# Antibody Bank: the Synthetic Cell Repertoire for Immunotherapy

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**Abstract**

Despite promising achievement, current immunotherapy is impeded by disease heterogeneity, cellular plasticity and treatment induced evolution. Here, we proposed a novel artificial bionic immune cell system based on synthetic-receptor library and genetic circuits. In this system, a pre-established population of genetically engineered lymphocyte repertoire was developed, and a particular antigen can activate only its specific counter cell, enable that specific cell to survival and multiply under specific artificial environment, destroying particular antigens. Two synthetic patterns were postulate based on CAR receptors and synNotch receptors, in which the antibody library took place of the antigen-recognition region. An inhibited expression of the inducible caspase-9 suicide gene is coupled with the activation of the synthetic receptor, allowing the lymphocyte repertoire to co-evolve with the dynamic expression of the antigens. In theory, if the diversity of lymphocyte repertoire is sufficient, we can use this artificial system for the treatment of any disease of mankind.

## Introduction

Significantly progresses have been made by modern immunotherapy over the past decade, which is driven by the targeted therapies that inhibit tumor angiogenesis and intrinsic drivers of cancer cell growth, immunomodulatory therapies that enhance host antitumor immunity, as well as genetically engineered autologous or allogeneic immune-cells expressing chimeric antigen receptors (CARs) or T-cell receptors (TCRs) as cellular therapy.

However, all the current immunotherapy relay on the development of “specific targets”. In one aspect, identification the underlying cell-autonomous, genetic driving targets of tumorigenesis led to the development of clinically important blockers that result in profound, but often not durable, tumour responses in genetically defined patient populations, such as EGFR or HER2 blockers. Another approach exporting the targets involved with protective tumour immunity has become a hot research area, most notably the ‘immune checkpoint’ antibodies that reverse the negative regulators of T cell function, that accomplish durable clinical responses in subsets of patients with various tumour types. Adoptive CAR based therapies, although shows very encouraging efficiency, also relay the construction of specific target binding domain, which is usually achieved by antigen-recognition regions in the form of a single-chain variable fragment (scFv) or a binding receptor/ligand in the extracellular domains. In fact, the theory “targeting a specific target” is a dilemma in the context of cellular plasticity and evolution, not to mention genetic or epigenetic heterogeneity of the disease at every level from molecular to human population.

Interestingly, our immune system never work dependent on “specific targets”. Our immune cells as the nature defenders to protect invading pathogens and make surveillance effect on tumorigenesis. One scientific theory that descripts the functions and procedures adopt by lymphocytes to hand wide variety of antigens that attack the body[1] is the Burnet's clonal selection theory. Introduced and promoted by Frank Macfarlane Burnet, the clonal selection theory explained the formation process of diverse antibodies or immunoglobulin-like protein in the initial stages of the immune response. In the immune system, Some of the lymphocytes with diverse immunoglobulin-like protein

are tolerant to self-tissues, while others are intolerant to self-tissues. However, notably, only cells that tolerate self-tissues survive the embryonic stage of development. In addition, the introduction of a nonself-tissue leads to the development of lymphocytes that include nonself-tissues as part of their self-tissues[2,3]. Frank Macfarlane Burnet proposed the clonal selection theory to explain and examine the functions of lymphocytes in the immune system and to assess how they respond to specific antigens that invade the body. Moreover, the theory provides a basis for understanding how the immune system responds to infections and how B and T lymphocytes are usually selected to destroy particular antigens.

In this report, we designed and characterized an artificial bionic Immune cell system based on synthetic-receptor library and genetic circuit. We postulates that in a preexisting group or population of genetically engineered lymphocytes, a particular antigen can activate only its specific counter cell, enable that specific cell to survival and multiply under specific artificial environment and to destroy particular antigens. When the diversity of genetically engineered lymphocytes is sufficient to accurately and precisely recognize any pathogen or disease-antigen, in theory the cell population has the captivity to combat any disease of mankind.

## Results

### Design and Characterize a synthetic Chimeric antigen receptor T cell library

Here we first proposed a design of a logic-gated Chimeric antigen receptor T cell library. ScFv library was used to fused to a CD8a hinge and transmembrane domain and the intracellular domains of human 4-1BB and CD3ζ (or z), consistent with the second generation CAR design. The scFv library can be customized. Unpaired cysteine 164 within the CD8a hinge region was replaced with a serine to increase CAR expression as reported previously[4]. The cDNA sequences containing the fusion constructs were cloned under control of the moderately strong constitutive human PGK promoter. We next constructed a lentiviral vector encoding a Gal4-KRAB transcription inhibitor under a control of a 6×NFAT-IL2min promoter. An inducible caspase-9 suicide gene was placed downstream of the Gal4-KRAB transcription inhibitor with under a control of a

UAS-SV40 combined promoter. Because the KRAB protein has been demonstrated to be capable of inhibiting all promoters within at least 3 kB [5] and the forward construct would therefore inhibit the 6×NFAT-IL2min promoter, we generated the opposite construct, where the two expression cassettes are cloned in a manner where both promoters are at the opposite ends at a long distance (Fig. 1A).

We sought to determine the kinetics of the genetic circuit, in which NFAT coupled with KRAB activation in engineered T cells after CAR stimulation. Therefore, we use engineered T cells with either a EGFR-specific or a HER2-specific CAR in our experiments first. We confirmed EGFR-CAR and HER2-CAR expression by staining with anti-Myc mAb, respectively (Fig. 1B). We then investigated the antitumour potential of the transduced T cells by standard $^{51}$Cr-release assays using MCF-7 cells (EGFR and HER2-negative cells), MCF-7 EGFR cells (a derivative engineered to express EGFR), MCF-7 HER2 cells (a derivative engineered to express HER2). The MCF-7 cells and derivative cells were characterized in our previously study[6]. CAR-T cells transduced with cetuximab scFv (termed CAR-T-CTX) efficiently lysed EGFR-positive cells, such as MCF-7 EGFR cells, but did not kill MCF-7 cells or MCF-7 HER2 cells. On the other hand, CAR-T cells transduced with trastuzumab scFv (termed CAR-T-TTZ) efficiently lysed HER2-positive cells, such as MCF-7 HER2 cells, but not MCF-7 cells or MCF-7 HER2 cells (Fig. 1C). Then, we performed co-culture experiments of engineered T cells and different MCF-7 stimulator cells to test whether the addition of the small molecule dimerizer AP1903 after different time would affect apoptosis of engineered T cells. Our data shows that the small molecule dimerizer AP1903 had no effect on the viability of CAR-T while AP1903 but induced significant apoptosis/necrosis of engineered T cells after 4h expose to stimulatior cells. However, 36 hour-stimulation of the engineered T cells to antigens notably alleviate the pro-apoptosis effect of AP1903 and both EGFR-specific or a HER2-specific CAR T cells shows resistant to AP1903 in 72 hours (Fig. 1D).

**Potent anti-tumor effect of Chimeric antigen receptor T cell library**

As a proof of concept, We therefore expressed a small library of 10 CAR constructs, comprising an antigen-specific scFv (CTX or TTZ) and 8 negative control scFvs derived

from 8 well-characterized antibodies, which showed negligible binding on MCF-7 cells and MCF-7 derivatives (Fig. S1 and Table S1). A CAR-T cell library of 10 control CAR constructs was also constructed and termed as CAR-$T_{Control}$ library. We confirmed uniform expression of each of the 10 negative control CAR construct on the cell surface in engineered T cells. Before the following experiments, The CAR-T therapeutic library is development that CAR-T cells were mixed that CAR-T CTX, CAR-T-TTZ and other 8 unique CAR-T cells were equally distributed in the populations, termed as CAR-$T_{CTX\text{-}TTZ}$ library.

Next, we sought to study the growth dynamics of the different CAR-T cell populations with different stimulator cells. We co-cultured the MCF-7 cells and MCF-7 derivatives with CAR-$T_{CTX\text{-}TTZ}$ library for 10 days and the CAR-T cell proliferation, the percentage αEGFR$^+$ CAR-T cells or αHER2$^+$ CAR-T cells as well as the cytotoxicity of CAR-T cells were analyzed every day (Fig. 2). Interesting, our data shows that also the cell number of CAR-$T_{CTX\text{-}TTZ}$ library were increased (Fig. 2A), the percentage of αEGFR$^+$ CAR-T cells or αHER2$^+$ CAR-T cells as well as the cytotoxicity effect did not significant evaluated as aspect (Fig. 2B and C). Next, we add AP1903 in the co-culture system at day 5. And our data shows that the cell number of CAR-T cells were markedly dropped in day 7, however, the percentage of αEGFR$^+$ CAR-T cells or αHER2$^+$ CAR-T cells were increased (Fig. 2D and E). At day 7, CAR-$T_{CTX\text{-}TTZ}$ library show notable cytotoxic effects on EGFR-expressing cells or HER2-expressing cells (Fig. 2F). These data suggested that AP1903 improved the artificial screening process of CAR-T cells.

**In vivo anti-tumor effect of Chimeric antigen receptor T cell library**

We next examined an anti-tumor ability of CAR-$T_{CTX\text{-}TTZ}$ library in vivo. Immunodeficient NSG mice xenografted with MCF-7, MCF-EGFR and MCF-HER2 were kept untreated until a formation of tumor at approximately 50 $mm^3$ size and then treated with $1\times 10^7$ CAR-$T_{Control}$ library and CAR-$T_{CTX\text{-}TTZ}$ library ($1\times10^6$ cells for each CAR construct containing T cells). One week later, three doses of AP1903 (50 μg each) were treated with mice intraperitoneally (i.p.) for every two days (Fig. 3A)..

For MCF-7 xenografts, the tumor growth kinetics was similar among mice receiving CAR-$T_{Control}$ library and CAR-$T_{CTX\text{-}TTZ}$ library. Tumors progressed rapidly, reaching a volume of 1,000$mm^3$ in less than 35 days. Compared with the MCF-7 xenografts, mice

receiving CAR-$T_{CTX\text{-}TTZ}$ library therapy significantly showed inhibited growth of MCF-7 EGFR tumors or MCF-7 HER2 tumors at about two weeks later after treatment, and the tumors shows almost complete regression at the end of the experiments. After the experiments, all mice were sacrificed and αEGFR$^+$ CAR-T cells or αHER2$^+$ CAR-T cells were measured by the frequencies of different CAR-T cells in CAR-positive T cells in blood, bone marrow, spleen and liver of animals by flow cytometry. Our data shows that administration of the small-molecule dimerizer resulted in a striking reduction in non-specific CAR-T cells in the blood and tissues of the treated mice (Fig. 3B and C).

**A Chimeric antigen receptor T cell library based on Phage Display Technology**

To construct a nonimmunized human single-chain variable fragment (scFv) library, blood samples from 200 nonimmunized, healthy volunteers were collected. B lymphocyte cDNA encoding variable fragment was used to construct a phage display scFv library that consisted of ~ $1\times10^8$ individual colonies. The scFv gene corresponded to the size of the insert of over 98% of the colonies. To confirm the heterogeneity of the individual clones from the library, we sequenced 50 randomly selected clones, and each clone showed a distinct scFv sequence.

To exclude phage binding to normal tissues, we used blood as the source of scFvs that did not bind to systemic organs. Four rounds of in vivo phage display in NSG mice were performed to collect this “negative” phage population (Fig. 4A). The DNA sequences of the isolates were analyzed; this confirmed that phage diversity was maintained. Previously, anti-EGFR CAR-T cells delayed the growth of SW480 colon tumors but unexpectedly led to the outgrowth of EGFR-negative tumor cells[7], therefore, we use this model as resistant model. A similar DNA analysis was performed after completion of four rounds of in vivo phage display in NSG mice with SW480 xenografts (Fig. 4B). About 450 unique scFv sequences, indicative of tumor binding scfvs, were identified in this manner. A CAR-T cell library was further development based on the CAR constructs derived from the scFvs, termed as CAR-$T_{sw480}$ library. As we use the standard transfection methods to generated CAR-T cell library, thus, the introduction of DNA encoding a repertoire of CAR genes has the potential to introduce multiple CAR genes into each cell.

Next, Immunodeficient NSG mice xenografted with SW480 cells were kept

untreated until a formation of tumor at approximately 50 $mm^3$ size and then treated with $1\times 10^7$ control T cells or CAR-T CTX cells (Fig, 4C). Consistent with previous report, tumor growth was significantly inhibited by anti-EGFR based cell therapy[7]. However, at about 3 weeks, mice treated with CAR-T CTX eventually succumbed to the tumor. Mice were then threated with $1\times 10^6$ CAR-$T_{sw480}$ library at Day 25, and one week later, three doses of AP1903 (50 μg each) were treated with mice for every two days. Interestingly, mice receiving CAR-$T_{sw480}$ library therapy significantly showed inhibited growth of tumors at about three weeks later after treatment, and the tumors shows complete regression in two weeks. After the experiments, all mice were also sacrificed and the $\alpha EGFR^+$ CAR-T cells was not detected in all the tested mice. CAR-positive T cells were sorted and sequenced and we are able to defined five unique scFv clones.

**Design a large-scale logic-gated synthetic receptor NK cell library**

We then aim to develop a large-scale synthetic cell library for immunotherapy (over $10^5$), therefore we sought to develop a novel library platform using NK-92 cells. As previously report[8] shows that genetically engineered NFAT or NFkB promoters shows leaky expression in “resting” non-activated NK-92 cells, we choose an alternative approach based on synNotch receptor. As show in Fig.5A, an antigen-sensing synNotch library releases orthogonal transcription factor that drives expression of a fused construct of anti-EpCAM CAR and a TetR-KRAB transcription inhibitor. An inducible caspase-9 suicide gene was placed downstream of the transcription inhibitor with under a control of a TRE-CMV combined promoter.

The anti-EpCAM scFv can binds to SW480 cells and anti-EpCAM CAR NK-92 cells also shows cytotoxicity to SW480 cells *in vitro* (Fig. 5B and C). Next, a NK-92 cell library contain $5\times 10^5$ individual synNotch constructs was developed based on the nonimmunized human scFv library, which has showed no binding to systemic organs of NSG mice in our above described experiments.

We next characterized the anti-tumor effect of NK-92 cell library in vivo (Fig. 5D). NSG mice xenografted with SW480 cells were treated of $1\times 10^7$ NK-92 library or parental NK-92 cells twice per week for one week, and one week later, three doses of AP1903

were treated with mice for every two days. Our data shows that tumors grew rapidly in control mice injected with medium, while with parental NK-92 cells, delay the growth of the tumor for 10 days. Tumors with NK-92 cell library treatment did not show any inhibition until about one month later, and the tumor growth is inhibited then. Completely regressed tumors were observed in 4 of the 8 tumors under therapy, and two mice was sacrificed during the 7 and 8 weeks for too large tumors. After the experiments, all mice were also sacrificed and the synNotch-positive NK-92 cells were sorted and sequenced and we are able to defined 11 unique scFv clones. Interestingly, one scFv clone is the same with the one we get form the CAR-$T_{sw480}$ library.

**Discussion**

Cancer remains a leading cause of mortality and poor health worldwide health worldwide[9]. Despite the enormous effort expended in the development of therapeutics, successful control and eradication of the disease have remained elusive[10,11]. In addition, risk factors such as physical inactivity, obesity, and smoking shows an increased worldwide prevalence, promoting the incidence of malignancy[12,13]. Moreover, our understanding of cancer genetics and biology has also been updated: cancer progression and development is an evolutionary processes driven by somatic cell mutations and subclonal selection [14], a groundbreaking theory developed by Peter Nowell and Macfarlane Burnet. Their unique theories have explicit parallels to Darwinian natural selection, in which cancer is viewed as an asexual process that produces single mutated cells and quasispecies [14,15]. Modern cancer genomics and biology have validated the concept that cancer development is a complex, adaptive, and Darwinian process.

In humans, husbands of millions of different antibodies (or immunoglobulin like proteins) are created by the immune system. Antibody display technologies, such as Phage, ribosome, yeast as well as mammalian cell, have turned into mainstream antibody and protein engineering platforms nowadays and also become one major technology for antibody discovery for therapeutic usage.

Antibody libraries can constructed based on different sources, such as immunized animals[16] or naturally immunized, or infected, humans[17], or naive immune system, which can be derived from nonimmune natural or computational-synthetic sources. When the

diversity is sufficiently large, the library has the capacity to generated antibodies against a large number of different antigens, including self, nonimmunogenic and toxic antigens, and for this reason these libraries are now extensively used in the industry and in academia[18,19]. Usually, panning of antibodies to a specific antigen using nonimmune libraries or from synthetic antibody libraries diversified in just one or two CDRs or in just one of the two chains results in the discovery of medium-affinity antibodies, which reflects the situation in transgenic mice with restricted antibody repertoires[20,21]. Interestingly, CARs constructed with high-affinity antibody fragments recognized target expressed at any level, including at levels in normal cells that were undetectable by flow cytometry, while CAR-T cells harbor medium-affinity CAR exhibited robust antitumor efficacy similar to high-affinity cells, but spared normal cells expressing physiologic target levels[22]. This suggested that the synthetic cell repertoire may not need the most successful antibody libraries in which the display (natural or synthetic) diversity in multiple CDRs and routinely yield single-digit nanomolar and sometimes subnanomolar affinity antibodies—the latter having affinities equal to the affinities of antibodies regularly isolated from immunized mice or from recombinant immune libraries. In general, the size of the library are proportional to the antibody affinities: up to 10 nM for libraries with $10^7$ to $10^8$ clones, and up to 0.1 nM for the best libraries with over $10^{10}$ members. Using such libraries, thousands of antibodies that bind distinct epitopes on the same target antigen can be achieved with high throughput screening [23,24]. Therefore, a synthetic cell repertoire with $10^6$ clones should be a very successful cell repertoire for therapeutic propose.

In this report, we postulate that the mating of the mammalian-cell antibody display library with synthetic genetic circuit builds a novel ‘molecular evolution machine’ which can function in vivo. With chimeric antigen receptors and immune cells, the new platform can directly be selected and screened for therapeutic effect but not only binding affinity. Moreover, the whole human antibody repertoire also can be preserved when one is healthy body condition, and use it to develop synthetic immune cell library for therapeutic usage when need, that is the definition of “antibody bank”, which may be the promised ‘land’ that Paul Ehrlich wrote about over 100 years ago[25]: “the land which […] will yield rich treasures for biology and therapeutics.

## Methods

### Cell lines

MCF-7, SW480 and 293T cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The identities of the cell lines were verified by STR analysis, and the cell lines were confirmed to be mycoplasma free. The cells were maintained in DMEM with 10% foetal bovine serum. Cell culture media and supplements were obtained from Life Technologies, Inc. Human NK-92 cells (ATCC) were propagated in X-VIVO 10 medium (Lonza) supplemented with 8% heat-inactivated human plasma (German Red Cross Blood Donation Service Baden-Württemberg–Hessen, Frankfurt, Germany) and 100 IU/ml IL-2 (Proleukin; Novartis Pharma, Nürnberg, Germany). Residues 1–619 of the extracellular domain of EGFR (EGFR-ECD) and residues 1–646 of HER2-ECD were prepared using the pcDNA3.4 expression vector (Invitrogen) and FreeStyle 293 expression system (Invitrogen).

### Vector construction

The sequence encoding the scFv antibodies generated from cetuximab, trastuzumab, CH65, 9.8B, 2F5, F10, 7D11, 8D6, omalizumab, TE33, R10 and HC33.8 was chemically synthesized. The information of the sequence can be found in the Protein Data Bank. The 4D5-5 scFv derived from trastuzumab was chosen for this study as we reported previously[6]. As shown in Fig. 1a, the CAR design contained the human CD8α signal peptide followed by the scFv linked in-frame to the hinge domain of the CD8α molecule, transmembrane region of the human CD8 molecule, and intracellular signalling domains of the CD137 and CD3ζ molecules. The constructs were further cloned into the pHR vector backbone under control of the PGK promoter. To generate CAR response elements, a CAR inducible promoter contains six NFAT-REs in a minimal IL-2 promoter4 were placed upstream of the transcription factor Gal4-KRAB. Moreover, the inducible caspase-9 suicide gene that induces apoptosis upon specific binding with the small molecule dimerizer CID AP1903 was placed downstream the expression under the control of SV40/UAS combined promoter. Because the KRAB protein has been demonstrated to be capable of inhibiting all promoters within at least 3 kB [26] and the

forward construct would therefore inhibit other promoters, we generated the opposite construct, where the two expression cassettes are cloned in a manner where both promoters are at the opposite ends at a distance over 4 kb. 2A peptide sequence was intercalated between iCASP9 and the GFP tag.

synNotch receptors and response elements were obtained from Addgene (Addgene plasmids #79123 and 79125). ScFv library with N-terminal CD8α signal peptide were fused onto the synNotch-Gal4VP64 receptor backbone (Addgene plasmid #79125) in place of the CD19-specific scFv. To generate response elements, the mCherry gene segment in Addgene plasmid #79123 was replaced with the described anti-EpCAM CAR transgenes. To express TetR-KRAB fusion protein concomitantly with CAR, 2A peptide sequence was intercalated between the two genes. ICASP9 and GFP fusion construct was laced downstream the expression under the control of the Tet-cytomegalovirus (CMV) promoter[26].

**Antibody Libirary**

The protocol for antibody library construction has been report in our previously report[27]. Brefly, Peripheral blood mononuclear cells were isolated from blood collected with Leucosep tubes (Greiner Bio-One, Kremsm¨unster, Austria). Total RNA was extracted, and nested PCR was used to clone genes with single domain antibodies consisting of the heavy chain and light chain variable domain. The phagemid vector pCANTAB5E (GE Healthcare) carried the final PCR products and was introduced into electrocompetent Escherichia coli TG1 cells that were freshly prepared. The cells were selected on lysogeny broth agar plates supplementedwith ampicillin and glucose cultured overnight at 37°C. After being scraped from the plates, the colonies were stored at 280°C in lysogeny broth supplemented with 20% glycerol.

**Cytotoxicity assays.**

Cytotoxicity of the CAR-expressing T cells or exosomes was tested in a standard 4-h $^{51}$Cr-release assay[6]. Target cells were labelled with 51Cr for 1 h at 37 °C. Radioactive $^{51}$Cr (50 μCi) was used to label $1 \times 10^6$ target cells. One hundred microliters of labelled target cells (n = 5000) as plated in each well of a 96- well plate. Effector cells were added at a volume of 100 μl at different E:T ratios. Exosomes were added at different concentrations. CAR-T cells or exosomes and targets were incubated together for 4 h at

37 °C. The supernatant (30 μl) from each well was collected and transferred to the filter of a LumaPlate. The filter was allowed to dry overnight. Radioactivity released into the culture medium was measured using a β-emission-reading liquid scintillation counter. The percentage of specific lysis was calculated as follows: (sample counts – spontaneous counts)/(maximum counts – spontaneous counts) × 100.

**Cell growth assays**

Cells were washed and labeled with AlamarBlue (Invitrogen) w and fluorescence was read using a 96-well fluorometer with excitation at 530 nm and emission at 590 nm. The results are expressed in relative fluorescence units (RFU) compared with the data of the first day.

**Activation of suicide gene in vitro and validation in vivo**

The small molecule dimerizer AP1903 (10nM, MCE) was added to indicated NK-92 cell cultures. The elimination of transduced cells was evaluated by Annexin-V staining. The efficacy of the suicide gene was tested in vivo by treating tumor-bearing mice that had received synthetic cell treatment with indicated doses of AP1903 (50 μg each) intraperitoneally (i.p.)[28].

**In vivo study**

In vivo experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Second Military Medical University, and mice were housed in a specific pathogen-free barrier facility. Cancer cells were inoculated into NSG mice. (Shanghai Model Organisms Center, Inc.) When the tumour volumes reached an average of approximately 50mm$^3$, the mice were randomly divided into groups of 8 mice each. The mice were injected i.p. with 200 mg/kg cyclophosphamide to further deplete the host lymphocyte compartments. The tumours were measured with digital callipers, and the tumour volumes were calculated by the following formula: volume = length × (width)$^2$/2. For cell treatment, genetically engineered cells were i.v. injected into mice with the indicated dose. Mice were i.p. injected with 2000 units of IL-2 twice a week following infusion. Mice were killed if the volume of the tumours reached 1500 mm$^3$.

**Statistical analysis**

Unless otherwise specified, Student's t-test was used to evaluate the significance of difference between 2 groups, and ANOVA was used to evaluate differences among 3 or

more groups. Differences between samples were considered statistically significant when $P < 0.05$.

## Conflicts of interest

Y.L., and M.D. declare they are employees of Pharchoice Therapeutics Inc. (Shanghai). M.D. is a shareholder at Pharchoice Therapeutics Inc. (Shanghai). The other authors declare no competing interests.

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## Figure Legends

**Figure 1. Design and characterize a synthetic chimeric antigen receptor T cell library. (A)** Map of lentiviral constructs encoding CAR library and CAR repressive iCASP9. **(B)** Membrane-bound CAR expression. Forty-eight hours after retroviral transduction, the expression of CAR on human T cells was detected by staining with anti-MYC antibody, followed by flow cytometry analysis. T cells without transduction were used as a negative control. The histograms shown in black correspond to the isotype controls, whereas the red histograms indicate the positive fluorescence. **(C)** Killing activity of CAR-T cells in response to tumour cells. The cytotoxic activity of CAR-T and control T cells against MCF-7 cells was assessed by a 51Cr-release assay at the indicated effector-to-target (E:T) ratios. **(D)** CAR-T cells were first stimulate with different MCF-7 cells for 24 or 72 hours. Addition of 10 nM of AP1903 to cultures induced apoptosis/necrosis of CAR-T as assessed by annexin-V staining in 4 independent experiments. The dimerizer did not induce apoptosis in T cells without transduction.

**Figure 2. In vitro anti-tumour effect of chimeric antigen receptor T cell library. (A and D)** Cell proliferation of CAR-T cells was measured using AlamarBlue staining. The results are expressed in relative fluorescence units (RFU). **(B and E)** The proportions of

cells with positive anti-EGFR or anti-HER2 expression are shown. **(C and F)** Killing activity of CAR-T cells in response to tumour cells. The cytotoxic activity of CAR-T and control T cells against MCF-7 cells was assessed by a $^{51}$Cr-release assay at the indicated E:T ratios.

**Figure 3. In vivo anti-tumor effect of chimeric antigen receptor T cell library. (A)** Tumour volumes of MCF-7 tumour xenografts after the indicated treatment. n= 8. **(B)** Frequencies of CAR-positive T cells in blood, bone marrow, spleen and liver of xenografts by flow cytometry. **(C)** The proportions of cells with positive anti-EGFR or anti-HER2 expression in CAR-positive T cells are shown.

**Figure 4. A Chimeric antigen receptor T cell library based on Phage Display Technology. (A)** Negative selection: an in vivo phage display was performed to collect non-binding phages to normal tissues in mice. **(B)** After negative selection, specific scFvs homing to the cancer cells were identified by in vivo phage display in NSG mice bearing SW480 xenografts. **(C)** Tumour volumes of SW480 tumour xenografts after the indicated treatment. n= 8. Arrows indicate the treatment point.

**Figure 5. Large-scale Logic-gated Chimeric antigen receptor NK cell library. (A)** Map of lentiviral constructs encoding anti-EpCAM CAR, SynNotch library and SynNotch repressive iCASP9. **(B)** Antibody-binding assay. Different scFvs were evaluated for their ability to binding to SW480 cells. **(C)** Killing activity of CAR-T cells in response to tumour cells. The cytotoxic activity of CAR-T and control T cells against MCF-7 cells was assessed by a 51Cr-release assay at the indicated E:T ratios**. (D)** Tumour volumes of SW480 tumour xenografts after the indicated treatment. n= 8.

Figure 1

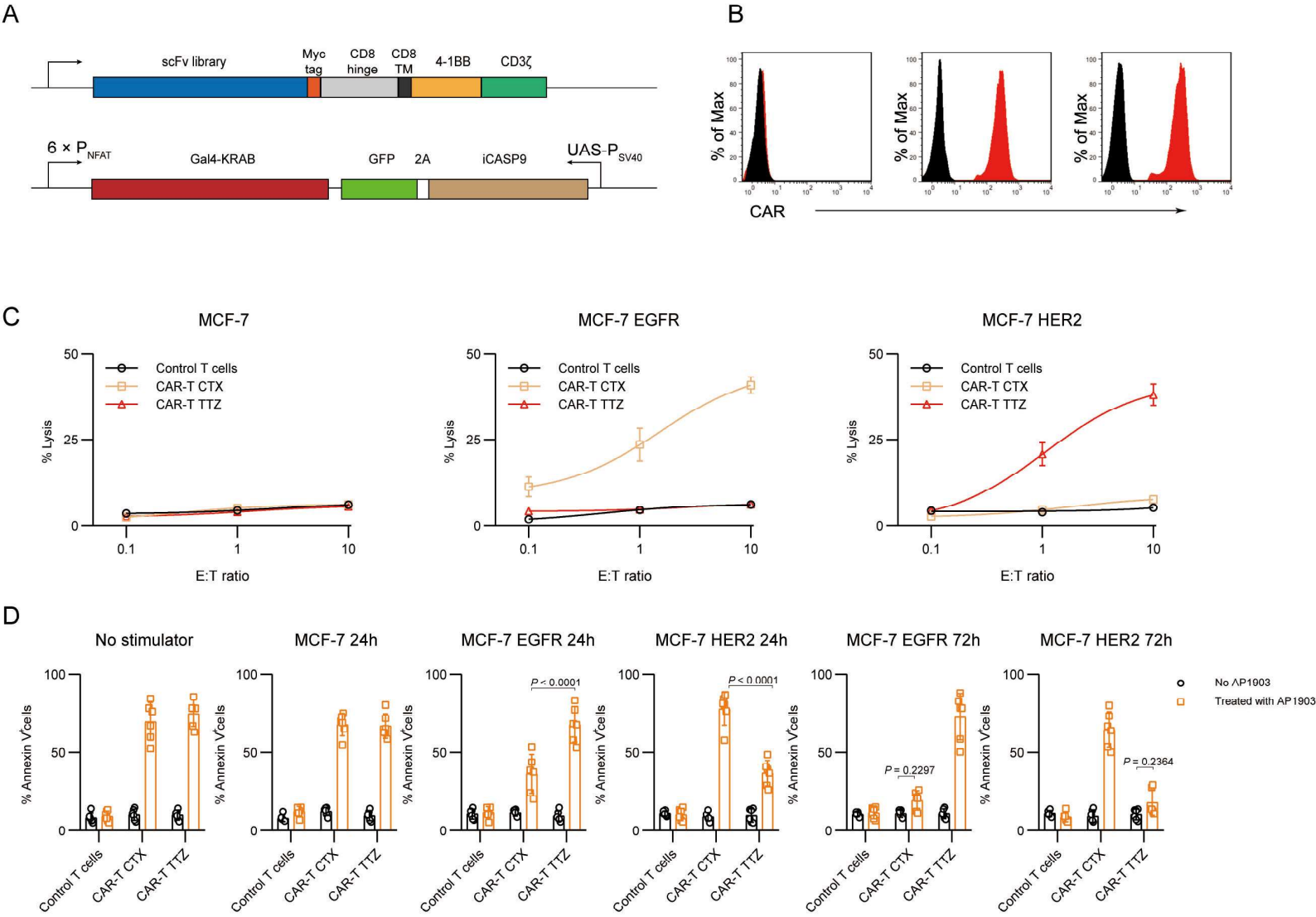

A
scFv library
Myc tag
CD8 hinge
CD8 TM
4-1BB
CD3ζ
6 × P NFAT
Gal4-KRAB
GFP
2A
iCASP9
UAS-P SV40
B
% of Max
CAR
C
MCF-7
MCF-7 EGFR
MCF-7 HER2
Control T cells
CAR-T CTX
CAR-T TTZ
% Lysis
E:T ratio
D
No stimulator
MCF-7 24h
MCF-7 EGFR 24h
MCF-7 HER2 24h
MCF-7 EGFR 72h
MCF-7 HER2 72h
% Annexin V+cells
P < 0.0001
P = 0.2297
P = 0.2364
No AP1903
Treated with AP1903

Figure 2

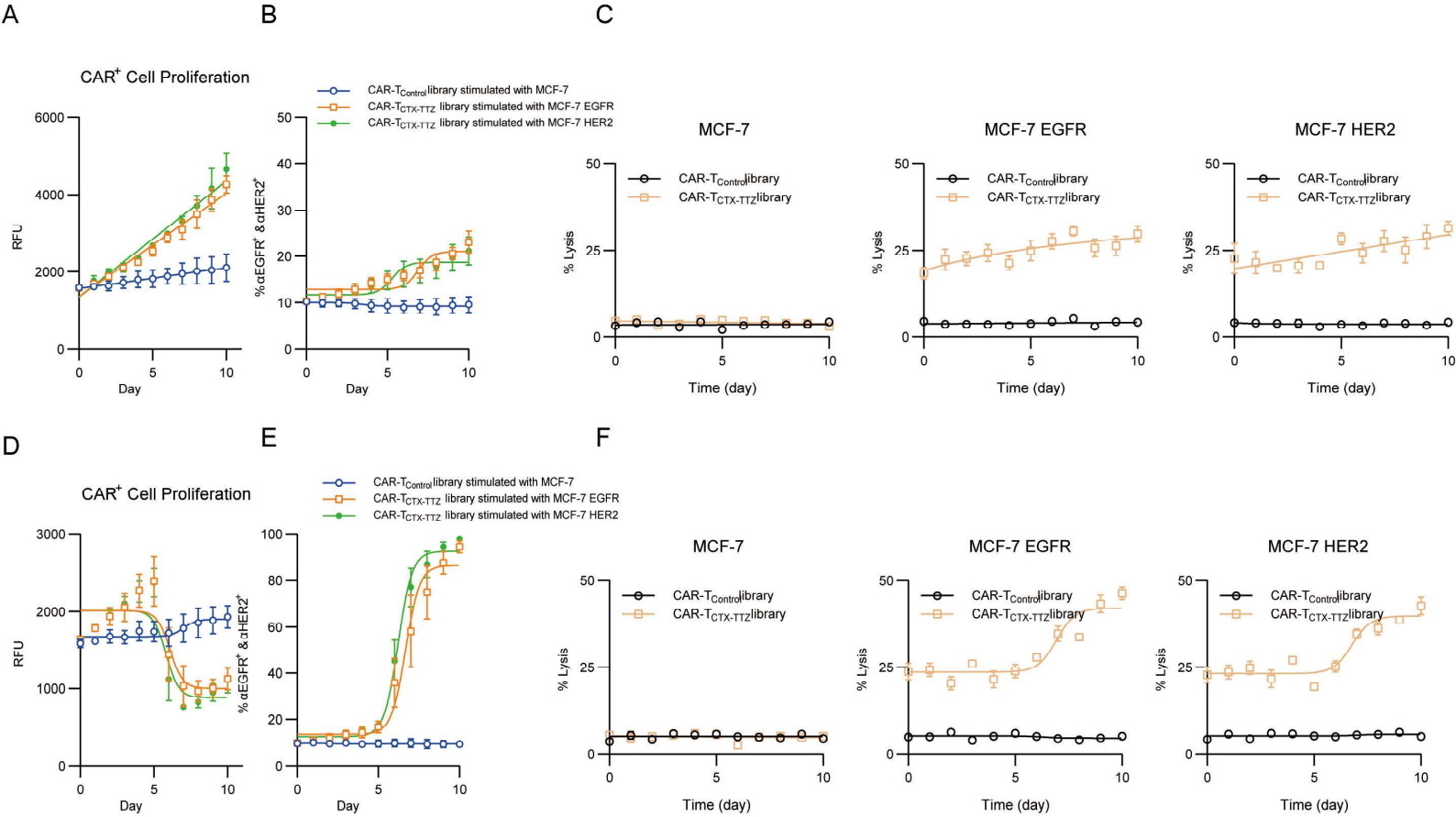
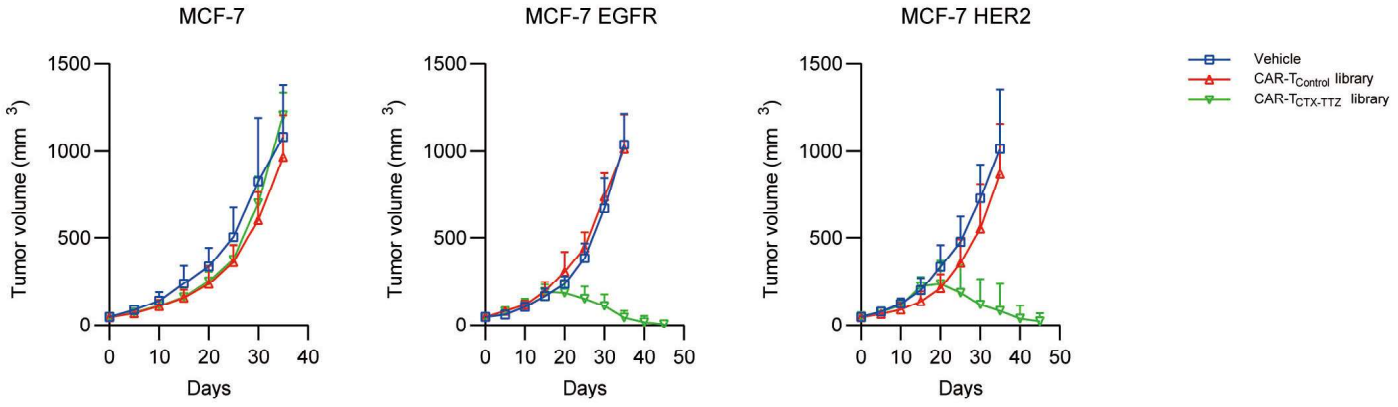
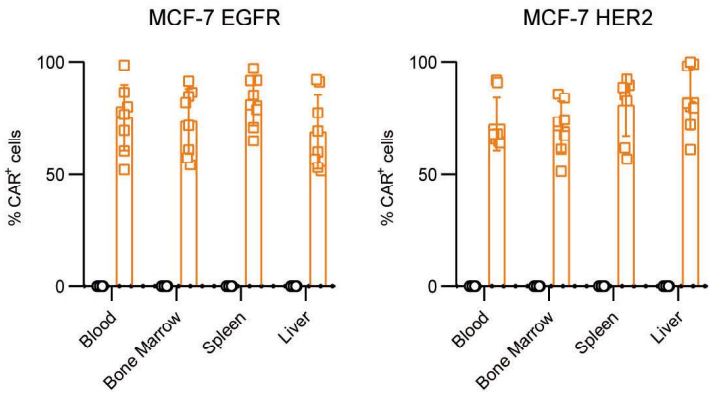

A
B
C
CAR+ Cell Proliferation
RFU
Day
CAR-TControl library stimulated with MCF-7
CAR-TCTX-TTZ library stimulated with MCF-7 EGFR
CAR-TCTX-TTZ library stimulated with MCF-7 HER2
% αEGFR+ & αHER2+
MCF-7
MCF-7 EGFR
MCF-7 HER2
CAR-TControl library
CAR-TCTX-TTZ library
% Lysis
Time (day)
D
E
F
CAR+ Cell Proliferation
RFU
Day
CAR-TControl library stimulated with MCF-7
CAR-TCTX-TTZ library stimulated with MCF-7 EGFR
CAR-TCTX-TTZ library stimulated with MCF-7 HER2
% αEGFR+ & αHER2+
MCF-7
MCF-7 EGFR
MCF-7 HER2
CAR-TControl library
CAR-TCTX-TTZ library
% Lysis
Time (day)

Figure 3

A


MCF-7
MCF-7 EGFR
MCF-7 HER2
Tumor volume (mm 3)
Days
Vehicle
CAR-TControl library
CAR-TCTX-TTZ library

B


MCF-7 EGFR
MCF-7 HER2
% CAR+ cells
Blood
Bone Marrow
Spleen
Liver

C

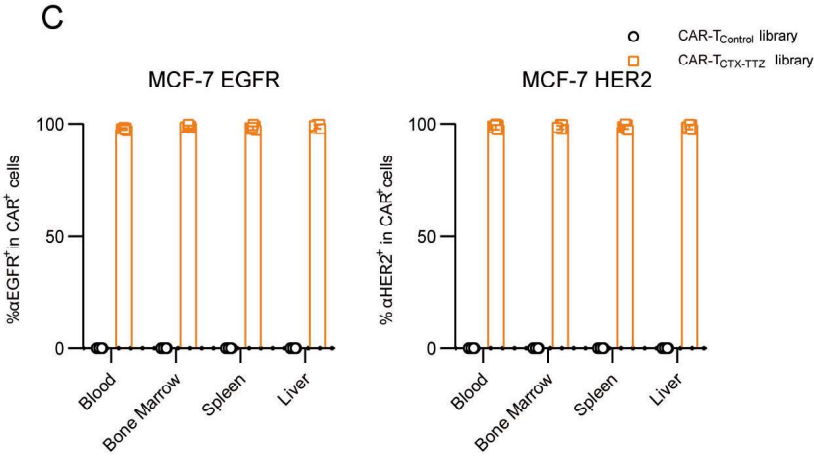
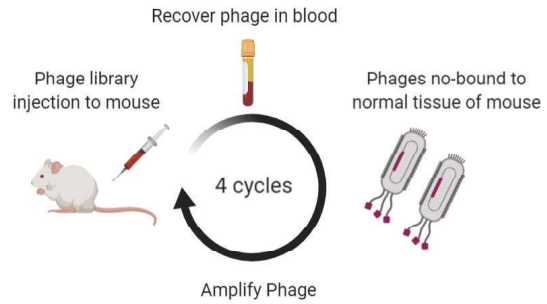
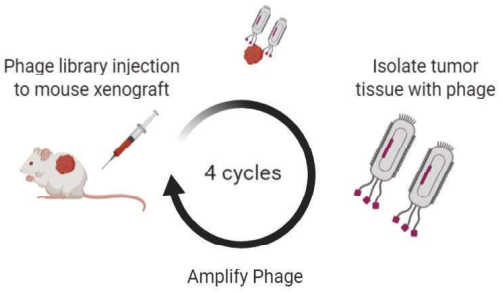

CAR-TControl library
CAR-TCTX-TTZ library
MCF-7 EGFR
MCF-7 HER2
%αEGFR+ in CAR+ cells
% αHER2+ in CAR+ cells
Blood
Bone Marrow
Spleen
Liver

Figure 4

A


Recover phage in blood
Phage library
injection to mouse
Phages no-bound to
normal tissue of mouse
4 cycles
Amplify Phage

B


Phage library injection
to mouse xenograft
Isolate tumor
tissue with phage
4 cycles
Amplify Phage

C

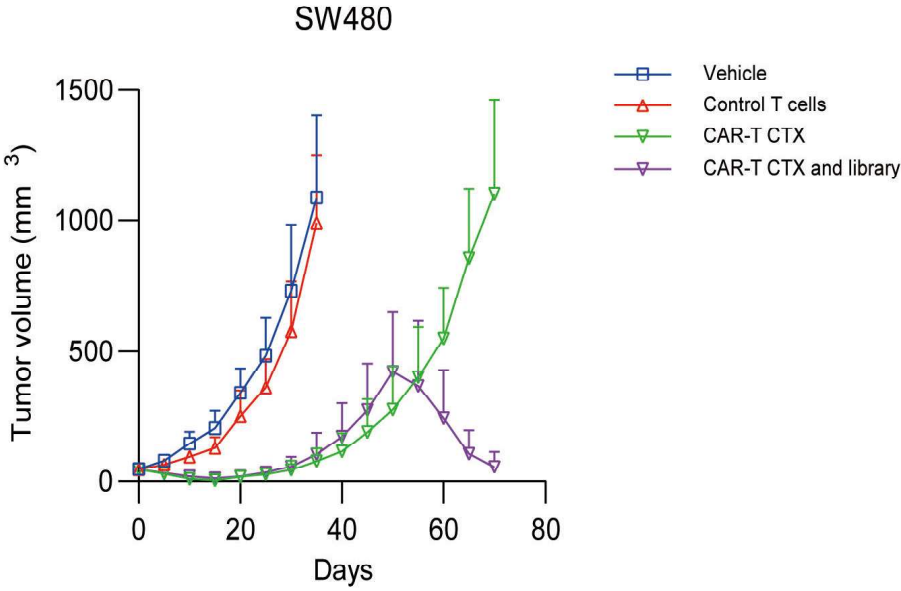
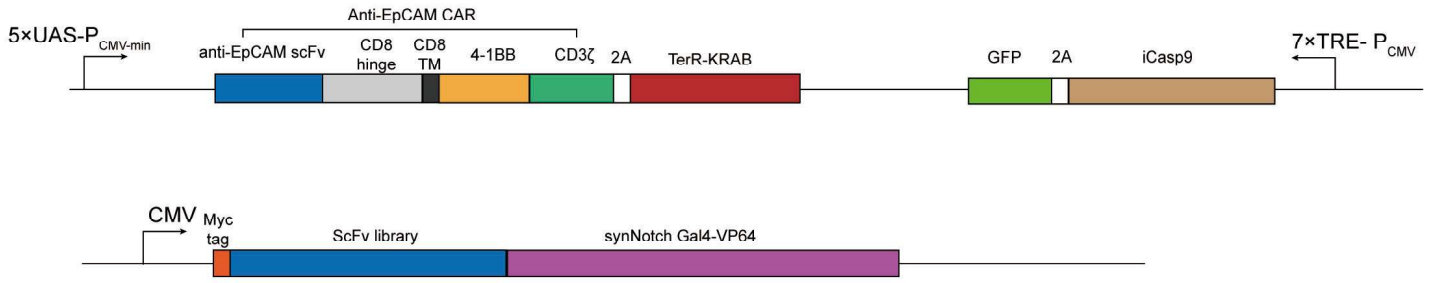
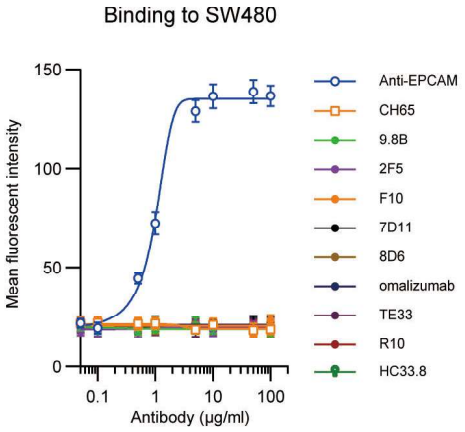

SW480
Tumor volume (mm 3)
1500
1000
500
0
0
20
40
60
80
Days
Vehicle
Control T cells
CAR-T CTX
CAR-T CTX and library

Figure 5

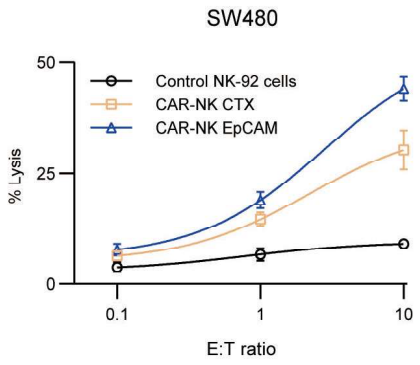
A

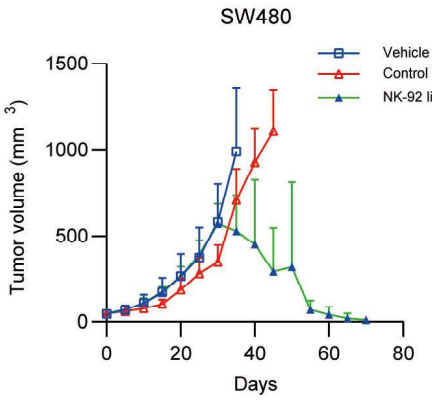



B




C




D