrate that SCG61-TGF-BB protects T cells from TGF-β-mediated suppression by blocking TGF-β-induced phosphorylation of SMAD2/3.

Example 25: Function Validation of SCG61-TGF-BB against Peptide-Loaded T2 Cells under TGF-β1 Stimulation

In this assay, T2 cells loaded with different concentrations of HPV-16 E7 epitope peptides were divided into two experimental groups, wherein one group was treated with 10 ng/mL TGF-β1. After 2 hours of incubation, effector cells were added, followed by 24-hour co-culture at 37 °C. Culture supernatants were harvested to measure secreted IFN-γ cytokines, so as to evaluate the inhibitory effect of TGF-β1 on T cell cytotoxicity.

Preparation of R10 Complete Medium: RPMI 1640 medium (Gibco, Cat. No. 22400-089) supplemented with 10% fetal bovine serum (FBS, Gibco, Cat. No. 10099141). Mixed thoroughly by gentle inversion and stored at 4 °C for later use.

Preparation of T Cell Culture Medium: CTS™ OpTmizer™ Medium with Supplement (Gibco, Cat. No. A379040-01), supplemented with 5% CTS™ Immune Cell Serum Replacement (SR, Gibco, Cat. No. A25961-01) and 2% GlutaMAX Supplement (Gibco, Cat. No. A12860-01). Mixed thoroughly by inversion and stored at 4 °C until use.

Target Cell Preparation: Well-grown T2 cells after several passages were collected and centrifuged at 500 g for 5 min, then the supernatant was discarded. Cell pellets were resuspended in R10 complete medium for counting. A total of 2.4×10^6 T2 cells were resuspended in 12 mL T cell culture medium (TCM). The cell suspension was added into U-bottom 96-well plates at 100μL per well, corresponding to 2×10^4 T2 cells per well. Peptide stock solutions were added in a dose-gradient manner to reach final concentrations of 100nM and 10nM. The plates were split into two groups: one treated with TGF-β1 and the other set as blank control. The U-bottom 96-well plates were incubated at 37 °C for 2 hours prior to the addition of effector cells.

Effector Cell Preparation: SCG61 TCR-T and SCG61-TGF-BB TCR-T cells were suspended homogeneously and counted. A total of 3.6×10^6 effector cells were resuspended in 6 mL T cell medium to adjust the density of SCG61-TGF-BB positive cells to 4×10^5 cells/mL. Effector cell suspension was dispensed at 50μL per well. After mixing, the plate was incubated at 37 °C. Following 24 hours of co-culture, supernatants were collected for flow cytometric detection of cytokine secretion.

The results demonstrated that in the absence of TGF-β1, SCG61 TCR-T and SCG61-TGF-BB TCR-T exhibited equivalent cytotoxic activity against T2 cells loaded with 100nM and 10nM HPV-16 E7 epitope peptides. Upon TGF-β1 treatment, the effector function of SCG61 TCR-T cells was markedly suppressed. In contrast, SCG61-TGF-BB cells displayed elevated cytokine secretion compared with the TGF-β1-free group (Figure 31).

T cells were genetically modified via lentiviral vector in the SCG61-TGF-BB construct. The lentiviral vector encodes two components: a TCR that specifically recognizes HPV E7 peptides presented by HLA-A*02:01 on tumor cells, and a chimeric switch receptor consisting of the extracellular domain of TGF-β receptor II fused to the intracellular signaling domain of 4-1BB. This engineered receptor converts inhibitory signal into a co-stimulatory signal after binding of the immunosuppressive cytokine TGF-β. Under high-TGF-β conditions mimicking the tumor microenvironment, SCG61-TGF-BB cells were not suppressed; instead, they exerted enhanced anti-tumor activity.

Example 26: In Vitro Tumor Cell Killing Capacity of SCG61-TGF-BB under TGF-β1 Treatment

Real-Time Cell Analysis (RTCA) was applied in this experiment to evaluate the tumor-killing efficacy of SCG61-TGF-BB against target cells in the presence of TGF-β1. CaSki cells were seeded onto 96-well RTCA plates as target cells and assigned into two groups, wherein one group was supplemented with 10 ng/mL TGF-β1. After approximately 16 hours of incubation, effector cells were added for co-culture. The co-culture system was continuously monitored for

around 48 hours, and real-time survival curves of target cells were plotted for comparative analysis. Upon completion of the killing assay, co-culture supernatants were harvested for cytokine quantification.

Preparation of R10 Complete Medium: RPMI 1640 medium (Gibco, Cat. No. 22400-089) supplemented with 10% fetal bovine serum (FBS, Gibco, Cat. No. 10099141). Mixed thoroughly by gentle inversion and stored at 4 °C for later use.

Preparation of T Cell Culture Medium: CTS™ OpTmizer™ Medium with Supplement (Gibco, Cat. No. A379040-01), supplemented with 5% CTS™ Immune Cell Serum Replacement (SR, Gibco, Cat. No. A25961-01) and 2% GlutaMAX Supplement (Gibco, Cat. No. A12860-01). Mixed thoroughly by gentle inversion and stored at 4 °C until use.

Day 0: Well-grown CaSki cells cultured in R10 complete medium were digested and adjusted to a cell density of 4×10^5 cells/mL for standby use. 50μL of the corresponding complete medium per well in a 96-well RTCA plate was used to measure the baseline. Subsequently, 50 μL of target cell suspension (4×10^5 cells/mL) was added to each well. Allow the plate to stand for approximately 5 minutes before placing it on the RTCA system, and continuously record cell growth curves for around 16 hours.

Day 1: Collect SCG61 TCR-T, SCG61-TGF-BB TCR-T and Mock T cells with confirmed positive rate and cell viability. Calculate the number of effector cells based on the positive rate, and add 50 μL of effector cell suspension to each well at an effector-to-target (E:T) ratio of 2.5:1 and 1:2. Place the plate on the RTCA system to continuously monitor cytotoxicity curves.

Day 2: Supernatants from 24-hour co-culture were collected to detect secreted cytokines.

Day 3: Stop the RTCA instrument and export recorded cell growth and cytotoxicity curves for analysis.

The results demonstrated that after 48 hours of co-culture under 10 ng/mL TGF-β1 stimulation, SCG61-TGF-BB exhibited prominent specific cytotoxicity against tumor cells, and the killing efficiency was positively correlated with the E:T ratio. In contrast, SCG61 TCR-T only showed obvious tumor inhibitory effects at the higher E:T ratio (2.5:1). Moreover, cytokine secretion from SCG61-TGF-BB groups was significantly higher than that from SCG61 groups at both tested E:T ratios (Figure 32). These results demonstrate that SCG61-TGF-BB confers superior anti-tumor activity under TGF-β1-rich conditions.

Example 27: The Tumor-Killing Capacity of SCG61-TGF-BB against Target Cells upon Multiple Rounds of Stimulation under TGF-β1 Treatment

This experiment recapitulates TGF-β1-mediated immune suppression within the tumor

microenvironment. Serial rounds of tumor cell stimulation were performed to mimic in vivo T cell exhaustion, and the tumor-killing capacity of SCG61 TCR-T and SCG61-TGF-BB TCR-T was compared to characterize the function of the TGF-BB auxiliary sequence.

Preparation of R10 Complete Medium：RPMI 1640 medium (Gibco, Cat. No. 22400-089) supplemented with 10% fetal bovine serum (FBS, Gibco, Cat. No. 10099141). Mixed thoroughly by gentle inversion and stored at 4 °C for subsequent use.

Preparation of T Cell Culture Medium：CTS™ OpTmizer™ Base Medium with Supplement (Gibco, Cat. No. A379040-01), supplemented with 5% CTS™ Immune Cell Serum Replacement (SR, Gibco, Cat. No. A25961-01) and 2% GlutaMAX Supplement (Gibco, Cat. No. A12860-01). Mixed thoroughly by gentle inversion and stored at 4 °C until use.

Day 0： Well-grown CaSki cells cultured in R10 complete medium were digested and resuspended to a density of 4×10^5 cells/mL for use. 50μL of the corresponding complete medium per well in a 96-well RTCA plate was used to measure the baseline. Subsequently, 50 μL of target cell suspension (4×10^5 cells/mL) was added to each well. Allow the plate to stand for approximately 5 minutes before placing it on the RTCA system, and continuously record cell growth curves for around 16 hours.

Day 1： Collect SCG61 TCR-T, SCG61-TGF-BB TCR-T and Mock T cells with confirmed positive rate and cell viability. Calculate the number of effector cells based on the positive rate, and add 50 μL of effector cell suspension to each well at an effector-to-target (E:T) ratio of 1:2. Place the plate on the RTCA system to continuously monitor cytotoxicity curves.

Day 3：A second RTCA plate was seeded with target cells following the identical procedure described on Day 0, then incubated at 37 °C for roughly 16 hours.

Day 4：The first RTCA plate was removed from the device, and effector cells were transferred to corresponding wells of the second RTCA plate, which was placed on the device for the detection of continuous cytotoxicity curves.

Day 7：Stop the RTCA instrument and export cytotoxicity curves for analysis. Supernatants harvested after 24-hour co-culture were subjected to cytokine quantification.

The results demonstrated that under treatment with 10 ng/mL TGF-β1, SCG61-TGF-BB exerted robust specific cytotoxicity against tumor cells upon the first stimulation, while SCG61 TCR-T merely produced notable tumor growth inhibition. During the second round of stimulation, the half-maximal killing time of SCG61-TGF-BB against tumor cells was markedly shortened, whereas the tumoricidal activity of SCG61 TCR-T was substantially

attenuated.

Across multiple rounds of tumor stimulation, SCG61-TGF-BB secreted significantly higher levels of cytokines than the SCG61 group both in the presence and absence of TGF-β1 (Figure 33), demonstrating superior anti-tumor potency of SCG61-TGF-BB under TGF-β1-rich conditions.

T cells were genetically modified via a lentiviral vector encoding two components: a TCR that recognizes HPV16 and HPV52 E7 epitope (residues 11–19) presented by HLA-A*02:01, and a chimeric switch receptor consisting of the extracellular domain of TGF-β receptor II (TGFBRII) fused to the intracellular signaling domain of 4-1BB. This fusion receptor converts inhibitory signal into a co-stimulatory signal after binding of the immunosuppressive cytokine TGF-β, thereby inhibiting T cell apoptosis, alleviating T cell exhaustion, and enhancing persistent anti-tumor function.

Collectively, these data confirmed that SCG61-TGF-BB sustains durable anti-tumor activity after multiple rounds of tumor stimulation that simulates the T cell exhaustion microenvironment.

Example 28: Proliferation Assay of SCG61-TGF-BB after Multiple Rounds of Stimulation under TGF-β1 Treatment

The proliferative expansion capacity of SCG61-TGF-BB upon repeated tumor cell stimulation was characterized in this study. SCG61 TCR-T, SCG61-TGF-BB TCR-T and Mock T cells were counted, resuspended in IL-2-free medium to a density of 1×10^6 cells/mL, and seeded into 24-well plates at 1 mL per well. Cells were treated with 10 ng/mL TGF-β1, followed by addition of HepG2-E7 target cells at an effector-to-target (E:T) ratio of 1:1.

After 5 days of primary stimulation, effector cells were counted and restimulated with an equivalent amount of target cells. Cell counting was performed every 2–3 days throughout the experiment to calculate the fold expansion of each group.

Experimental data revealed that SCG61-TGF-BB exhibited significantly higher proliferation fold than SCG61 TCR-T and Mock T cells after each round of tumor cell stimulation (Figure 34).

Example 29: Cross-Reactivity Assessment of SCG61-TGF-BB in Human Peptide Library

The human TCR library exhibits inherent cross-reactivity; merely ~10^8 unique TCRs are capable of recognizing over 10^{15} potential peptide antigens. In clinical trials, TCR cross-reactivity may trigger off-target recognition of healthy human tissues and induce severe toxicities. The critical amino acid residues at positions 2/4/5/6 of the HPV16 E711-19 epitope

peptide targeted by SCG61-TGF-BB have been identified.

Bioinformatic algorithm prediction and BLAST sequence alignment in a human peptide library identified a total of 18 peptide sequences sharing six consistent amino acid residues with the E7 epitope (five identical residues plus one conserved residue). Expitope 2.0 software was utilized to analyze the four key fixed motifs spanning residues 2/4/5/6: two motifs harbored four mismatched amino acid residues, while fourteen motifs contained five mismatched residues.

In this experiment, T2 cells loaded with the aforementioned peptides were co-cultured with SCG61-TGF-BB cells. The concentration of IFN-γ in culture supernatants was quantified to investigate potential cross-reactivity between SCG61-TGF-BB and these human-derived peptides, evaluate the risk of off-target effects, and provide supporting evidence for non-clinical safety evaluation.

Preparation of R10 Complete Medium: RPMI 1640 medium (Gibco, Cat. No. 22400-089) supplemented with 10% fetal bovine serum (FBS, Gibco, Cat. No. 10099141). Mixed thoroughly by gentle inversion and stored at 4 °C for standby use.

Preparation of T Cell Culture Medium: CTS™ OpTmizer™ Medium with Supplement (Gibco, Cat. No. A379040-01), supplemented with 5% CTS™ Immune Cell Serum Replacement (SR, Gibco, Cat. No. A25961-01) and 2% GlutaMAX Supplement (Gibco, Cat. No. A12860-01). Mixed thoroughly by gentle inversion and stored at 4 °C until use.

Peptide Preparation: Each of the 36 peptides numbered 1 to 36 (2 mg in one vail) was dissolved in 150 μL to 200 μL DMSO to prepare stock solutions at a final concentration of 10 mM. Detailed information is listed in the table below.

No.	Peptide Name	Peptide Sequence	Purity
1	E7-11-19	YMLDLQPET(SEQ ID NO:31)	>98%
2	E7-SH3GLB1	YMLDLQKQL(SEQ ID NO:32)	>98%
3	E7-SBK3	GLLDLDPET(SEQ ID NO:33)	>98%
4	E7-ZNF236	AMLDLEPQH(SEQ ID NO:34)	>98%
5	E7-GLIS1	SGLGLQPET(SEQ ID NO:35)	>98%
6	E7-TNS1	LMLDLEPAS(SEQ ID NO:36)	>98%
7	E7-SNAP91	DLLDLQPDF(SEQ ID NO:37)	>98%
8	E7-GCN1	MGLDLQPDL(SEQ ID NO:38)	>98%
9	E7-LPIN3	AGADLQPDT(SEQ ID NO:39)	>98%
10	E7-GHDCI3	LGLNLQPEQ(SEQ ID NO:40)	>98%
11	E7-GHDCI1	ELLNLQPEQ(SEQ ID NO:41)	>98%
12	E7-PCDHA9	LSYELQPET(SEQ ID NO:42)	>98%
13	E7-ITGA2B	YILDIQPQG(SEQ ID NO:43)	>98%
14	E7-TRIM66	PVSDMQPET(SEQ ID NO:44)	>98%
15	E7-L1CAM	TQWDLQPDT(SEQ ID NO:45)	>98%
16	E7-PTPRS	VITNLQPET(SEQ ID NO:46)	>98%
17	E7-COL12A1	MEINLQPET(SEQ ID NO:47)	>98%
18	E7-SACS	NRLDLQPDL(SEQ ID NO:48)	>98%

19	E7-AHNAK2	ISGDLQPDT(SEQ ID NO:49)	>98%
20	E7-ITSN2	YMADLQLVV(SEQ ID NO:50)	>98%
21	E7-ERC2	TMLDLQAQL(SEQ ID NO:51)	>98%
22	E7-ACTN1	AMEDLQDTF(SEQ ID NO:52)	>98%
23	E7-GOLGA3	AMTDLQNML(SEQ ID NO:53)	>98%
24	E7-ACTN2	AMEDLQDMF(SEQ ID NO:54)	>98%
25	E7-UTP14A	QMIDLQNLL(SEQ ID NO:55)	>98%
26	E7-KAT2B	FMADLQRVF(SEQ ID NO:56)	>98%
27	E7-PCNT	EMEDLQNQF(SEQ ID NO:57)	>98%
28	E7-ARHGEF17	GMEDLQAPL(SEQ ID NO:58)	>98%
29	E7-CD68	FMQDLQQKV(SEQ ID NO:59)	>98%
30	E7-ITIH2	QMDDLQDFL(SEQ ID NO:60)	>98%
31	E7-FAM71B	SMGDLQRQL(SEQ ID NO:61)	>98%
32	E7-SLC9A3	LMGDLQIGL(SEQ ID NO:62)	>98%
33	E7-DAB2IP	SMVDLQDAR(SEQ ID NO:63)	>98%
34	E7-DHRSX	NMDDLQSSA(SEQ ID NO:64)	>98%
35	E7-ICA1	NMKDLQASL(SEQ ID NO:65)	>98%
36	S20-28	FLLTRILTI(SEQ ID NO:66)	>98%

Target Cell Preparation: Well-grown T2 cells after multiple passages were harvested and centrifuged at 500 g for 5 min, followed by supernatant removal. Cell pellets were resuspended in R10 complete medium and counted. The cell suspension was dispensed into U-bottom 96-well plates at 100μL per well, corresponding to 2×10^4 T2 cells per well. Then 11μL of peptide stock solutions numbered 1 to 36 were added separately to achieve final peptide concentrations of 1μM and 100nM, corresponding to stock concentrations of 10μM and 1μM respectively. The U-bottom 96-well plate was incubated at 37 °C for 2 hours prior to the addition of effector cells.

Effector Cell Preparation: SCG61-TGF-BB cell suspension was homogenized by gentle pipetting and counted. Cell density was adjusted to 4.0×10^5 cells/mL according to the positive transduction rate. Effector cells were added into the plate at 50μL per well, mixed thoroughly, and the plate was incubated at 37 °C. After approximately 24 hours of co-culture, cells were harvested for flow cytometric quantification of cytokine levels.

The results demonstrated that at both tested peptide concentrations (1μM and 100nM), T2 cells loaded with $E7_{11–19}$ peptide robustly stimulated SCG61-TGF-BB to secrete IFN-γ. In contrast, no detectable cytokine secretion was observed for the negative control peptide S20-28 or any of the 34 experimental human-derived peptides.

These data demonstrate that SCG61-TGF-BB exhibits no cross-reactivity against human endogenous peptides with the highest sequence homology to $E7_{11–19}$ (i.e., peptides sharing six identical amino acids, or five identical residues plus one conserved residue) (Figure 35), nor against peptides containing the four critical amino acid motifs of the target epitope.

Collectively, these findings indicate that SCG61-TGF-BB carries a minimal risk of potential off-target toxicity directed against human endogenous antigenic peptides.

Example 30: In Vitro Tumor-Killing Function of SCG61-TGF-BB batches prepared for

animal studies against Target Cells

Real-Time Cell Analysis (RTCA) was adopted in this experiment to evaluate the in vitro cytotoxic function of SCG61-TGF-BB batches prepared for animal studies. CaSki-E7 and HepG2-E7 cells were seeded onto 96-well RTCA plates as target cells. Effector cells were supplemented after approximately 16 hours of incubation for co-culture. Culture supernatants harvested at the 24-hour co-culture time point were subjected to cytokine detection, followed by continuous monitoring for an additional 48–60 hours. Real-time survival curves of target cells were plotted for comparative analysis.

Preparation of M10 Complete Medium: DMEM (Gibco, Cat. No. 11965-092) supplemented with 10% FBS (Gibco, Cat. No. 10099141), 1% Sodium Pyruvate (Gibco, Cat. No. 11360070), 1% HEPES (Gibco, Cat. No. 15630080) and 1% NEAA (Gibco, Cat. No. 11140-050). Mixed thoroughly by gentle inversion and stored at 4 °C until use.

Preparation of R10 Complete Medium: RPMI 1640 medium (Gibco, Cat. No. 22400-089) supplemented with 10% FBS (Gibco, Cat. No. 10099141). Mixed thoroughly by gentle inversion and stored at 4 °C for standby use.

Preparation of T Cell Culture Medium: CTS™ OpTmizer™ Medium with Supplement (Gibco, Cat. No. A379040-01), supplemented with 5% CTS™ Immune Cell Serum Replacement (SR, Gibco, Cat. No. A25961-01) and 2% GlutaMAX Supplement (Gibco, Cat. No. A12860-01). Mixed thoroughly by gentle inversion and stored at 4 °C for subsequent experiments.

Day 0: Well-grown human cervical cancer CaSki-E7 cells (cultured in complete R10 medium) and HepG2-E7 cells (cultured in complete M10 medium) were digested and adjusted to a cell density of 4×10^5 cells/mL for use. 50μL of the corresponding complete medium per well in a 96-well RTCA plate was used to measure the baseline. Subsequently, 50 μL of target cell suspension (4×10^5 cells/mL) was added to each well. Allow the plate to stand for approximately 5 minutes before placing it on the RTCA system, and continuously record cell growth curves for around 16 hours.

Day 1: Collect SCG61-TGF-BB TCR-T and Mock T cells with confirmed positive rate and cell viability. Calculate the number of effector cells based on the positive rate, and add 50 μL of effector cell suspension to each well at an effector-to-target (E:T) ratio of 2:1, 1:2 and 1:8. Place the plate on the RTCA system to continuously monitor cytotoxicity curves.

Day 2: Supernatants collected after 24 hours of co-culture were analyzed for cytokine secretion.

Day 3: Stop the RTCA instrument, and exported recorded cell growth and cytotoxicity curves

for data analysis.

The results showed that SCG61-TGF-BB exerted significant specific cytotoxicity against CaSki-E7 cells at effector-to-target (E:T) ratios of 2:1 and 1:2, and the killing efficiency was positively correlated with the E:T ratio. At E:T ratios of 2:1, 1:2 and 1:8, SCG61-TGF-BB completely eliminated HepG2-E7 cells, with both killing efficiency and the rate of target cell clearance positively associated with the E:T ratio. The measured IFN-γ levels secreted by SCG61-TGF-BB at all the above E:T ratios were highly consistent with the cytotoxicity profiles (Figure 36), whereas Mock T cells exhibited no anti-tumor activity against either target cell line.

In conclusion, SCG61-TGF-BB possesses robust in vitro cytolytic and anti-tumor activity against both CaSki-E7 and HepG2-E7 cells. These data provide supportive evidence and dosing references for subsequent non-clinical studies.

Example 31: In Vivo Cytotoxicity, Expansion and Persistence of SCG61-TGF-BB in Immunodeficient Mice Bearing CaSki-E7 Xenografts

To characterize the in vivo tumoricidal activity, expansion and persistence of SCG61-TGF-BB against CaSki-E7 tumor cells, a total of 23 female NPG immunodeficient mice were used in this study. On Day -7, all mice were inoculated with CaSki-E7 cells. The animals were randomly assigned into five groups: three SCG61-TGF-BB treatment groups receiving low, medium and high doses of the product respectively. On Day 0, mice were administered a single injection in tail vein of SCG61-TGF-BB at doses of 2×10^6, 7×10^6 and 2×10^7 positive T cells per mouse in sequence. The negative control group received a single injection in tail vein of 2×10^7 Mock T cells per mouse. Meanwhile, mice in the model control group were injected with an equal volume of vehicle solution via the tail vein in a single administration. The observation period lasted for 4 weeks post dosing.

The long and short diameters of tumors were measured twice weekly to calculate tumor volume. Peripheral blood samples were collected from animals every week to monitor the expansion and persistence of SCG61-TGF-BB TCR-T cells.

No.	Group Name	Test Article	Administration Dose (Positive T cells/mouse)	Number of Animals
1	Model Control Group	Vehicle	N/A	4
2	Negative Control Group	Mock T cells	2×10^7	4
3	SCG61-TGF-BB Low-Dose Group	SCG61-TGF-BB	2×10^6	5
4	SCG61-TGF-BB Mid-Dose Group	SCG61-TGF-BB	7×10^6	5
5	SCG61-TGF-BB High-Dose Group	SCG61-TGF-BB	2×10^7	5

"NA" stands for not applicable.

The results showed that on Day 22, the tumor volumes of the model control group, negative control group, and low-, medium-, and high-dose SCG61-TGF-BB groups were 1266.94±104.49, 1229.65±225.45, 1117.53±165.11, 581.24±249.42 and 117.73±31.32 mm^3, respectively. The tumor volume inhibition rates of the low-, medium-, and high-dose SCG61-TGF-BB groups were 11.79%, 54.12% and 90.71%, indicating a dose-dependent inhibitory effect of SCG61-TGF-BB on tumor volume.

One week after administration, the high dose of SCG61-TGF-BB (2.0×10^7 TCR-T cells per mouse) significantly suppressed tumor cell proliferation (P=0.00001, P<0.05). The medium dose (7×10^6 TCR-T cells per mouse) also exerted a notable inhibitory effect on tumor proliferation (P=0.055), yet tumor regrowth was observed in 2 out of 5 mice two weeks post-treatment. The low dose (2×10^6 TCR-T cells per mouse) restrained tumor growth within the first two weeks, but tumors eventually progressed in all 5 mice (Figure 37). On Day 7 after cell infusion, the expansion of SCG61-TGF-BB TCR-T cells in peripheral blood peaked; cell counts were positively correlated with the administered dose and gradually declined over time (Figure 38).

Under the present experimental conditions, intravenous administration of SCG61-TGF-BB at doses ranging from 0.7×10^7 to 2.0×10^7 TCR-T cells per mouse significantly inhibited the proliferation of CaSki-E7-LG tumor cells, with the minimum effective dose were determined as 0.7×10^7 TCR-T cells per mouse.

Example 32: In Vivo Cytotoxicity, Expansion and Persistence of SCG61-TGF-BB in Immunodeficient Mice Bearing HepG2-E7-LG Xenografts

To investigate the in vivo tumoricidal activity, cellular expansion and persistence of SCG61-TGF-BB against HepG2-E7-LG tumor cells, a total of 25 female NPG immunodeficient mice were utilized in this experiment. All mice were inoculated with HepG2-E7-LG cells on Day -7. The animals were randomly assigned to five experimental groups, among which three groups received low, medium and high doses of SCG61-TGF-BB respectively. On Day 0, a single injection in tail vein of SCG61-TGF-BB was administered to mice at doses of 2×10^6, 7×10^6 and 2×10^7 positive T cells per mouse in sequence. The negative control group received a single injection in tail vein of 2×10^7 Mock T cells per mouse. Meanwhile, mice in the model control group were injected with an equal volume of test article vehicle via a single administration in tail vein. The observation period lasted for 4 weeks following dosing.

The long and short diameters of tumors were measured twice every week to calculate tumor volume. Peripheral blood samples were collected from animals weekly to assess the expansion and persistence of SCG61-TGF-BB TCR-T cells.

No.	Group Name	Test Article	Dose (Positive T cells per mouse)	Number of Animals
1	Model Control Group	Vehicle	N/A	5
2	Negative Control Group	Mock T cells	2×10^7	5
3	SCG61-TGF-BB Low-Dose Group	SCG61-TGF-BB	2×10^6	5
4	SCG61-TGF-BB Medium-Dose Group	SCG61-TGF-BB	7×10^6	5
5	SCG61-TGF-BB High-Dose Group	SCG61-TGF-BB	2×10^7	5

"NA" stands for not applicable.

The results demonstrated that on Day 22, the tumor volumes of the model control group, negative control group, and low-, medium-, and high-dose SCG61-TGF-BB groups were 2400.34±726.22, 1986.19±720.41, 1252.83±202.74, 439.02±365.64 and 132.34±175.87 mm^3, respectively. The tumor volume inhibition rates of the low, medium and high SCG61-TGF-BB dose groups were 47.81%, 81.71% and 94.49% (Figure 39). The medium and high doses of SCG61-TGF-BB significantly suppressed tumor proliferation ($P < 0.05$) in a dose-dependent manner.

On Day 7 after cell infusion, the expansion of SCG61-TGF-BB TCR-T cells in the peripheral blood of mice reached its peak. The absolute cell count was positively correlated with the administered dose and gradually declined over time (Figure 40).

Under the experimental conditions, intravenous administration of SCG61-TGF-BB at doses ranging from 0.7×10^7 to 2.0×10^7 TCR-T cells per mouse markedly inhibited the proliferation of HepG2-E7-LG tumor cells, with the minimum effective dose were determined as 0.7×10^7 TCR-T cells per mouse.

This novel generation of HPV-targeted TCR-T therapy is that transduces T cells with a T cell receptor specific for HPV-16 E7 together with a co-stimulatory fusion receptor derived from PD-1 or TGFβRII. The engineered T cells specifically recognize and eliminate HPV-16-infected cells, and are applicable to multiple malignancies induced by human papillomavirus (HPV), including cervical cancer, head and neck cancer, anal cancer, vaginal cancer, vulvar cancer and penile cancer. The therapy eliminates tumor cells by targeting antigens uniquely expressed in HPV-infected cells while simultaneously clearing viral infection.

In vitro studies revealed that this enhanced HPV-targeted TCR-T cell exhibits specific TCR expression with a low mispairing rate. The engineered T cells can bind to key epitope-binding residues of HPV-16 and HPV-52 E7 peptides with robust binding activity. They exert specific cytotoxicity against tumor cells positive for both HLA-A*02:01 *and* HPV-16/52 E7; higher effector-to-target ratios correspond to stronger tumoricidal capacity and greater cytokine secretion. No cytotoxicity was detected against tumor cells lacking HLA-A*02:01 or HPV-16 E7 expression, confirming high target specificity and favorable safety profiles. Moreover, the enhanced HPV-targeted TCR-T cells retain potent anti-tumor activity and proliferative capacity after multiple rounds of tumor antigen stimulation.

In vivo studies showed that different doses of enhanced HPV-targeted TCR-T cells all showed remarkable tumor growth suppression in NCG mouse CDX models bearing distinct HPV-16 E7-positive tumor xenografts. Mice receiving the high dose (2×10^7 cells) displayed prominent anti-tumor efficacy, while moderate tumor inhibitory effects were also observed in medium- and low-dose cohorts, verifying sustained anti-cancer activity of the engineered TCR-T cells in vivo.

In summary, preclinical data preliminarily validate the favorable safety profile and potent cytotoxic activity of enhanced HPV-targeted TCR-T cells against HPV-16 E7-positive target cells. No obvious toxic or adverse reactions were observed in preclinical animal studies even at the maximum tested dose of 2×10^7 enhanced HPV-targeted TCR-T cells, supporting further clinical investigation to characterize human safety and tolerable dose ranges.

The present application has been described in detail above, with specific examples elaborating the principles and embodiments thereof. The descriptions of the foregoing examples are merely intended to facilitate comprehension of the technical solutions and core concepts of the application. Those skilled in the art will recognize that various modifications may be made to the specific embodiments and scope of application in accordance with the inventive concept disclosed herein. Accordingly, the content of this specification shall not be construed as limiting the scope of the present application.

Claims

1. A modified immune cell, wherein the modified immune cell comprises a T cell receptor (TCR) targeting HPV E7 antigen and a chimeric switch receptor.

2. The modified immune cell of claim 1, wherein the TCR comprises a TCR α chain variable domain and a TCR β chain variable domain; wherein the amino acid sequence of αCDR3 of the TCR α chain variable domain is shown as SEQ ID NO: 3, or a variant thereof in which one or two amino acids are substituted with other amino acids; and the amino acid sequence of βCDR3 of the TCR β chain variable domain is shown as SEQ ID NO: 6, or a variant thereof in which one or two amino acids are substituted with other amino acids.

3. The modified immune cell of claim 2, wherein the TCR α chain variable domain comprises complementarity-determining regions αCDR1, αCDR2 and αCDR3, and the TCR β chain variable domain comprises complementarity-determining regions βCDR1, βCDR2 and βCDR3, wherein:

αCDR1, αCDR2 and αCDR3 are shown as SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3 respectively, and βCDR1, βCDR2 and βCDR3 are shown as SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6 respectively; or CDR variants thereof in which one or two amino acids in one or more CDRs are substituted with other amino acids.

4. The modified immune cell of claim 2, wherein the TCR α chain variable domain comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 7; and/or the TCR β chain variable domain comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 8.

5. The modified immune cell of claim 1, wherein the modified immune cell expresses the following elements:

A. a T cell receptor (TCR), which specifically targets HPV E7 antigen peptide;

preferably, the TCR is capable of binding to HPV E7 antigen peptide presented by HLA-A*02; more preferably, the TCR is capable of binding to HPV E7 antigen peptide presented by HLA-A*02:01; further preferably, the peptide comprises the amino acid sequence YMLDLQPET or YILDLQPET;

and

B. a chimeric switch receptor, which comprises an extracellular domain (ECD), a transmembrane region (TM) and an intracellular domain (ICD), wherein the ECD is selected from ECDs of immunosuppressive molecules, and the ICD is selected from ICDs of co-stimulatory molecules.

6. The modified immune cell of claim 5, wherein the chimeric switch receptor comprises an extracellular domain (ECD) of an immunosuppressive protein fused to an intracellular domain (ICD) of a co-stimulatory molecule that mediates immune cell activating signals; wherein binding of the ECD of the immunosuppressive protein to its immunosuppressive factor or ligand generates an activating signal rather than an inhibitory signal in the modified immune cell.

7. The modified immune cell of claim 6, wherein the immunosuppressive protein is any one or a combination of PD-1, CTLA4, BTLA, TIM3, TIGIT, TGFβ receptor and any other proteins with immunosuppressive functions or associated with immunosuppressive signaling pathways, and the ECD sequence of the immunosuppressive protein may contain at least one amino acid mutation; and/or

the co-stimulatory molecule is any one or a combination of CD28, 4-1BB, ICOS, CD27, IL-12R, CD3 and OX40 proteins, and the ICD sequence of the co-stimulatory molecule may contain at least one amino acid mutation.

8. The modified immune cell of claim 6, wherein the ECD is TGFβ receptor II ECD; and the sequence of the TGFβ receptor II ECD is shown as SEQ ID NO: 12.

9. The modified immune cell of claim 6, wherein the ECD is PD-1 ECD; more preferably, the PD-1 ECD sequence carries one amino acid mutation where alanine at position 132 is mutated to leucine; most preferably, the amino acid sequence of the ECD is shown as SEQ ID NO: 9.

10. The modified immune cell of claim 6, wherein the ICD is 4-1BB ICD; more preferably, the amino acid sequence of the 4-1BB ICD is shown as SEQ ID NO: 11.

11. The modified immune cell of claim 5, wherein the TCR comprises a TCR α chain and a TCR β chain, the variable domain of the TCR α chain comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 7; and/or

the variable domain of the TCR β chain comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 8.

12. The modified immune cell of claim 6, wherein the ECD and the ICD are linked via a transmembrane sequence; preferably, the transmembrane region comprises a transmembrane domain of a protein selected from the group consisting of TCR α chain, TCR β chain, TCR ζ chain, CD28, CD3ε, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154 and combinations thereof, and the transmembrane sequence may contain at least one amino acid mutation; more preferably, the transmembrane sequence is a CD8 transmembrane sequence; further preferably, the CD8 transmembrane sequence is shown

as SEQ ID NO: 10.

13. The modified immune cell of claim 12, wherein the immune cell comprises the HPV E7 antigen-targeted TCR and a chimeric switch receptor with the structure of PD1(ECD)-CD8(TM)-4-1BB(ICD); more preferably, the immune cell comprises an amino acid sequence shown as SEQ ID NO: 15.

14. The modified immune cell of claim 12, wherein the immune cell comprises the HPV E7 antigen-targeted TCR and a chimeric switch receptor with the structure of TGFβRII(ECD)-CD8(TM)-4-1BB(ICD); more preferably, the immune cell comprises an amino acid sequence shown as SEQ ID NO: 17.

15. The modified immune cell of claim 3 or claim 7, wherein the immune cell is selected from lymphocytes, dendritic cells, monocytes/macrophages, granulocytes and mast cells; preferably, the immune cell is a T cell.

16. A nucleic acid molecule, wherein the nucleic acid molecule comprises a nucleic acid sequence encoding the TCR in any one of claims 1 to 4.

17. The nucleic acid molecule of claim 16, wherein the nucleic acid molecule further comprises a nucleic acid sequence encoding the chimeric switch receptor in any one of claims 1 to 14.

18. A vector, wherein the vector comprises the nucleic acid molecule of claim 16 or claim 17.

19. A pharmaceutical composition, wherein the composition comprises a pharmaceutically acceptable carrier and the modified immune cell in any one of claims 1 to 15, the nucleic acid molecule in claim 16 or 17, or the vector in claim 18.

20. Use of the modified immune cell in any one of claims 1 to 15, the nucleic acid molecule of claim 16 or 17, the vector in claim 18, or the pharmaceutical composition of claim 19 in the manufacture of a medicament for preventing or treating diseases associated with HPV infection.

21. The use of claim 20, wherein the HPV infection-associated diseases include one or more malignant tumors induced by human papillomavirus (HPV), selected from the group consisting of cervical cancer, oropharyngeal cancer, head and neck cancer, vaginal cancer, vulvar cancer, penile cancer and anal cancer.

22. A T cell receptor targeting HPV E7 antigen or a fragment thereof, wherein the T cell receptor or fragment thereof comprises a TCR α chain variable domain and a TCR β chain variable domain selected from the following group:

(1) the TCR α chain variable domain comprises the following three CDRs: αCDR1 shown as SEQ ID NO: 1, αCDR2 shown as SEQ ID NO: 2, and αCDR3 shown as SEQ ID NO: 3, or a variant of the foregoing CDRs with substitution of 1 or 2 amino acids therein; and

(2) the TCR β chain variable domain comprises the following three CDRs: βCDR1 shown as SEQ ID NO: 4, βCDR2 shown as SEQ ID NO: 5, and βCDR3 shown as SEQ ID NO: 6, or a variant of the foregoing CDRs with substitution of 1 or 2 amino acids therein.

23. The T cell receptor or fragment of claim 22, wherein the TCR α chain variable domain comprises the following three CDRs: αCDR1 shown as SEQ ID NO: 1, αCDR2 shown as SEQ ID NO: 2, and αCDR3 shown as SEQ ID NO: 3; and

the TCR β chain variable domain comprises the following three CDRs: βCDR1 shown as SEQ ID NO: 4, βCDR2 shown as SEQ ID NO: 5, and βCDR3 shown as SEQ ID NO: 6.

24. The T cell receptor or fragment of claim 22, wherein the TCR α chain variable domain comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 7; and/or

the TCR β chain variable domain comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 8.

25. The T cell receptor or fragment of claim 22, wherein it is capable of binding to HPV E7 antigen peptide presented by HLA-A02; more preferably, it is capable of binding to HPV E7 antigen peptide presented by HLA-A*02:01.

26. The T cell receptor or fragment of claim 25, wherein the peptide comprises the amino acid sequence YMLDLQPET or YILDLQPET.

27. A nucleic acid molecule, wherein the nucleic acid molecule comprises a nucleic acid sequence encoding the T cell receptor or fragment in any one of claims 22 to 26, or a complementary sequence thereof.

28. A vector, wherein the vector comprises the nucleic acid molecule of claim 27, wherein the vector is selected from plasmids, binary vectors, DNA vectors, mRNA vectors, retroviral vectors, lentiviral vectors, transposon-based vectors and artificial chromosomes.

29. An isolated polypeptide encoded by the nucleic acid molecule of claim 27 or the vector of claim 28.

30. An isolated cell, wherein the cell contains the T cell receptor or fragment thereof in any one of claims 22 to 26, the nucleic acid molecule of claim 27, the vector of claim 28 or the polypeptide of claim 29; preferably, the cell comprises an amino acid sequence shown as SEQ

ID NO: 13.

31. A pharmaceutical composition, wherein the composition comprises a pharmaceutically acceptable carrier and the T cell receptor or fragment thereof in any one of claims 22 to 26, the nucleic acid molecule of claim 27, the vector of claim 28, the polypeptide of claim 29, or the cell of claim 30.

32. Use of the T cell receptor or fragment thereof in any one of claims 22 to 26, the nucleic acid molecule of claim 27, the vector of claim 28, the polypeptide of claim 29, the cell of claim 30, or the pharmaceutical composition of claim 31 in the manufacture of a medicament for preventing or treating diseases associated with HPV infection.

33. The use of claim 32, wherein the HPV infection-associated diseases include one or more malignant tumors induced by human papillomavirus (HPV), selected from the group consisting of cervical cancer, oropharyngeal cancer, head and neck cancer, vaginal cancer, vulvar cancer, penile cancer and anal cancer.