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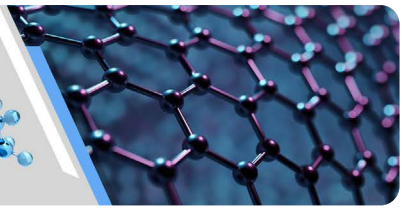

Development of Novel NKp46-based Chimeric Antigen Receptors for Cancer Immunotherapy

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Abstract

Chimeric antigen receptor (CAR)-T cell therapy has been highly successful in the treatment of hematological malignancies, yet its effectiveness in solid tumors remains limited. To develop CAR-T cell therapy for solid tumors, identifying new target antigens is crucial. NKp46, a critical natural cytotoxicity receptors (NCRs) on NK cells, play a pivotal role in their antitumor function. Interaction between NKp46 and its ligands activates NK cells, prompting the release of antitumor effector molecules. We found that NKp46 ligands (NKp46L) were specifically expressed in various tumor cells but absent in normal tissue cells, warranting further investigation. Consequently, we have developed novel second-generation CAR constructs featuring NKp46 extracellular immunoglobulin-like structural domains and demonstrated their *in vitro* cytotoxic activities against tumor cells. In conclusion, NKp46-based chimeric antigen receptors show promise in cancer immunotherapy but require further explorations.

Keywords: Chimeric antigen receptor; NKp46; Natural cytotoxicity receptors; NKp46L; Immunotherapy

Introduction

Cancer has emerged as a significant threat to human health, necessitating the pursuit of innovative treatments. In recent years, chimeric antigen receptor (CAR)-T cell therapy, a notable emerging from of cancer immunotherapy, has garnered substantial research attention as a potential cancer treatment. It now stands as the fourth major treatment modality for tumors, alongside surgery, radiotherapy and chemotherapy [1]. Globally, several CAR-T cell products have been approved for clinical use in treating tumors; however, these primarily target CD19 or BCMA and are exclusively used for hematological cancers [2]. While research of target antigens for CAR-T cell therapy is extensive, the majority focuses on tumor-associated antigens (TAAs) that are overexpressed in tumor tissues but present in low levels in normal tissues. Tumor-specific antigens (TSAs), which are exclusively overexpressed in tumors and absent in normal tissues, are exceptionally scarce, thus hampering the safety and efficacy of CAR-T cell therapy [3]. Therefore, discovering novel target antigens for CAR-T cells holds immense promise for advancing cancer therapy. Natural killer (NK) cells are belong to the lymphocytes of the innate immune system in the body, which possesses the capability to recognize tumor cells and virus-infected cells, directly attacking these cells and participating in the regulation of various cellular immune responses [4]. NK cells primarily execute their functions through the expression of activating receptors that identify their ligands, thereby mediating antitumor immunity. These activating receptors consist mainly of CD16, NKG2D, and natural cytotoxicity receptors (NCRs), which include NKp30, NKp44, and NKp46

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[5]. When these activating receptors on NK cells (NK) are stimulated, they can kill infected, cancerous and stressed cells [6]. A significant proportion of NK cells and some innate lymphoid cells (ILCs) express the activating receptor NKp46, encoded by the *NCR1* gene, which is the most evolutionarily conserved NK cell receptor [7, 8]. The NKp46 receptor is a vital component of the immune system, particularly in recognizing and eliminating infected or transformed cells [9]. Studies have indicated that blockade of NKp46 impairs the tumoricidal effect of NK cells [10]. NKp46 is a crucial NCR on NK cells, and its expression levels are closely linked to the progression of various immune-related diseases, including viral infections and tumors [11]. During the recognition and attack of tumor cells by NK cells, NKp46 interacts with its ligands, activating NK cells and prompting them to release various antitumor effector molecules [12]. Previous study has shown that NKp46 recognizes externalized calreticulin (ecto-CRT), which translocate from endoplasmic reticulum (ER) to the cell membrane during ER stress. This recognition plays a pivotal role in the immune response against cancer cells and viral infection, such as Zika virus (ZIKV) infection. Furthermore, NKp46 recognition of ecto-CRT also controls the invasion of B16 melanoma and RAS-driven lung cancer in mice, suggesting its critical role in controlling tumor growth and viral infection by enhancing NK cell degranulation and cytokine secretion [13].

Although recent study has confirmed that ecto-CRT serve as an endogenous ligand for NKp46, playing a pivotal role in NK cell recognition, viral infection control, ER stress alleviation, and senescent cell elimination, and the control of melanoma and lung cancer in mice, a comprehensive exploration of ecto-CRT expression in human tumors and its function in tumor control is still lacking. In addition, the discovery of eco-CRT was made in a model of Zika virus infection, not within tumor cells, suggesting the existence of other tumor-associated ligands beyond eco-CRT that remained unexplored. The identification of ecto-CRT, along with potential undiscovered ligands for NKp46, holds promise for advancing NKp46-based CAR-T cell therapies. Currently, reported CAR-T cell therapies leveraging NK cell-activated receptors encompass CD16 CAR-T [14], NKG2D-based CAR-T [15], NKp30-based CAR-T [16], and NKp44-based CAR-T [17]. However, NKp46-based CAR-T cell therapies have yet to been reported, and the antitumor effects of such a therapy remain uncertain and warrants further investigation.

Materials and Methods

Cell Lines and Cell Culture

Unless specified otherwise, all cell lines were obtained from the American Typical Culture Collection (ATCC, Manassas, VA, USA), including lung cancer cells (A549, Calu-3), hepatocellular carcinoma cells (Huh-7, HepG2, Hep3B), myeloid leukemia cells (Kasumi-1, K562), lymphoma cells (Raji, Daudi, SU-DHL-2,), acute B-lymphoblastic leukemia cells (SUP-B15, NALM6), osteosarcoma cells (H929, U266, KMS-2), glioblastoma cells (SF268), esophageal carcinoma cells (KYSE150), breast cancer cells (ZR-75-1) colorectal cancer cells (HCT116, RKO), head and neck cancer cells (SCC47), oral squamous cell carcinoma (SCC090), cervical cancer cells (SiHa), and human embryonic kidney cell (HEK293T). These cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. They were cultures in DMEM or RPMI1640 medium (Corning, USA) supplemented with 10% fetal bovine serum (FBS; Corning, USA), and 1% Penicillin-Streptomycin-Neomycin-Solution (PSN; Yeasen, China).

Construction and Preparation of NKp46-based CAR-T Cells

The construction and preparation of NKp46-based CAR-T cells involved multiple steps, including gene synthesis, plasmid construction, lentiviral preparation, T-cell activation, lentiviral transduction, CAR-T cells expansion and phenotypic analysis. The brief protocol outlines are as follows:

(1) The extracellular region of NKp46 was applied to construct the second-generation CAR incorporating 4-1BB costimulatory domain and CD3ξ signaling domain. Various NKp46-based CARs were genetically synthesized and cloned into the lentiviral expression plasmid (pLV-EF1α-MCS-P2A-EGFP), ultimately yielding the recombinant lentiviral expression plasmid;

(2) The recombinant lentiviral expression plasmid, along with psPAX2 and pVSVG helper plasmids, were co-transfected into HEK293T cells to package the lentiviral vectors;

(3) Peripheral blood mononuclear cells (PBMC) were cultured in serum-free X-VIVO15 medium (Lonza, USA), supplemented with 50 IU/mL interleukin-2 (IL-2), 5 ng/ml recombinant human interleukin-7 (IL-7), and 10 ng/ml interleukin-15 (IL-15) (Novoprotein, China). These PBMCs were pre-activated using CD3/CD28 antibody-coated magnetic beads for a period of 72 hours;

(4) Following pre-activation, lentiviral vectors were introduced to the T cells through centrifugation at 1000×g, for 1.5 hours at 32°C. The culture medium was then replaced with fresh medium the next day;

(5) After a 10-14 day expansion period, the NKp46-based CAR-T cells were harvested and their CAR expression levels were assessed using flow cytometry.

Flow Cytometry Analysis

The expression levels of NKp46 ligands (NKp46L), NKp46-based CARs and surface markers were detected utilizing BD FACSymphony A3 flow cytometer (BD

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Biosciences, USA). The reagents used were as follows PE Streptavidin, Pacific Blue anti-human CD335 (NKp46) antibody, PE anti-human IgG Fc antibody (Biolegend, USA), CoraLite® Plus 647-conjugated calreticulin Polyclonal antibody, CoraLite® Plus 647-conjugated Rabbit IgG control Polyclonal antibody (Proteintech, USA), recombinant Human NKp30/NCR3 (C-Fc), recombinant human NKp46/NCR1 (C-Fc), recombinant Human NKG2D (N-Fc) (Novoprotein, China), and biotinylated-recombinant human NKp44/NCR2 Protein (His Tag) (SinoBiological, China). One million cells were harvested from culture flasks and then washed twice with cold FACS buffer (PBS containing 0.02% FBS). These cells were centrifuged at 400×g for 5 minutes. Subsequently, these cells were stained with indicated antibodies or recombinant proteins for 20 minutes at room temperature. Following, staining, the cells underwent a single wash with FACS buffer, were resuspended in 300 µL of same buffer, and then subjected to flow cytometric analysis. The collected data were subsequently analyzed using Flowjo version 10.0 software.

Cytotoxicity Assay of NKp46-based CAR-T Cells

For the cytotoxicity assay, we utilized Cell Counting Kit-8 reagent (CCK-8; MCE, USA) to evaluate the cytolytic activity against cancer cells. Specifically, tumor cells (1.5×10^4 cells) and NKp46-based CAR-T cells were plated in flat-bottomed 96-well plates at the indicated effector-to-target (E: T) ratios. These plates were then incubated for 24 hours under standard conditions of 37°C and 5% CO_2. At the end of incubation, the supernatant was discarded, and the cells were gently washed twice with 1×PBS. Subsequently, 100 µL of fresh medium was added to each well, followed by the addition of 10 µL of CCK-8 reagent. The optical density (OD) of each well was then measured at a wavelength of 450 nm using a Varioskan LUX multi-mode microplate reader (ThermoFisher Scientific, USA). The control groups comprised a tumor-only group and a medium-only group. To quantify the percentage of cytotoxicity, we assessed the proportion of live cells in each experimental condition. The percentage of specific lysis (cytotoxicity (%)) was calculated using the following formula: cytotoxicity (%) = (1 - (the experimental group (OD value) – the medium-only group (OD value)) / (the tumor-only group (OD value) – the medium-only group (OD value)) × 100.

Statistical Analyses

Statistical analyses were conducted utilizing GraphPad Prism version 8 software. To evaluate he statistical significance of differences between two groups, the Student's t-test was utilized. For comparisons encompassing three or more groups, a one-way ANOVA was employed. Statistical significance was assigned to all values with a p-value less than 0.05, denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

Results

Expression Levels of Ligands for NCRs and NKG2D in Cancer Cells

It has been demonstrated that the activated receptors of NK cells predominantly encompass NKp46, NKp44, NKp30, and NKG2D. Currently, CAR-T cell therapies leveraging NK cell activation receptors include NKG2D-based CAR-T, NKp30-based CAR-T, and NKp44-based CAR-T, whereas NKp46-based CAR-T remains unreported. To explore novel potential ligands for NK cell activation receptors on tumor cells, we attempted to detect potential ligands on tumor cells using recombinant human NKp30-Fc, NKp44-Biotin, NKp46-Fc, and NKG2D-Fc. The findings revealed that NKp46 ligands were ubiquitously expressed across various tumor cells, notably lung cancer cells (A549, Calu-3), hepatocellular carcinoma cells (HepG2, Hep3B), myeloid leukemia cells (K562), and lymphoma cells (Raji). Notably, the positive rate of NKp46 ligands (NKp46L) was more than 90% in lymphoma cells. Furthermore, NKG2D ligands are also expressed to varying degrees in these cancer cells, with MICA/B and ULBP1/2/3 reported as endogenous ligands for NKG2D [15,18]. However, no ligands binding to NKp44 or NKp30 were detected in any of the aforementioned cancer cells (Figure 1).

Comparison of NKp46 and NKG2D Ligands in Hematological and Solid Tumors

After initial screening, NKp46 and NKG2D ligands displayed significant expression across multiple cancer cell lines, prompting an expanded investigation into various tumor types. Initially, we conducted a NKp46 and NKG2D ligand assay on prevalent hematological cancer cells. The results indicated robust expression of NKp46L in lymphoma cells, notably in diffuse large B-cell lymphoma (DLBCL: SU-DHL-2) and Burkitt's lymphoma (Raji, Daudi). Conversely, NKp46L was scarcely detected in acute B-lymphoblastic leukemia cells (SUP-B15, NALM6). NKp46L was found expressed in some certain leukemia cells (Kasumi-1, K562) and osteosarcoma cells (U266, KMS-2). Meanwhile, NKG2D ligands (NKG2DL) were selectively expressed in specific acute B-lymphoblastic leukemia cells (NALM6), myeloid leukemia cells (Kasumi-1, K562), and osteosarcoma cells (H929, U266, KMS-2) (Figure 2A). Next, we extended our analysis in common solid tumor cells. Our results revealed that NKp46L was expressed in lung cancer cells (A549, Calu-3), hepatocellular carcinoma cells (Huh-7, HepG2, Hep3B), glioblastoma cells (SF268), esophageal carcinoma cells (KYSE150), and breast cancer cells (ZR-75-1). However, it was absent in colorectal cancer cells (HCT116, RKO), head and neck carcinoma cells (SCC47), oral squamous cell carcinoma (SCC090), and cervical cancer cells (SiHa). NKG2DL was expressed in some lung cancer cells (A549), hepatocellular carcinoma cells (Huh-7), glioblastoma cells (SF268), esophageal carcinoma cells (KYSE150), breast

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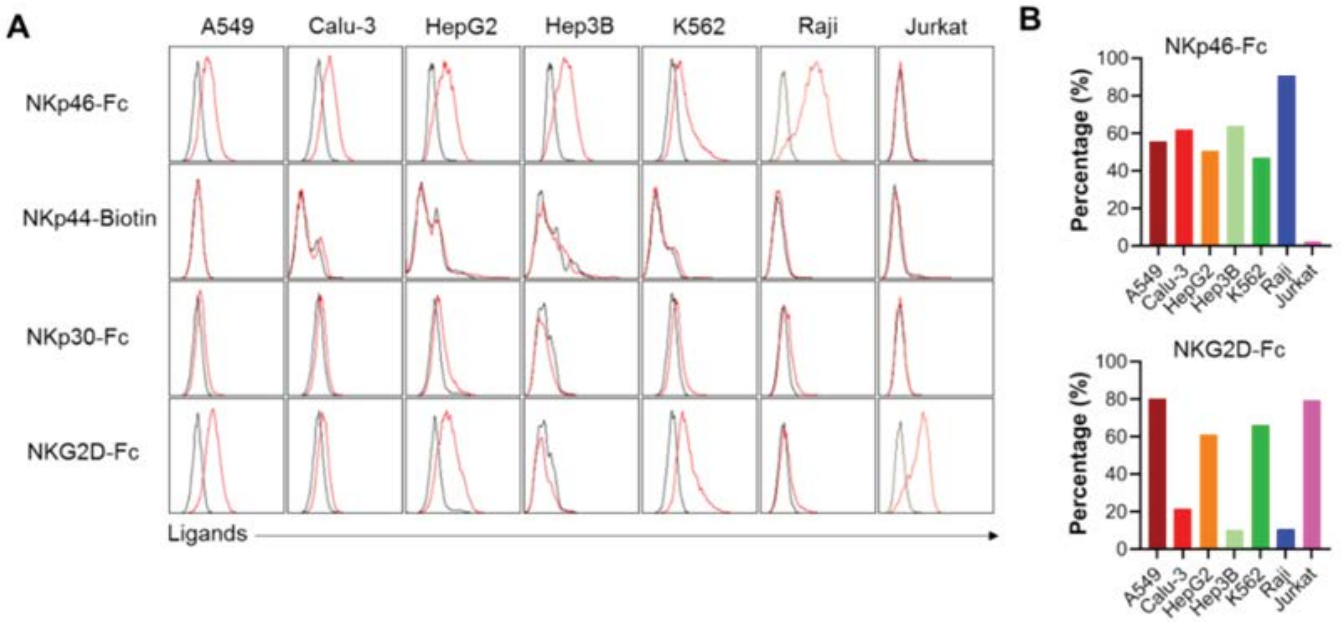

Figure 1: Expression levels of ligands for natural cytotoxicity receptors (NCRs) and NKG2D in various cell lines. (**A**) Expression levels of ligands for NKp30, NKp44, NKp46 and NKG2D in lung cancer cells (A549, Calu-3), hepatocellular carcinoma cells (HepG2, Hep3B), myeloid leukemia cells (K562), and lymphoma cells (Raji). (**B**) Statistical plots of the expression levels of NKp46 and NKG2D ligands in cancer cell lines.

cancer cells (ZR-75-1), head and neck cancer cells (SCC47), oral squamous cell carcinoma (SCC090), and cervical cancer cells (SiHa). Additionally, a relatively low level of NKG2DL expression was detected in colorectal cancer cells (HCT116, RKO) (Figure 2B).

Tumor-associated NKp46 Ligands beyond ecto-CRT

Prior experimental findings have established that NKp46 and NKG2D ligands exhibit differential expression patterns across various hematological and solid tumors. Notably, B-cell lymphoma stands out with elevated levels of NKp46L

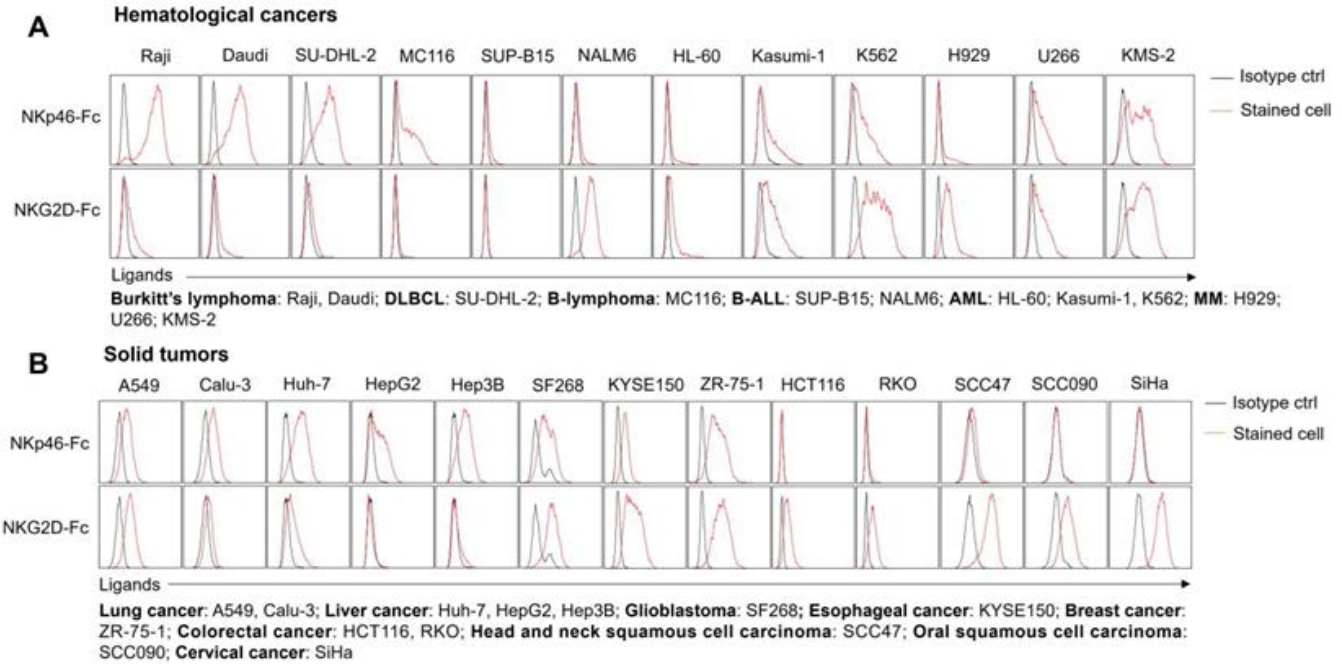

Figure 2: Expression levels of NKp46 and NKG2D ligands in hematological and solid Tumors. (A) Expression levels of NKp46 and NKG2D ligands in hematological cancers. (B) Expression levels of NKp46 and NKG2D ligands in solid tumors.

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expression. To delve deeper into the specificity of NKp46L expression in lymphoma versus its absence in normal tissues, we conducted an analysis involving peripheral blood mononuclear cells (PBMCs) sourced from both lymphoma patient and healthy donor. Through assays targeting NKp46 and NKG2D ligands assay, we observed strong staining signals in PBMCs from lymphoma patient, indicative of high NKp46L expression in primary lymphoma cells. Conversely, PBMCs from healthy individual showed no expression of NKp46L, highlighting its tumor-associated specificity (Figure 3A). Despite the robust NKp46L expression in B-cell lymphomas, its classification as a novel ligand for NKp46 remained unconfirmed. Recent study has illuminated externalized calreticulin (ecto-CRT) as an endogenous ligand for NKp46, functioning as a danger-associated molecular pattern that aids NK cells in recognizing and eliminating infected, malignant, stressed or senescent cells [9]. To ascertain whether the identified NKp46 ligands in lymphoma cells (Raji and Daudi) were indeed ecto-CRT, we utilized an anti-human CRT antibody. However, our findings revealed no significant expression of ecto-CRT in either Raji or Daudi cells, contrasting sharply with the results obtained using the recombinant NKp46 protein (Figure 3B). This discrepancy underscores the existence of novel NKp46 ligands beyond ecto-CRT and necessitates a comprehensive screen of tumor cells to identify these new ligands. In summary, NKp46L emerges as a promising new tumor target antigen, exhibiting a distinct expression profile that does not overlap with NKG2D ligands. It is predominantly expressed in B-cell lymphomas and is absent in normal lymphocytes, while also showing upregulation in solid tumors, including lung, liver, and breast cancers.

The Successful Expression of NKp46-based CARs on Primary T Cells

Based on prior experimental findings, we hypothesized that NKp46 ligands could represent novel potential targets for cancer therapy, and CAR-T cells targeting these ligands

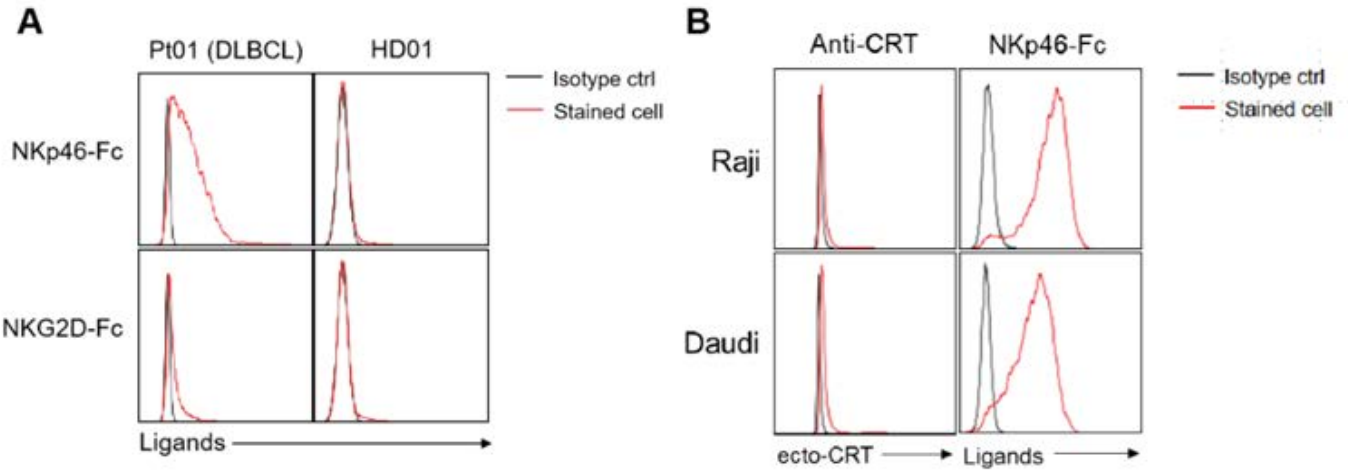

Figure 3: Existence of potential NKp46 ligands beyond ecto-CRT. (**A**) Expression of NKp46 and NKG2D ligands in lymphoma patient (left) and healthy donor (right). (**B**) Expression of ecto-CRT in lymphoma cell lines.

might exhibit antitumor effects. Using the extracellular domain (ECD) of NKp46 as the recognition module for CARs, we designed four different second-generation CAR constructs, each incorporating the NKp46 ECD along with 4-1BB costimulatory domain and CD3ζ signaling domain (Figure 4A). These constructs were then introduced into primary T cells via lentiviral vectors to generate CAR-T cells. Flow cytometric analysis revealed that CAR variant featuring the transmembrane region of NKp46 (NKp46-TM-BBz) failed to stably express on T cells, while NKp46-based CARs with the CD8 transmembrane region could be successfully expressed on T-cell membrane (Figure 4B, C).

In Vitro Cytotoxicity of NKp46-based CAR-T Cells Against Tumor Cells

To further assess the antitumor capabilities of NKp46-based CAR-T cells, we conducted a preliminary test to evaluate their tumor-killing effects. Expanded NKp46-based CAR-T cells were co-incubated with lung cancer cells (A549) at effector-to-target ratio of 1:2, 1:1, and 2:1 for a duration of 24 hours to measure their cytotoxic activities. The results from the cytotoxicity assays indicated that the various NKp46-based CAR-T cells exhibited different levels of efficacy in killing cancer cells. Notably, two of the NKp46-based CARs demonstrated comparable cytotoxicity against cancer cells. However, modifications such as replacing the NKp46 transmembrane region or deleting the NKp46 hinger region significantly impaired cytotoxic activity. These findings suggest that NKp46-based CAR-T cells possess *in vitro* antitumor effects, warranting further investigation into their *in vivo* antitumor efficacy.

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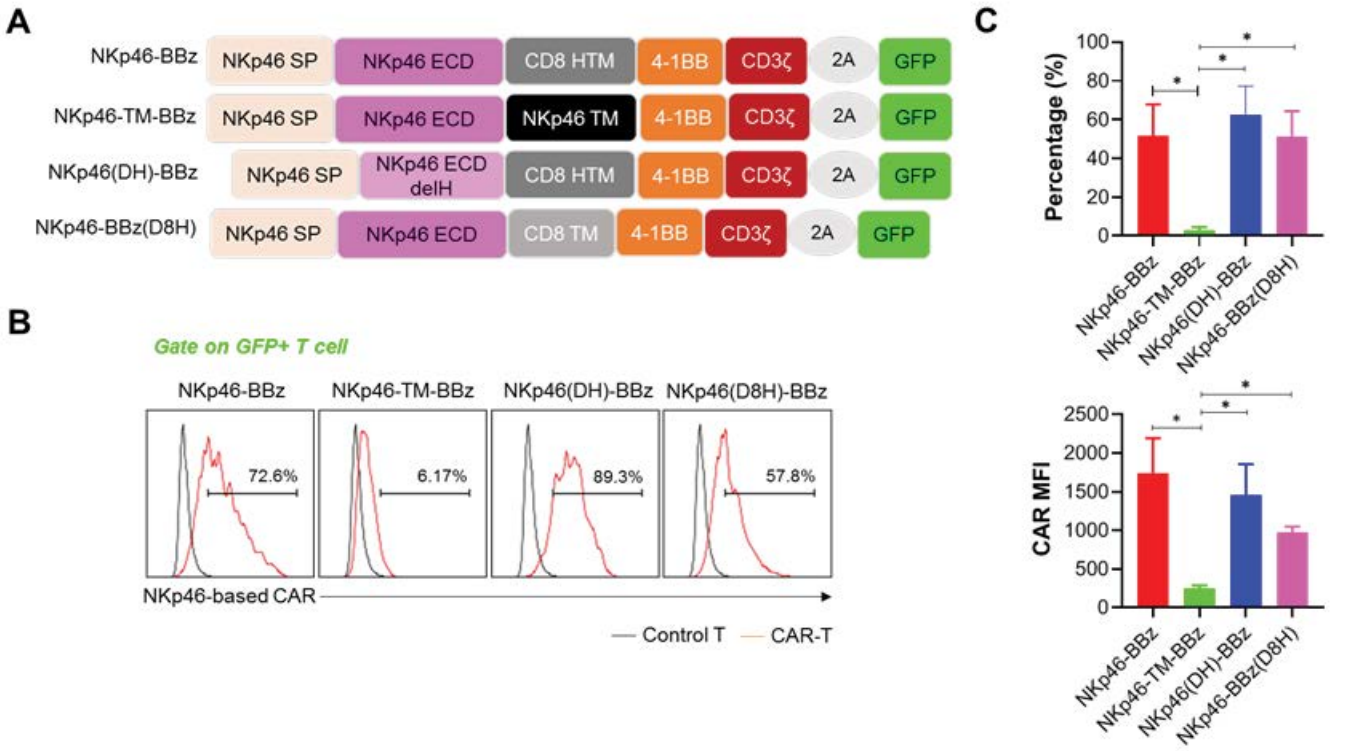

Figure 4: The construction and preparation of NKp46-based CAR-T cells. (**A**) Four different NKp46-based CAR constructs were designed using the NKp46 extracellular domain (ECD) as the CAR recognition module. (**B**) Flow cytometric analysis of NKp46-based CAR expression in T cells. (**C**) Statistical plots of CAR positive rate (upper panel) and mean fluorescence intensity (MFI) (lower panel) of NKp46-based CAR-T cells. Statistical significance was assigned to all values with a p-value less than 0.05, denoted as follows: *p < 0.05.

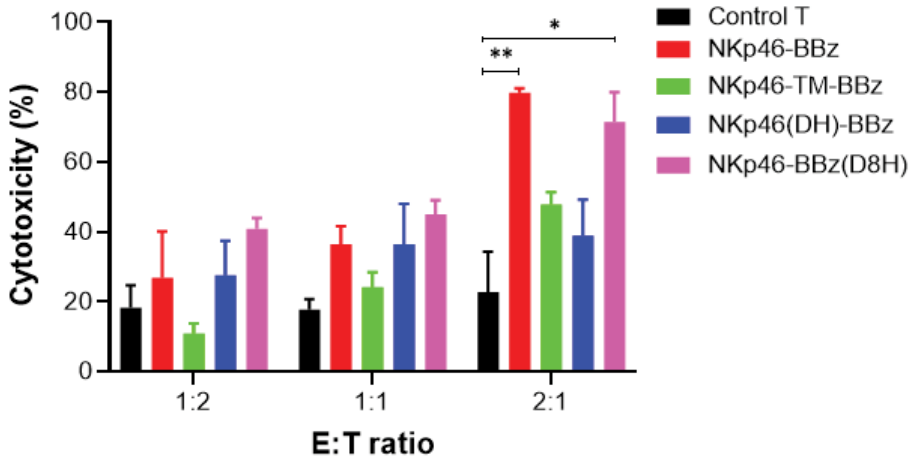

Figure 5: The Killing effect of NKp46-based CAR-T cells on lung cancer cells. Statistical plots of cytotoxicity of NKp46-based CAR-T cells against cancer cells. Statistical significance was assigned to all values with a p-value less than 0.05, denoted as follows: *p < 0.05, **p < 0.01.

Discussion

In this study, we initially employed recombinant proteins of NKp30, NKp44, NKp46, and NKG2D, binding to potential ligands present on diverse tumor cells. Subsequently, we narrowed our focus to conduct an expanded expression analysis specifically for NKp46 ligands across various tumor cell lines. Our findings revealed that NKp46 ligands are expressed on both hematological and solid tumors, with notably higher expression levels observed in B-cell lymphomas. By comparing these ligands with those already known, we excluded the possibility that the tumor-expressed ligand was externalized calreticulin (ecto-CRT). This suggests the presence of novel, yet undiscovered NKp46 ligands on tumor cells, potentially heralding new therapeutic targets for cancers. Subsequently, we constructed four distinct NKp46-

based CARs, utilizing the extracellular domain of NKp46 as the antigen recognition module. We then generated CAR-T cells through lentiviral transduction of T cells and examined the expression profiles and *in vitro* antitumor efficacy of NKp46-based CAR-T cells. From this screen, we identified the most promising constructs. However, further exploration is warranted to delineate the specific NKp46 ligands expressed on tumor cells and refine the design of NKp46-based CAR-T cells for optimal therapeutic outcomes.

Four main lysis receptors have been identified in NK cells, including human CD16 and the natural cytotoxicity receptors NKp46, NKp44, and NKp30, which play a pivotal role in modulating tumor progression through their interaction with molecules featuring immunoreceptor tyrosine-based activation motifs (ITAMs) [19]. NKp46 stands as the first natural cytotoxicity receptor discovered in NK cells. It is constitutively expressed on the surface of NK cells, irrespective of their activation status, and its expression level is intricately linked with viral infections and tumor invasions [12, 20]. NKp46, alternatively known as natural cytotoxicity receptor 1 (NCR1), is encoded by the *NCR1* gene. It is a ~46 kDa Type 1 transmembrane protein and a member of the immunoglobulin superfamily. Across mammals, NKp46 is highly conserved and comprises two extracellular C2-type Ig-like domains, followed by a stalk region, and it associates with CD3ζ or FcRγ at the cell membrane [21, 22]. To date, most of the known ligands for NKp46 are derived from pathogen products, such as influenza virus haemagglutinin [23], herpes simplex virus ICP0 [24], fungal adhesins [25], an undefined surface ligand on pancreatic β cells [26], and soluble complement factor P [27]. Furthermore, NKp46 recognizes monocytes infected with mycobacterium tuberculosis by waveform proteins, and a 57-kDa molecule, vimentin, has been identified as a ligand for NKp46 via mass spectrometry [28]. Recent study has validated ecto-CRT as an endogenous ligand for NKp46, which is involved in NK cell recognition and control of Zika virus infection, the removal of endoplasmic reticulum stress, and senescent cells. However, this study did not delve into the expression of ecto-CRT in human tumors or its role in human tumor control [13]. Therefore, further validation is imperative to ascertain whether the identified ligand is a potential tumor-associated ligand for NKp46 and to explore the existence of other, yet-to-be-identified, tumor-associated ligands.

In summary, despite the existence of several studies on NKp46 ligands, which have identified specific candidates, these findings have yet to be confirmed at the tumor cell level. Our research revealed that NKp46 ligands are ubiquitously expressed in both hematologic malignancies and solid tumors, while their expression in normal tissue cells is scare. We utilized the potential interaction between NKp46 and ligands naturally expressed by tumor cells to identify tumor-associated ligands for NKp46. These ligands may differ from the currently discovered ecto-CRT, necessitating further investigation to pinpoint specific ligands. In addition, CAR-T cells engineered to target NKp46 ligands exhibit anti-tumor activity *in vitro*. However, the variations in anti-tumor efficacy among theses NKp46-baed CAR-T cells require more in-depth exploration, particularly *in vivo* studies to assess their therapeutic potential.

Conclusion

Through this study, we have discovered the existence of novel ligands for NKp46 on tumor cells, distinct from ecto-CRT. Our findings reveal that NKp46-based CAR-T cells exhibit significant anti-tumor effects on certain solid tumors. This suggests that these newly identified NKp46 ligands may represent a promising new target for cancer therapy, with the potential to advance the development of targeted CAR-T cell therapies. By providing candidate therapeutic targets for both antibody-based and cellular therapies targeting tumors, we anticipate facilitating the creation of new drugs that address these targets, ultimately benefiting patients with various types of cancer. As our next step, we plan to use recombinant NKp46 protein to specifically bind the NKp46 ligands present on tumor cells. Following this, we will employ immunoprecipitation coupled with mass spectrometry to determine which ligands interact with NKp46. Additionally, we will further validate the identity of these potential ligands of NKp46 through the use of NKp46- blocking antibodies or CRISPR-Cas9 knockdown assays.

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Ethics Approval and Consent to Participate

This study was approved by the Ethical Committee of Shenzhen Third People's Hospital (No. 2023-069-02). All donors provided written informed consent prior to the collection of blood samples.

Consent for Publication

All authors have approved the final paper for publication.

Availability of Data and Materials

Data supporting the findings of this study are included in the published article and its supplementary materials. Additional data are available from the corresponding author upon reasonable request.

Conflict of interests

The authors declare that there is no conflict of interests.

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