Development and Characterization of an Artificial Bionic Immune Cell System based on a Synthetic Receptor Repertoire

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Abstract

A functional and effective cell-based immunosurveillance system requires two necessary components: a repertoire of receptors derived from immunoglobulin-like molecules for diverse molecular recognition and a signalling pathway-based activation mechanism for sorting signals and enabling effective activation. Besides the MHC restricted recognition pattern adopt by the adaptive immune cells, such as T cells, and the “missing-self” mechanism discovered in innate immune, such as NK cells, we proposed another novel artificial bionic immune cell system based on a synthetic receptor repertoire and genetic circuits. For this system, a pre-established population of genetically engineered lymphocyte repertoires was developed in which a particular antigen can exclusively activate its specific counter cell, enabling that specific cell to survive and multiply under a specific artificial environment and to destroy particular antigens. Two synthetic patterns were postulated based on CAR receptors and synNotch receptors, in which the antibody library took the place of the antigen-recognition region. The inhibited expression of the inducible caspase-9 suicide gene was coupled with the activation of the synthetic receptor, allowing the lymphocyte repertoire to co-evolve with the dynamic expression of antigens. Synthetic cell repertoire shows significant anti-tumour effect both in vitro and in vivo. Tumour specific killer cells were enriched within one month in vivo even for large repertoire with abundant diversity. In theory, if the diversity of the lymphocyte repertoire is sufficient, then this artificial system can be used for the treatment of any human disease.

Introduction

Over the past decade, significant progress has been made in modern immunotherapy driven by targeted therapies that inhibit tumour angiogenesis and intrinsic drivers of cancer cell growth. These immunomodulatory therapies enhance host antitumour immunity and the basis of genetically engineered autologous or allogeneic immune cells expressing chimeric antigen receptors (CARs) or T-cell receptors (TCRs).

However, all current immunotherapies rely on the development of “specific targets”. In one respect, identification of the underlying cell-autonomous, gene-driven targets of tumorigenesis led to the development of clinically important blockers, such as EGFR or HER2 blockers, that resulted in profound, but often not durable, tumour responses in genetically defined patient populations. 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 action of negative regulators of T cell function that has led to durable clinical responses in subsets of patients with various tumour types. Adoptive CAR-based therapies, although showing very encouraging efficiency, also depend on the construction of a specific target binding domain, which is usually achieved by development of antigen-recognition regions in the form of a single-chain variable fragment (scFv) or a binding receptor/ligand in the extracellular domain. In fact, the theory of “targeting a specific target” creates a dilemma in the context of cellular plasticity and evolution, not to mention genetic or epigenetic heterogeneity of the disease, at every level from the molecule to the human population.

The human immune system never functions by depending on “specific targets”. Human immune cells act as natural defenders to protect the body from invading pathogens and to provide surveillance and respond to tumorigenesis. One scientific theory that describes the functions and processes adopted by lymphocytes to handle a wide variety of antigens that attack the body[1] is Burnet's clonal selection theory. Introduced and promoted by the Frank Macfarlane Burnet, the clonal selection theory explains the formation process of diverse antibodies and immunoglobulin-like proteins in the initial stages of the immune response. In the immune system, some lymphocytes with diverse immunoglobulin-like proteins are tolerant of self-tissues, while others are

intolerant of self-tissues. However, notably, only cells that tolerate self-tissue 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.

The immunosurveillance hypothesis posits that because of the ability of host immune cells to recognize and destroy nascent malignancies, tumours arise at more frequent intervals than they are detected[4]. This hypothesis is further evidenced by the link of immune incompetence with an increased incidence of cancers[5]. Tumour-associated antigens (TAAs), which are mutated self-proteins expressed by some tumours, are capable of eliciting a tumour-specific immune response [6]; however, the immune unresponsiveness hypothesis suggests that failure of the immune system to recognize TAAs leads to the evolution of manifested cancerous disease[7]. Accumulated [8]evidence has led to the development of the cancer immunoediting hypothesis, which unifies the tumour protective immunosurveillance and tumour-promoting immune unresponsiveness aspects of immunity[9]. A corollary of this hypothesis, that the immune system shapes tumour immunogenicity, indicates that, to be effective, cancer immunotherapies must involve large numbers of high-quality effectors that can eliminate tumours and tumour-induced immune suppressors.

T cells, known to be key effectors of antitumour effects, are attractive candidates for cancer immunotherapy[10]. Theoretically, reconstruction of a healthy, tumour-responsive immune system in patients results in recognition and destruction of the TAA-expressing tumour cells. There is also suggestive evidence for this theory. Transplantation of haematopoietic cells from allogeneic donors supplemented with mature donor T cells can cure minimal residual disease and prevent leukaemia relapse [11]. In a path-breaking trial by Hans Kolb and colleagues [12], adoptively transferred peripheral blood mononuclear cells (PBMCs) derived from the peripheral blood of donors were used to treat three patients who suffered from chronic myeloid leukaemia (CML) relapse after bone marrow

transplantation. All three patients achieved haematologic and cytogenetic remission that persisted up to 7 months, thus documenting the first trial of allogeneic T-cell-based immunotherapy. Of note, two of the three patients treated with allogeneic PBMCs developed graft-versus-host disease (GVHD).

CAR-T cells, in contrast to conventional effector T cells, can be activated through the recognition of antigens irrespective of the MHC presentation. Interestingly, recent reports show that CAR-T cells originating from a single clone have the potential to show antitumour activity accompanied by complete remission in patients. We hypothesize that a CAR-T cell repertoire (or T cell CAR display) that has the potential to recognize a variety of nonself antigens can be directly used as a therapeutic agent. In this report, we designed and characterized an artificial bionic immune cell system that is based on synthetic receptor libraries and genetic circuits. We postulate that, in a pre-existing group or population of genetically engineered lymphocytes, a particular antigen can exclusively activate its specific counter cell, enabling that specific cell to survive and multiply under a 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 capacity to combat any disease in humans.

Results

Design and characterization of a synthetic chimeric antigen receptor T cell library

We first propose the design of a logic-gated chimeric antigen receptor T cell library. The ScFv library was fused to a CD8a hinge and transmembrane domain and the intracellular domains of human 4-1BB and CD3ζ (or z), consistent with second generation CAR design. The scFv library could be customized. Unpaired cysteine 164 within the CD8a hinge region was replaced with a serine to increase CAR expression, as reported previously[13]. The cDNA sequences containing the fusion constructs were cloned under the control of a moderately strong constitutive human PGK promoter. We next constructed a lentiviral vector encoding a Gal4-KRAB transcription inhibitor under the control of a 6×NFAT-IL2 min promoter. An inducible caspase-9 suicide gene was placed

downstream of the Gal4-KRAB transcription inhibitor under the control of a combined UAS-SV40 promoter. Because the KRAB protein has been demonstrated to be capable of inhibiting all promoters within at least 3 kB [14] and because the forward construct would therefore inhibit the 6×NFAT-IL2 min promoter, we also generated the opposite construct, in which the two expression cassettes were cloned in such a manner that both promoters were at the opposite ends and at a long distance (Fig. 1A).

We sought to determine the kinetics of the genetic circuit in which NFAT was coupled with KRAB activation in engineered T cells after CAR stimulation. Therefore, we first used engineered T cells with either an EGFR-specific or a HER2-specific CAR in our experiments. We confirmed the EGFR-CAR and HER2-CAR expression by staining with anti-Myc mAb (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), and the MCF-7 HER2 cells (a derivative engineered to express HER2). The MCF-7 cells and the derivative cells were characterized in our previous study[15]. CAR-T cells transduced with cetuximab scFv (termed CAR-T-CTX) efficiently lysed the EGFR-positive cells, that is, the MCF-7 EGFR cells, but did not kill the MCF-7 cells or the MCF-7 HER2 cells. On the other hand, the CAR-T cells transduced with trastuzumab scFv (termed CAR-T-TTZ) efficiently lysed the HER2-positive cells, that is, the MCF-7 HER2 cells, but not the MCF-7 cells or MCF-7 HER2 cells (Fig. 1C). Then, we performed coculture experiments with the engineered T cells and different MCF-7 stimulator cells to determine whether treatment with the small molecule dimerizer AP1903 after different time would affect the apoptosis rate of the engineered T cells. Our data show that the small molecule dimerizer AP1903 had no effect on the viability of the CAR-T cells, while AP1903 caused significant apoptosis/necrosis of the engineered T cells after 4 h of exposure to the stimulatory cells. However, after 36 hours of stimulation with antigens, the engineered T cells were notably affected by the reduced pro-apoptotic effect of AP1903, and both the EGFR-specific and HER2-specific CAR T cells were resistant to AP1903 72 hours after antigen stimulation (Fig. 1D).

Potent antitumour effect of the chimeric antigen receptor T cell library

For proof of concept, we 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 to MCF-7 cells or MCF-7 derivatives (Fig. S1 and Table S1). A CAR-T cell library of 10 control CAR constructs was also constructed and termed the CAR-$T_{Control}$ library. We confirmed the uniform expression of each of the 10 negative control CAR constructs on the cell surface of engineered T cells. Before conducting the experiments, a CAR-T therapeutic library was developed in which CAR-T cells were mixed, and CAR-T CTX, CAR-T-TTZ and 8 other unique CAR-T cells were equally distributed in the population; this library was termed the CAR-$T_{CTX\text{-}TTZ}$ library.

Next, we sought to study the growth dynamics of the different CAR-T cell populations in the presence of different stimulator cells. We cocultured MCF-7 cells and MCF-7 derivative cells with the CAR-$T_{CTX\text{-}TTZ}$ library for 10 days, and the CAR-T cell proliferation rate, percentage of $\alpha EGFR^+$ CAR-T cells or $\alpha HER2^+$ CAR-T cells and the cytotoxicity induced in the CAR-T cells were analysed every day (Fig. 2). Interestingly, our data showed that the cell number of the CAR-$T_{CTX\text{-}TTZ}$ library was increased (Fig. 2A), while the percentage of $\alpha EGFR^+$ CAR-T cells and $\alpha HER2^+$ CAR-T cells, as well as the cytotoxicity effect, were not significantly increased (Fig. 2B and C). Next, on day 5, we added AP1903 to the coculture system. Our data showed that the number of CAR-T cells had markedly decreased by day 7; however, the percentage of $\alpha EGFR^+$ CAR-T cells and $\alpha HER2^+$ CAR-T cells had increased (Fig. 2D and E). On day 7, the CAR-$T_{CTX\text{-}TTZ}$ library showed notable cytotoxic effects on the EGFR-expressing cells and the HER2-expressing cells (Fig. 2F). These data suggest that AP1903 enhanced the artificial screening process of CAR-T cells.

In vivo antitumour effect of the chimeric antigen receptor T cell library

We next examined the antitumour ability of the CAR-$T_{CTX\text{-}TTZ}$ library in vivo. Immunodeficient MCF-7, MCF-EGFR and MCF-HER2 xenograft NSG mice were maintained without treatment until a tumour of approximately 50 mm^3 formed, and then, the mice were treated with 1×10^7 CAR-$T_{Control}$ library and the CAR-$T_{CTX\text{-}TTZ}$ library (1×10^6 cells for each CAR construct containing T cells). One week later, the mice were treated with three doses of AP1903 intraperitoneally (i.p.) every two days as indicated (Fig. 3A).

For the MCF-7 xenograft mice, the tumour growth kinetics were similar among the mice that received the CAR-$T_{Control}$ library and CAR-$T_{CTX-TTZ}$ library. The tumours progressed rapidly, reaching a volume of 1,000 mm^3 in fewer than 35 days. In contrast to the tumour growth in the MCF-7 xenograft mice, the mice that received the CAR-$T_{CTX-TTZ}$ library therapy showed significantly inhibited growth of the MCF-7 EGFR tumours or the MCF-7 HER2 tumours approximately two weeks after treatment, and the tumours showed almost complete regression at the end of the experiment. After the experiment, all the mice were sacrificed, and the αEGFR$^+$ CAR-T cells or αHER2$^+$ CAR-T cells were measured by the frequencies of the different CAR-T cells among the CAR-positive T cells in the blood, bone marrow, spleen and liver of the animals, as determined by flow cytometry. Interestingly, our data showed that, regardless of whether the small-molecule dimerizer was administered or not, the number of non-specific CAR-T cells in the blood and tissues of the treated mice was dramatically reduced (Fig. 3B and C), suggesting that the iCASP9 suicide gene is not essential for the antigen-based evolution of CAR-T cells but is necessary for the elimination of non-specific CAR-T cells.

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 a variable fragment was used to construct a phage display scFv library that consisted of ~ 1×10^8 individual colonies. The scFv gene corresponded to the size of the insert of more than 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 prevent phage binding to normal tissues, we used blood that did not bind to systemic organs as the source of the scFvs. Four rounds of in vivo phage display were performed in the NSG mice to collect this “negative” phage population (Fig. 4A). The DNA sequences of the isolates were analysed, and the results confirmed that phage diversity was maintained, and this library termed $library_{NSG}$. Previously, we found that anti-EGFR CAR-T cells delayed the growth of SW480 colon tumours but unexpectedly induced the outgrowth of EGFR-negative tumour cells[16]; therefore, we used the SW480

cells as resistance models. A similar DNA analysis was performed after the completion of four rounds of in vivo phage display in NSG mice injected with SW480 xenograft cells (Fig. 4B). Approximately 450 unique scFv sequences, indicative of tumour-binding scfvs, were identified in this manner for use in further research. A CAR-T cell library was also developed based on the CAR constructs derived from the scFvs, and it was termed the CAR-T_{sw480} library. As we used standard transfection methods to generate CAR-T cell libraries, the introduction of DNA encoding a repertoire of CAR genes has the potential to introduce multiple CAR genes into each cell.

To further evaluate the therapeutic role of the CAR-T_{sw480} library in vivo, the SW480 cells were injected subcutaneously into the NSG mice, and the T cells were administered when the tumour volume reached approximately 50 mm^3. The mice were then injected (i.v.) with 1×10^7 control T cells or CAR-T CTX cells (Fig. 4C). Consistent with the findings of a previous report, tumour growth was significantly inhibited by the anti-EGFR-based cell therapy[16]. However, at approximately 3 weeks, the mice treated with the CAR-T CTX cells eventually succumbed to the tumour. The mice were then treated with 1×10^6 CAR-T_{sw480} libraries on day 25. Interestingly, the mice receiving CAR-T_{sw480} library therapy showed significantly inhibited tumour growth approximately three weeks after treatment, and the tumours showed complete regression two weeks later. After the experiments, all the remaining mice were sacrificed, and no $\alpha EGFR^+$ CAR-T cells were detected in any of the tested mice. The CAR-positive T cells were sorted and sequenced, and we were able to define five unique scFv clones.

Design of a large-scale CAR-T Repertoire

We aimed to develop a large-scale synthetic cell library for immunotherapy, which raised concern that the limited expansion and persistence of the CAR-T clones may diminish the antitumour effect. Therefore, a CAR-T cell library containing 5×10^5 individual constructs was developed based on the $library_{NSG}$, containing candidates that showed no binding to systemic organs in NSG mice, as explained in the experiments described above. We first investigated the antitumour efficacy of the CAR-T_{NSG} cell library in the NSG mice xenografted with SW480 cells. However, we did not observe any antitumour effect during the experiments and observed no signs of CAR-T cell expansion at the end of the experiments (Fig. S2).

A previous study showed that a single *TET2*-modified CAR-T cell had the capacity to induce leukaemia remission and that CAR-T cells exhibited a central memory phenotype. We therefore used *TET2*-knockdown CAR-T cells (referred to as CAR-T_{shTET2} cells) to construct a synthetic cell library (Fig. 4D). The experimental knockdown of *TET2* in the T cells resulted in a central memory phenotype in the $CD8^+$ and $CD4^+$ T cells (Fig. 4E), and a CAR-T_{shTET2} cell library containing 5×10^5 individual constructs based on the $library_{NSG}$ was also developed.

The CAR-T_{shTET2} cell library or parental T_{shTET2} cells were used to treat the mice bearing the established subcutaneous SW480 xenograft tumours. Our data showed that tumours grew rapidly in the control mice injected with medium or parental T_{shTET2} cells; similarly, the tumours treated with the CAR-T_{shTET2} cell library did not show any signs of inhibition until approximately one month later, at which time, tumour growth was inhibited (Fig. 4F). Completely regressed tumours were observed in 5 of the 8 tumours in the mice receiving therapy, although three mice in the CAR-T_{shTET2} cell library treatment group were sacrificed during the 7th week because the tumours had become excessively large.

Next, the potential for the CAR-T_{shTET2} cell library to eradicate tumours in mouse xenografts was further examined (Fig. 4F). Mice xenografted with SK-BR-3 cancer cells serving as a models of $HER2^+$ breast cancer were treated with a transfer of the CAR-T_{shTET2} cell library or parental T_{shTET2} cells. Tumours in the mice treated with the CAR-T_{shTET2} cell library were significantly inhibited and then eliminated compared with those in the mice treated with parental T_{shTET2} cells, although four mice in the CAR-T_{shTET2} cell library treatment group were sacrificed during the 7^{th} and 8^{th} weeks for the outgrowth of tumours. The therapeutic effect of the CAR-T_{shTET2} cell library was further examined in another model using SK-OV-3 cells, an ovarian $HER2^+$ cancer cell line. Transfer of the CAR-T_{shTET2} cell library significantly inhibited tumour growth approximately one month later, and the tumours were eradicated in 3 of 8 mice. However, five mice were sacrificed for tumours that were excessively large.

After the experiments, all the SK-BR-3- and SK-OV-3-free mice were sacrificed, and the CAR^+ T cells were further sorted to study the treatment of a second cohort of established tumours (termed CAR-$T_{shTET2\text{-}SKBR3}$ cells and CAR-$T_{shTET2\text{-}SKOV3}$ cells) (Fig.

S3). The SK-BR-3 xenograft and SK-OV-3 xenograft mice remained untreated until tumours of approximately 500 mm^3 size formed, and then, they were treated with 5×10^5 CAR-$T_{shTET2\text{-}SKBR3}$ cells or CAR-$T_{shTET2\text{-}SKOV3}$ cells. Our data showed that the treatment of CAR-$T_{shTET2\text{-}SKBR3}$ cells and CAR-$T_{shTET2\text{-}SKOV3}$ cells significantly inhibited the growth of established tumours, and the tumours were eliminated after 2 weeks of treatment.

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

We next aimed to develop a novel library platform using NK-92 cells. As previously report[17] showed that genetically engineered NFAT or NFkB promoters have leaky expression in "resting" non-activated NK-92 cells, we choose an alternative approach based on the synNotch receptor. As shown in Fig. 5A, an antigen-sensing synNotch library releases an orthogonal transcription factor that drives the expression of a fused construct consisting of an anti-EpCAM CAR and a TetR-KRAB transcription inhibitor. An inducible caspase-9 suicide gene was placed downstream of the transcription inhibitor under the control of a TRE-CMV combined promoter.

The anti-EpCAM scFv binds to SW480 cells, and the anti-EpCAM CAR NK-92 cells induce cytotoxicity in SW480 cells in vitro (Fig. 5B and C). Next, a NK-92 cell library containing 5×10^5 individual synNotch constructs was developed based on the nonimmunized human scFv library, which showed no binding to the systemic organs of NSG mice, as in the experiments described above. We next characterized the antitumour effect of the NK-92 cell library in vivo (Fig. 5D). NSG mice xenografted with SW480 cells were treated with the 1×10^7 NK-92 library or parental NK-92 cells twice per week for one week, and one week later, three doses of AP1903 were administered to the mice every two days. Our data showed that the tumours grew rapidly in the control mice injected with medium, while in the mice injected with parental NK-92 cells, tumour growth was delayed by 10 days. Tumours treated with the NK-92 cell library did not show any sign of inhibition until approximately one month later, and then, tumour growth was inhibited. Completely regressed tumours were observed in 4 of the 8 tumours in mice under therapy, and two mice were sacrificed during the 7th and 8th week because of excessively large tumours. After the experiment, all the remaining mice were sacrificed, and the synNotch-positive NK-92 cells were sorted and sequenced. We were able to define 11 unique scFv clones. Interestingly, one scFv clone was the same as one we

obtained from the CAR-T_{sw480} library.

Expanding the therapeutic role of the Synthetic Receptor Repertoire to include Non-malignant diseases

To further investigate the therapeutic role of the synthetic receptor repertoire, we tested whether the CAR-T_{shTET2} cell library could prevent the formation of endometriosis in vivo. Human endometrial tissue obtained from women undergoing surgery for benign conditions was implanted in NSG female mice. The animals were distributed into vehicle, control T_{shTET2} cells or CAR-T_{shTET2} cell groups and the respective library was administered twice for one week.

After 45 days, the human endometrial implants were excised. For the CAR-T_{shTET2} cell library-treated animals, all the parietal peritoneum in which the endometrial tissue had been implanted was fully extracted because macroscopic lesions were not identified. The results of the observations are listed in Table S2.

Discussion

Cancer remains a leading cause of mortality and poor health worldwide[18]. Despite the enormous effort expended in the development of therapeutics, successful control and eradication of the disease remain elusive[19,20]. In addition, risk factors such as physical inactivity, obesity, and smoking have shown increased prevalence worldwide, promoting the incidence of malignancy[21,22]. Moreover, our understanding of cancer genetics and biology has also been updated: cancer progression and development are evolutionary processes driven by somatic cell mutations and subclonal selection[23], a ground-breaking 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 quasi-species [23,24]. Modern cancer genomics and biology have validated the concept that cancer development is a complex, adaptive, and Darwinian process.

In humans, hundreds of millions of different antibodies (or immunoglobulin-like

proteins) are created by the immune system. Antibody display technologies using phage, ribosomes, yeast and mammalian cells, have become mainstream antibody and protein engineering platforms and constitute major technology for antibody discovery and therapeutic use.

Antibody libraries can be constructed based on different sources, such as immunized animals[25] or naturally immunized or infected humans[26], or naive immune systems, which can be derived from nonimmune natural or computational and synthetic sources. When the diversity is sufficiently large, the library has the capacity to generate 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 industry and academia[27,28]. Usually, panning for antibodies to a specific antigen using nonimmune libraries or based on synthetic antibody libraries that are diversified in merely one or two CDRs or in only one of the two chains results in the discovery of medium-affinity antibodies, reflecting the situation of transgenic mice with restricted antibody repertoires[29,30]. Interestingly, CARs constructed with high-affinity antibody fragments recognize targets expressed at any level, including in normal cells in which they were undetectable by flow cytometry, while CAR-T cells harbouring medium-affinity CAR exhibited robust antitumour efficacy similar to that exhibited by high-affinity cells but normal cells were spared from expressing physiologic target levels[31]. These findings suggested that a synthetic cell repertoire may not need the most successful antibody libraries to display (natural or synthetic) diversity in multiple CDRs and routinely yield single-digit nanomolar and sometimes sub-nanomolar 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 is 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 retrieved with high-throughput screening [32,33]. Therefore, a synthetic cell repertoire with 10^6 clones should be a very successful cell repertoire for therapeutic purposes.

In this report, we postulate that the mating of the mammalian-cell antibody display library with a synthetic genetic circuit builds a novel ‘molecular evolution machine’ that

can function in vivo. With chimeric antigen receptors and immune cells, the new platform can be directly selected and screened for therapeutic effects, not merely binding affinity. Moreover, the whole human antibody repertoire can also be preserved when one is healthy and use it to develop a synthetic immune cell library for therapeutic use when need. This definition of the "antibody bank" may represent the promised 'land' that Paul Ehrlich wrote about over 100 years ago[34]: "the land which […] will yield rich treasures for biology and therapeutics.

Methods

Cell lines

MCF-7, SW480, SK-BR-3, SK-OV-3, 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 sequence information can be found in the Protein Data Bank. The 4D5-5 scFv derived from trastuzumab was chosen for this study, as we reported previously[15]. 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 the intracellular signalling domains of the CD137 and CD3ζ molecules. The constructs were further cloned into the pHR vector

backbone under the control of a PGK promoter. To generate CAR response elements, a CAR inducible promoter containing six NFAT-REs in a minimal IL-2 promoter[17] was 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 of the expression under the control of the combined SV40/UAS promoter. Because the KRAB protein has been demonstrated to be capable of inhibiting all promoters within at least 3 kB [35] and because the forward construct would therefore inhibit other promoters, we generated the opposite construct, in which the two expression cassettes were cloned in a manner such that both promoters were at the opposite ends at a distance greater than 4 kb. The 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). The ScFv library with the N-terminal CD8α signal peptide was fused to the synNotch-Gal4VP64 receptor backbone (Addgene plasmid #79125) in place of the CD19-specific scFv. To generate the response elements, the mCherry gene segment in Addgene plasmid #79123 was replaced with the described anti-EpCAM CAR transgene. To express the TetR-KRAB fusion protein concomitantly with CAR, the 2A peptide sequence was intercalated between the two genes. The ICASP9 and GFP fusion construct was placed downstream of the expression under the control of the Tet-cytomegalovirus (CMV) promoter[35].

In vitro T cell transduction and cultures

Negative selection using RosetteSep kits (Stem Cell Technologies) was adopted to isolate primary human T cells from healthy volunteer donors following leukapheresis. All specimens were collected under an approved protocol by the Second Military Medical University Review Board, and written informed consent was obtained from each donor. Bulk primary human T cells were activated with paramagnetic beads coated with anti-CD3 and anti-CD28 monoclonal antibodies as previously described[15] and transduced with lentiviral vectors encoding the indicated CAR, genetic circuits or shRNA hairpin sequences targeting *TET2* or a scrambled control coexpressing BFP (GeneChem). Following 9-10 days in culture, the T cells were FACS sorted to >95% purity with the

following markers: untransduced T cells: myc^{-}; CAR-T cells: myc^{+}; logic-gated CAR-T cells: $myc^{+}GFP^{+}$; and logic-gated CAR-T_{shTET2} cell: $myc^{+}GFP^{+}BFP^{+}$. Sorted T cells were subsequently expanded for 3 days in medium (RPMI 1640 with 10% human serum, 2 mM L-glutamine, 25 mM HEPES, penicillin/streptomycin (100 U/ml), and 50 mM b-mercaptoethanol (Sigma)) with 50 U/ml recombinant human IL-2 (Prometheus) with 50 U/ml human IL-2 prior to in vitro assays or adoptive transfer. Knockdown efficiency in the T cells following shRNA transduction was determined by real-time quantitative PCR with TaqMan gene expression assays (Applied Biosystems) for *TET2* (assay Hs00325999_m1) and *GAPDH* (assay (Hs03929097_g1), which served as a loading and normalization control.

Antibody library

The protocol for antibody library construction was described in our previous report[36]. Briefly, peripheral blood mononuclear cells were isolated from blood. 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 domains. 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 supplemented with ampicillin and glucose cultured overnight at 37°C. After being scraped from the plates, the colonies were stored at -80°C in lysogeny broth supplemented with 20% glycerol.

Cytotoxicity assays

The cytotoxicity of the CAR-expressing T cells or exosomes was tested in a standard 4-h ^{51}Cr-release assay[15]. Target cells were labelled with 51Cr for 1 h at 37°C. Radioactive ^{51}Cr (50 μCi) was used to label 1×10^{6} target cells. One hundred microliters of labelled target cells (n = 5000) was 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. The 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. The 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

The cells were washed and labelled with alamarBlue (Invitrogen), 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 (RFUs) and compared with the data obtained on the first day.

Activation of the suicide gene in vitro and validation in vivo

The small molecule dimerizer AP1903 (10 nM, MCE) was added to the indicated cell cultures. The extent to which the transduced cells were eliminated was evaluated by Annexin-V staining. The efficacy of the suicide gene was determined in vivo by treating the tumour-bearing mice that had received synthetic cell treatment with the indicated doses of AP1903 (50 μg each) intraperitoneally (i.p.)[37].

In vivo study

In vivo experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Second Military Medical University, and the mice were housed in a specific pathogen-free barrier facility. The cancer cells were inoculated into NSG mice. (Shanghai Model Organisms Center, Inc.) When the tumour volume reached an average of approximately 50 mm^3, the mice were randomly divided into groups of 8 mice.

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-based therapy, the mice were injected intravenously with the indicated dose or 1×10^7 control cells or cell libraries twice per week for one week, and one week later, three doses of AP1903 were administered to the mice treated with cell library every two days to eliminate the non-activated synthetic cells in all experiments unless otherwise specified. Mice were injected i.p. with 2000 units of IL-2 twice a week following infusion of the T cell-based therapy. The mice were sacrificed when the volume of the tumours reached 1300 mm^3.

For the experimental model of endometriosis, the menstrual endometrium was obtained by aspiration using Cornier cannula for endometrial biopsy during the

proliferative phase (from 5 to 10 days) from 6 women undergoing surgery for benign conditions (3 myomas and 3 simple cysts). The endometrium samples were preserved in physiological saline and implanted immediately to prevent cell death. The patients were of reproductive age (23-45 years old) and had not received hormone treatment within 8 weeks prior to sample collection. The medical history of endometriosis or adenomyosis was not available; however, an extensive preoperative evaluation was performed for all the patients, and it included clinical exploration, MRI, and transvaginal sonography to exclude both endometriosis and adenomyosis. The absence of endometriotic lesions was confirmed during laparoscopy conducted for the determination of benign disease. All specimens were collected under an approved protocol by the Second Military Medical University Review Board, and written informed consent was obtained from each donor.

Statistical analysis

Unless otherwise specified, Student's t-test was used to evaluate the significance of differences between 2 groups, and ANOVA was used to evaluate differences among 3 or more groups. Differences between samples were considered 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 characterization of a synthetic chimeric antigen receptor T cell library. (A) Map of lentiviral constructs encoding the CAR library and CAR repressive iCASP9. **(B)** Membrane-bound CAR expression. Forty-eight hours after retroviral transduction, the expression of CARs on human T cells was measured by staining with anti-MYC antibody, followed by flow cytometry analysis. T cells without transduction were used as negative controls. The histograms shown in black corresponds to the isotype controls, whereas the red histograms indicate positive fluorescence. **(C)** Killing activity of the CAR-T cells in response to tumour cells. The cytotoxic activity of the CAR-T and control T cells against MCF-7 cells was assessed by a ^{51}Cr-release assay at the indicated effector-to-target (E:T) ratios. **(D)** CAR-T cells were first stimulated with different MCF-7 cells for 24 or 72 hours. The addition of 10 nM AP1903 to the cultures induced apoptosis/necrosis of the CAR-T cells, as assessed by annexin-V staining in 4 independent experiments. The dimerizer did not induce apoptosis in the untransduced T cells.

Figure 2. In vitro antitumour effect of the chimeric antigen receptor T cell library. (A and D) Cell proliferation of the CAR-T cells was measured using alamarBlue staining. The results are expressed in relative fluorescence units (RFUs). **(B and E)** The proportion of the cells with positive anti-EGFR or anti-HER2 expression is shown. **(C and F)** Killing activity of CAR-T cells in response to tumour cells. The cytotoxic activity of the 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 antitumour effect of the chimeric antigen receptor T cell library. (A) Tumour volumes of different MCF-7 tumour xenografts after the indicated treatment; n= 8. **(B)** Frequencies of the CAR-positive T cells in the blood, bone marrow, spleen and liver of the xenograft mice, as determined by flow cytometry. **(C)** The proportion of the cells with positive anti-EGFR or anti-HER2 expression in CAR-positive T cells is 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 nonbinding 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 the NSG mice bearing SW480 xenograft cells. **(C)** Tumour volumes of SW480 xenograft tumours after the indicated treatment; n= 8. Arrows indicate the point in which the CAR-Tsw480 library was used for treatment. **(D)** *TET2* expression in the T cells transduced with a scrambled shRNA (control) or TET2 shRNA. **(E)** Frequencies of central memory $CD8^+$ T cells (left) and $CD4^+$ T cells (right) following shRNA-mediated knockdown of TET2 (n = 12; pooled results from 4 independent experiments). **(F)** Volumes of the SW480 (left), SK-BR-3 (middle), and SK-OV-3 (right) xenograft tumours after the indicated treatment; n= 8.

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

Figure 1

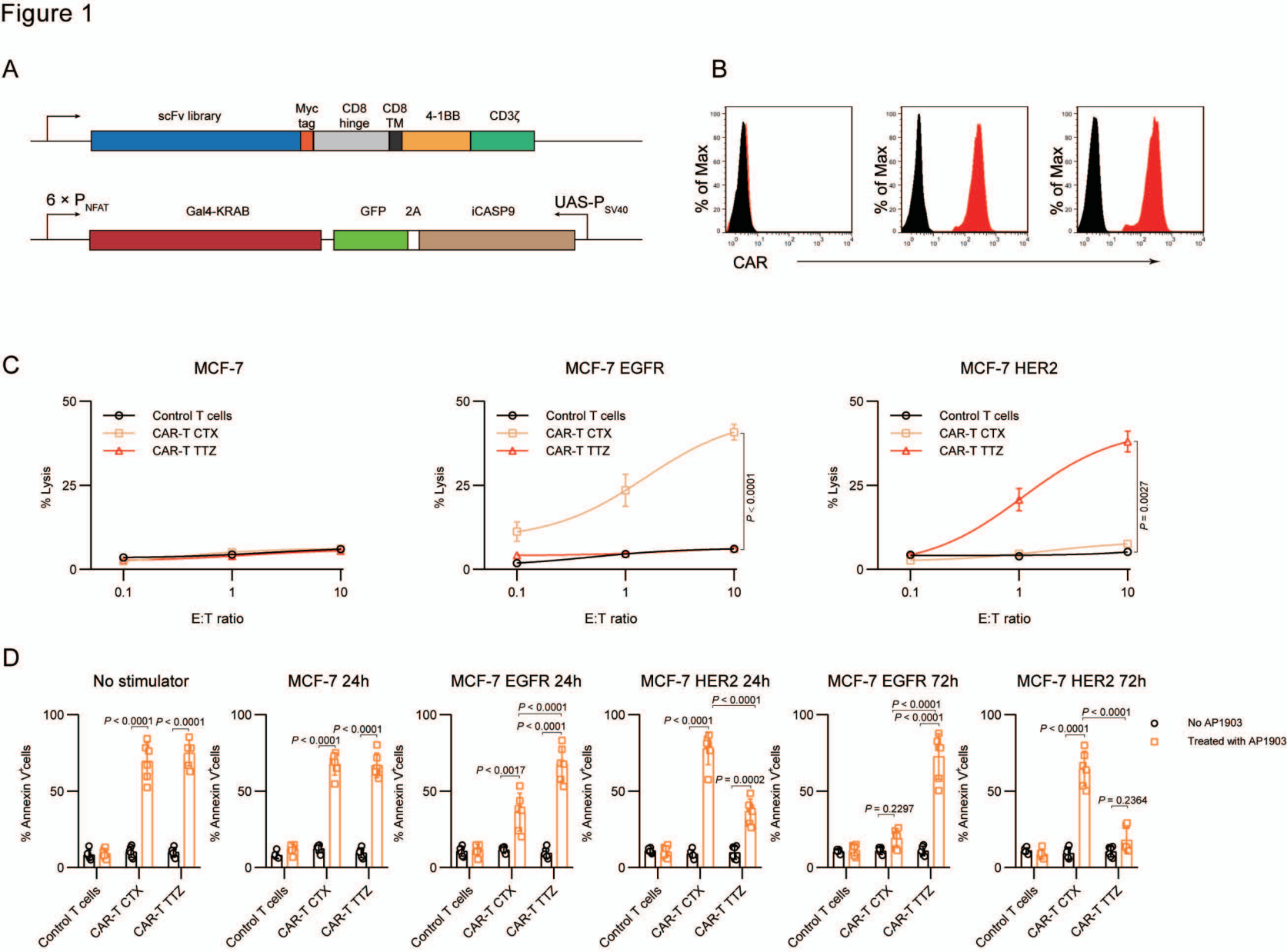

A
scFv library
Myc tag
CD8 hinge
CD8 TM
4-1BB
CD3ζ
6 × P NFAT
Gal4-KRAB
GFP
2A
iCASP9
UAS-P SV40
B
% of Max
CAR
C
MCF-7
MCF-7 EGFR
MCF-7 HER2
Control T cells
CAR-T CTX
CAR-T TTZ
% Lysis
E:T ratio
P < 0.0001
P = 0.0027
D
No stimulator
MCF-7 24h
MCF-7 EGFR 24h
MCF-7 HER2 24h
MCF-7 EGFR 72h
MCF-7 HER2 72h
% Annexin V+cells
P < 0.0001
P < 0.0017
P = 0.0002
P = 0.2297
P = 0.2364
No AP1903
Treated with AP1903

Figure 2

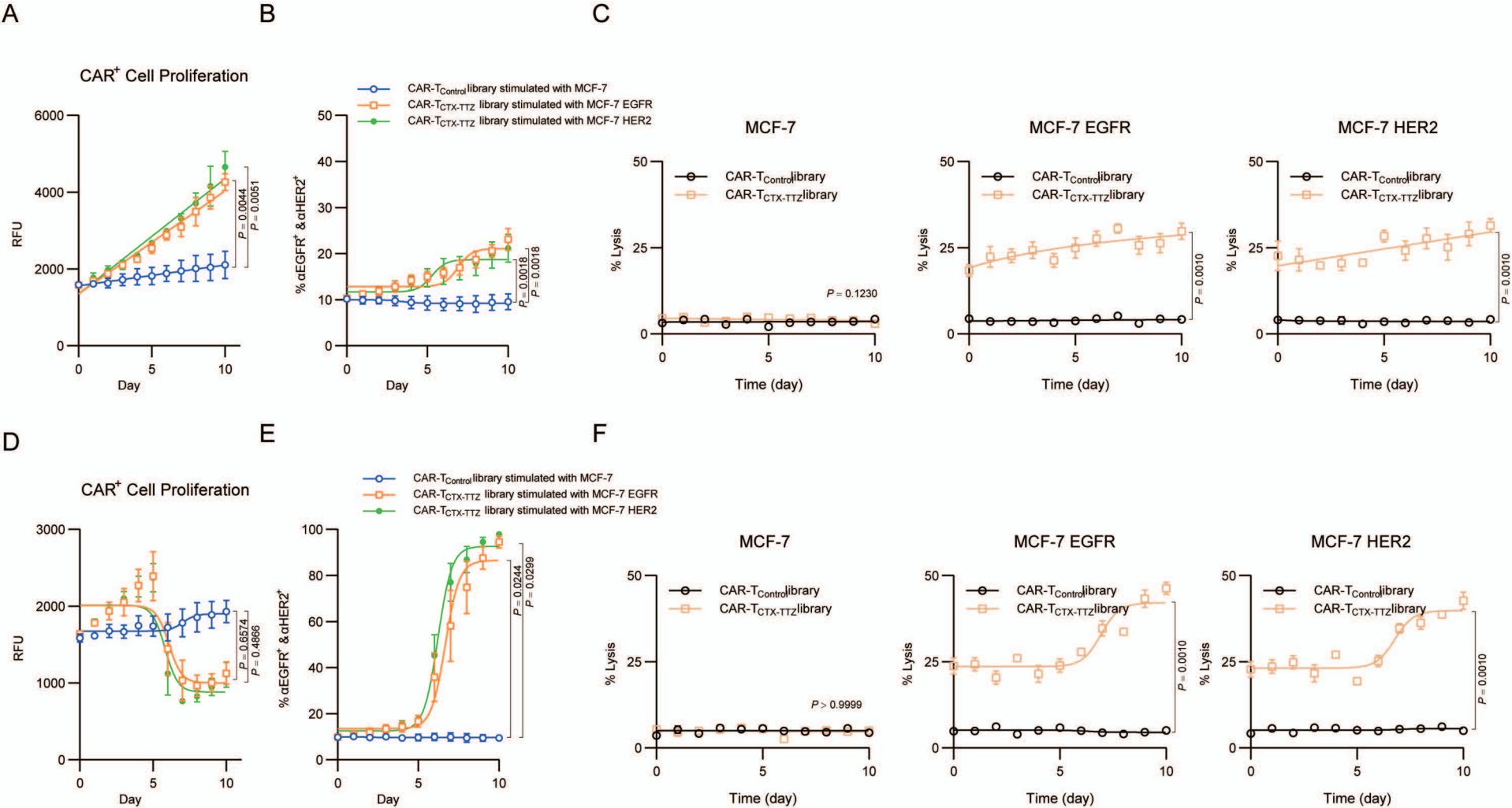
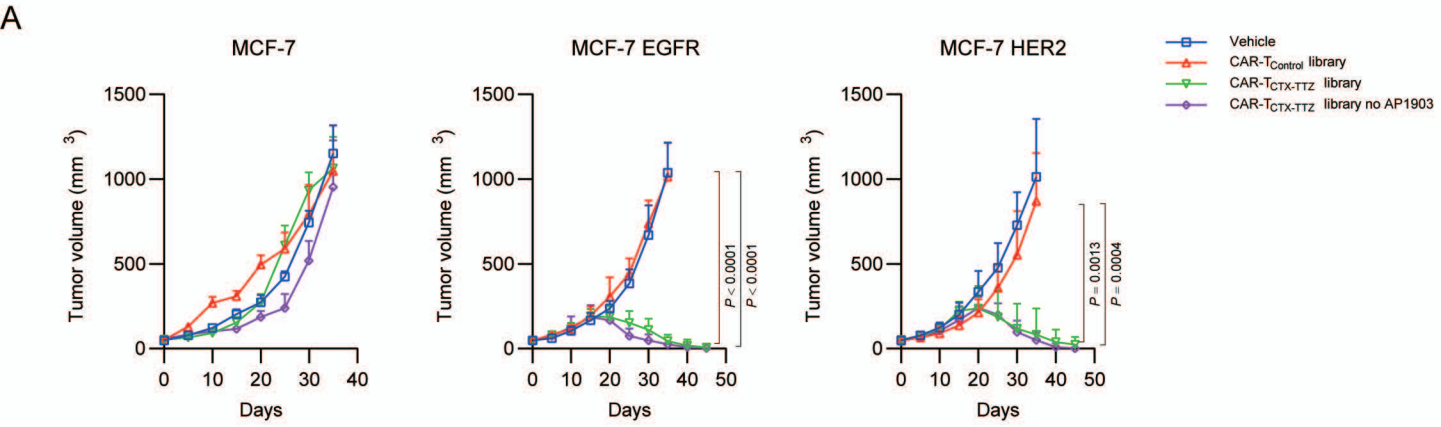
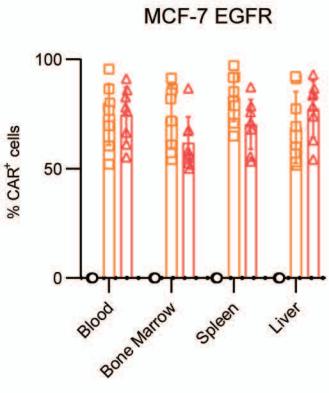
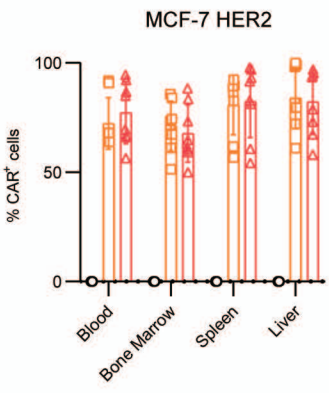

A
CAR+ Cell Proliferation
RFU
Day
P = 0.0044
P = 0.0051
B
CAR-TControl library stimulated with MCF-7
CAR-TCTX-TTZ library stimulated with MCF-7 EGFR
CAR-TCTX-TTZ library stimulated with MCF-7 HER2
% αEGFR+ &αHER2+
Day
P = 0.0018
P = 0.0018
C
MCF-7
MCF-7 EGFR
MCF-7 HER2
CAR-TControl library
CAR-TCTX-TTZ library
% Lysis
Time (day)
P = 0.1230
P = 0.0010
P = 0.0010
D
CAR+ Cell Proliferation
RFU
Day
P = 0.6574
P = 0.4866
E
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+
Day
P = 0.0244
P = 0.0299
F
MCF-7
MCF-7 EGFR
MCF-7 HER2
CAR-TControl library
CAR-TCTX-TTZ library
% Lysis
Time (day)
P > 0.9999
P = 0.0010
P = 0.0010

Figure 3


A
MCF-7
MCF-7 EGFR
MCF-7 HER2
Tumor volume (mm 3)
Days
Vehicle
CAR-TControl library
CAR-TCTX-TTZ library
CAR-TCTX-TTZ library no AP1903
P < 0.0001
P < 0.0001
P = 0.0013
P = 0.0004


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


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

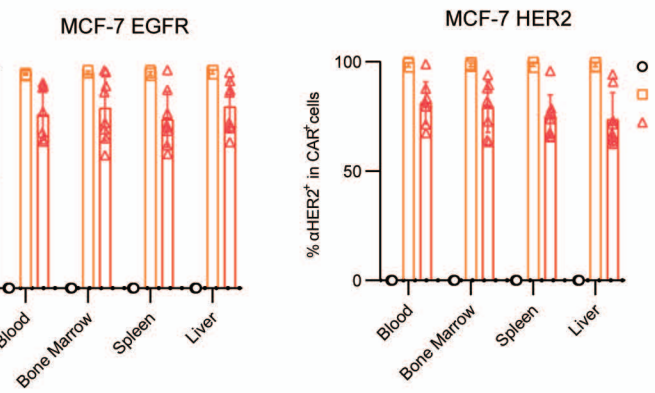

C
MCF-7 EGFR
%αEGFR+ in CAR+ cells
MCF-7 HER2
% αHER2+ in CAR+ cells
Blood
Bone Marrow
Spleen
Liver
CAR-TControl library
CAR-TCTX-TTZ library
CAR-TCTX-TTZ library no AP1903

Figure 4

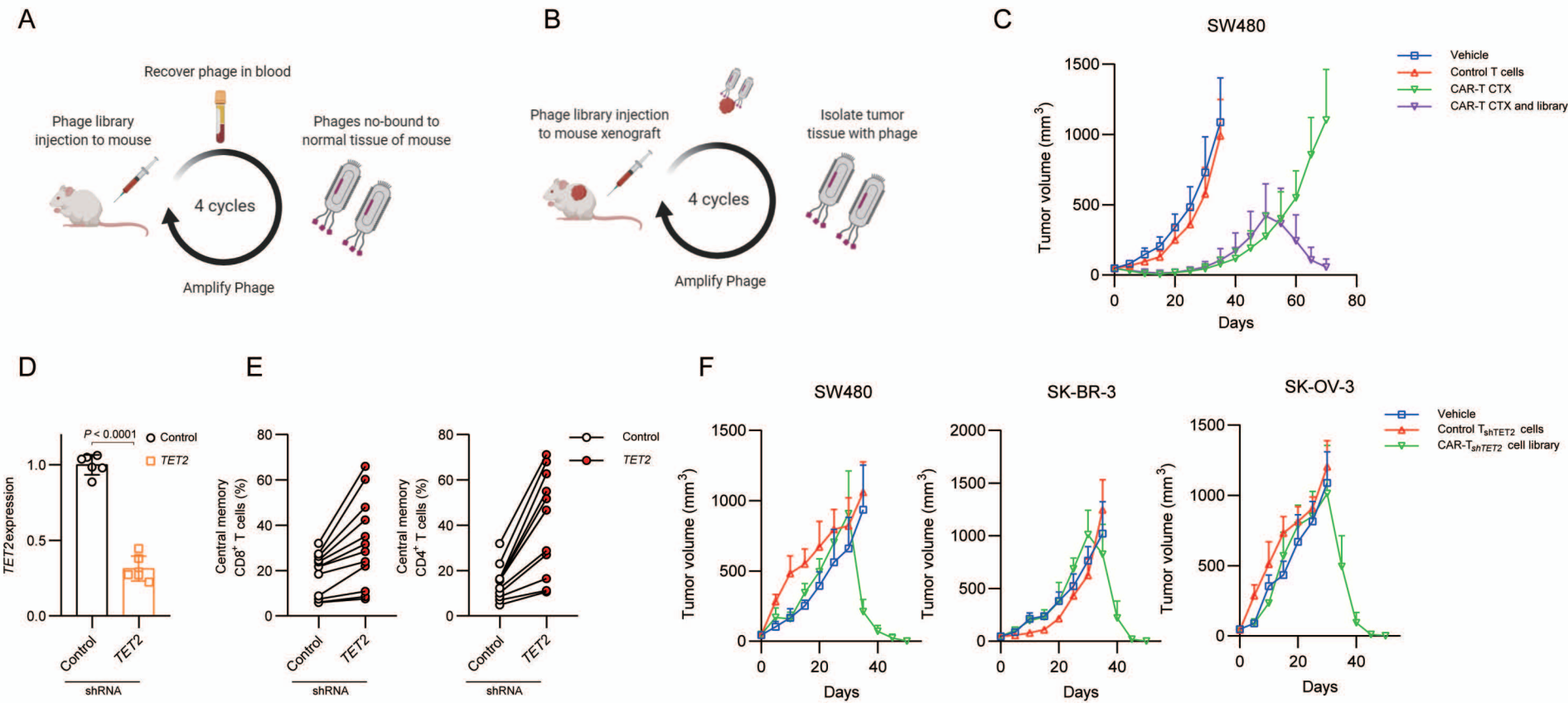
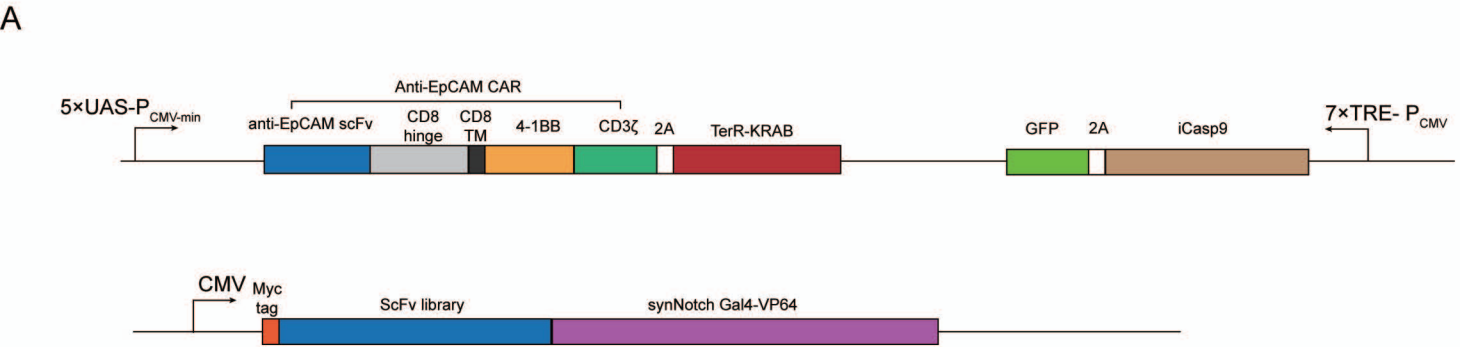
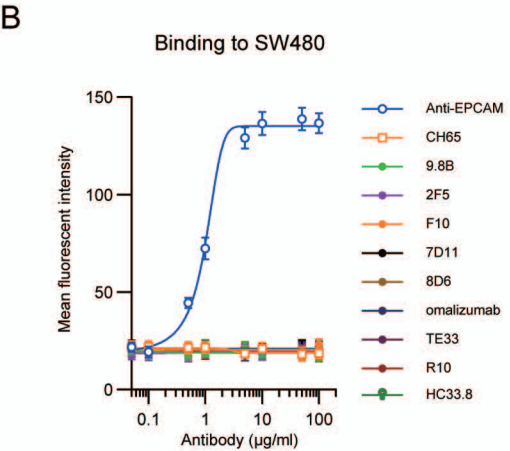
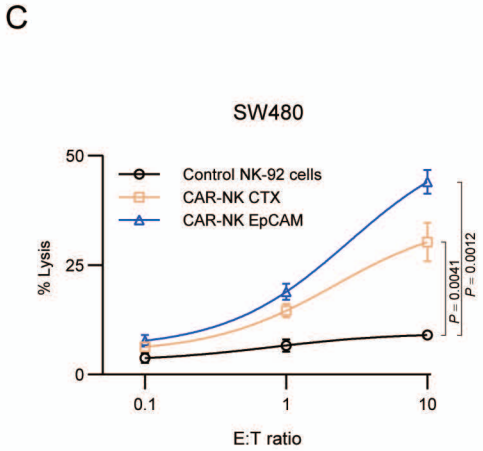
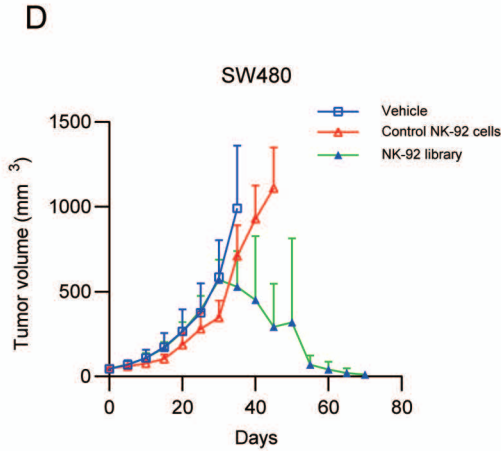

A
Recover phage in blood
Phage library injection to mouse
Phages no-bound to normal tissue of mouse
4 cycles
Amplify Phage
B
Phage library injection to mouse xenograft
Isolate tumor tissue with phage
4 cycles
Amplify Phage
C
SW480
Vehicle
Control T cells
CAR-T CTX
CAR-T CTX and library
Tumor volume (mm3)
Days
D
P < 0.0001
Control
TET2
TET2expression
shRNA
E
Central memory CD8+ T cells (%)
Central memory CD4+ T cells (%)
F
SW480
SK-BR-3
SK-OV-3
Vehicle
Control TshTET2 cells
CAR-TshTET2 cell library
Tumor volume (mm3)
Days

Figure 5


A
5×UAS-P CMV-min
Anti-EpCAM CAR
anti-EpCAM scFv
CD8 hinge
CD8 TM
4-1BB
CD3ζ
2A
TerR-KRAB
GFP
2A
iCasp9
7×TRE- P CMV
CMV
Myc tag
ScFv library
synNotch Gal4-VP64


B
Binding to SW480
Mean fluorescent intensity
Antibody (μg/ml)
Anti-EPCAM
CH65
9.8B
2F5
F10
7D11
8D6
omalizumab
TE33
R10
HC33.8


C
SW480
% Lysis
E:T ratio
Control NK-92 cells
CAR-NK CTX
CAR-NK EpCAM
P = 0.0041
P = 0.0012


D
SW480
Tumor volume (mm 3)
Days
Vehicle
Control NK-92 cells
NK-92 library