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Protocol

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Generating allogeneic CAR-NKT cells for off-the-shelf cancer immunotherapy with genetically engineered HSP cells and feeder-free differentiation culture

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Abstract

The clinical potential of current chimeric antigen receptor-engineered T (CAR-T) cell therapy is hampered by its autologous nature that poses considerable challenges in manufacturing, costs and patient selection. This spurs demand for off-the-shelf therapies. Here we introduce an ex vivo feeder-free culture method to differentiate gene-engineered hematopoietic stem and progenitor (HSP) cells into allogeneic invariant natural killer T (^{Allo}NKT) cells and their CAR-armed derivatives (^{Allo}CAR-NKT cells). We include detailed information on lentivirus generation and titration, as well as the five stages of ex vivo culture required to generate ^{Allo}CAR-NKT cells, including HSP cell engineering, HSP cell expansion, NKT cell differentiation, NKT cell deep differentiation and NKT cell expansion. In addition, we describe procedures for evaluating the pharmacology, antitumor efficacy and mechanism of action of ^{Allo}CAR-NKT cells. It takes ~2 weeks to generate and titrate lentiviruses and ~6 weeks to generate mature ^{Allo}CAR-NKT cells. Competence with human stem cell and T cell culture, gene engineering and flow cytometry is required for optimal results.

Key points

- This protocol describes an ex vivo feeder-free culture method to differentiate genetically engineered hematopoietic stem and progenitor cells into allogeneic CAR-NKT cells for off-the-shelf cancer immunotherapy. In addition, procedures are provided for evaluating their pharmacology, antitumor efficacy and mechanism of action.
- This approach overcomes challenges in manufacturing, costs and patient selection that hamper the clinical potential of current autologous CAR-T cell therapies.

Key references

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Introduction

Adoptive cell therapy has brought about a profound transformation in cancer immunotherapy, specifically through the application of chimeric antigen receptor (CAR)-engineered T (CAR-T) cell therapy. This form of treatment has shown outstanding effectiveness in addressing hematological malignancies[1,2]. The successful clinical outcomes achieved through US Food and Drug Administration-approved CAR-T cells for treating B cell malignancies and multiple myeloma (MM) provide hope for individuals with other types of cancer[3]. However, the current autologous approach of generating CAR-T cell products is associated with high costs, prolonged manufacturing time and limited accessibility to patients[2,4]. In particular, patients with progressive disease or those who have received prior treatments may not have adequate or functional T cells for CAR-T cell production. As such, there is a growing demand for allogeneic products that are accessible and affordable to all patients. To develop allogeneic cellular products, two distinct approaches are being explored. One approach involves utilizing conventional αβ T cells and abrogating their endogenous T cell receptor (TCR) expression to minimize the risk of graft-versus-host disease (GvHD)[5,6]. The other approach involves using other cell types that inherently pose low GvHD risk, such as natural killer (NK) cells, unconventional T cells and macrophages, to produce off-the-shelf cellular products[7–9].

Invariant natural killer T (NKT) cells, named after their usage of an invariant TCR α-chain (TRAV10–TRAJ18 in human and TRAV11–TRAJ18 in mice) and their co-expression of NK receptors (NKRs), are a unique type of αβ T cells that are relatively rare in the bloodstream of humans, comprising only 0.001–1% of circulating T cells[10–16]. Despite their low abundance, NKT cells have demonstrated promising characteristics that make them an attractive option for use as a carrier for off-the-shelf CAR-directed cell therapy. Compared with conventional CAR-T cells, CAR-engineered NKT (CAR-NKT) cells can attack tumor cells using multiple mechanisms and at higher efficacy, can more effectively traffic to and infiltrate solid tumors, can alter solid tumor immunosuppressive microenvironment and, most importantly, do not induce GvHD[17–23]. In clinical studies, the use of GD2-targeting CAR-NKT cells has shown impressive responses in treating relapsed or resistant neuroblastoma without causing noticeable toxicity or cytokine release syndromes[8,24]. However, the use of NKT cells is limited by their scarcity in human blood and expanding the number of NKT cells from peripheral blood mononuclear (PBM) cells can be challenging and may result in the inclusion of bystander conventional T cells that could trigger GvHD[19]. Thus, an advanced method that can reliably generate a pure population of CAR-NKT cells at large quantities is thus pivotal to developing an off-the-shelf NKT cell-based cancer therapy.

We have successfully overcome this critical hurdle by establishing an ex vivo feeder-free hematopoietic stem and progenitor (HSP) cell-derived CAR-NKT cell culture method, that can produce therapeutic levels of pure and potent allogeneic CAR-NKT (^{Allo}CAR-NKT) cells suitable for off-the-shelf cell therapy[25,26] (Figs. 1 and 2). We demonstrated that these cells exhibit potent antitumor efficacy, showing effective tumor homing, clonal expansion, and persistence in vivo[25]. Impressively, ^{Allo}CAR-NKT cells can alter the tumor microenvironment by selectively depleting immunosuppressive tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs), and can antagonize tumor immune evasion by deploying CAR/TCR/NKR triple-targeting mechanisms[25]. ^{Allo}CAR-NKT cells also demonstrate an appealing safety profile with low GvHD and cytokine release syndrome risks, and exhibit a stable 'hypoimmunogenic' phenotype attributed to epigenetic and signaling regulations[25] (Fig. 2). So far, a diverse array of ^{Allo}CAR-NKT cell products have been generated, including those targeting blood cancers (i.e., B cell maturation antigen (BCMA) and CD19-targeting ^{Allo}CAR-NKT cells) and solid tumors (i.e., disialoganglioside (GD2)-, glypican-3 (GPC3)- and epidermal growth factor receptor variant III (EGFRvIII)-targeting ^{Allo}CAR-NKT cells) (Table 1). Additionally, allorejection-resistant allogeneic CAR-NKT cell products have been developed by using clustered regularly interspaced short palindromic repeats (CRISPR) gene editing to eliminate HLA-I and HLA-II expressions on the CAR-NKT cells[26]. To better enable

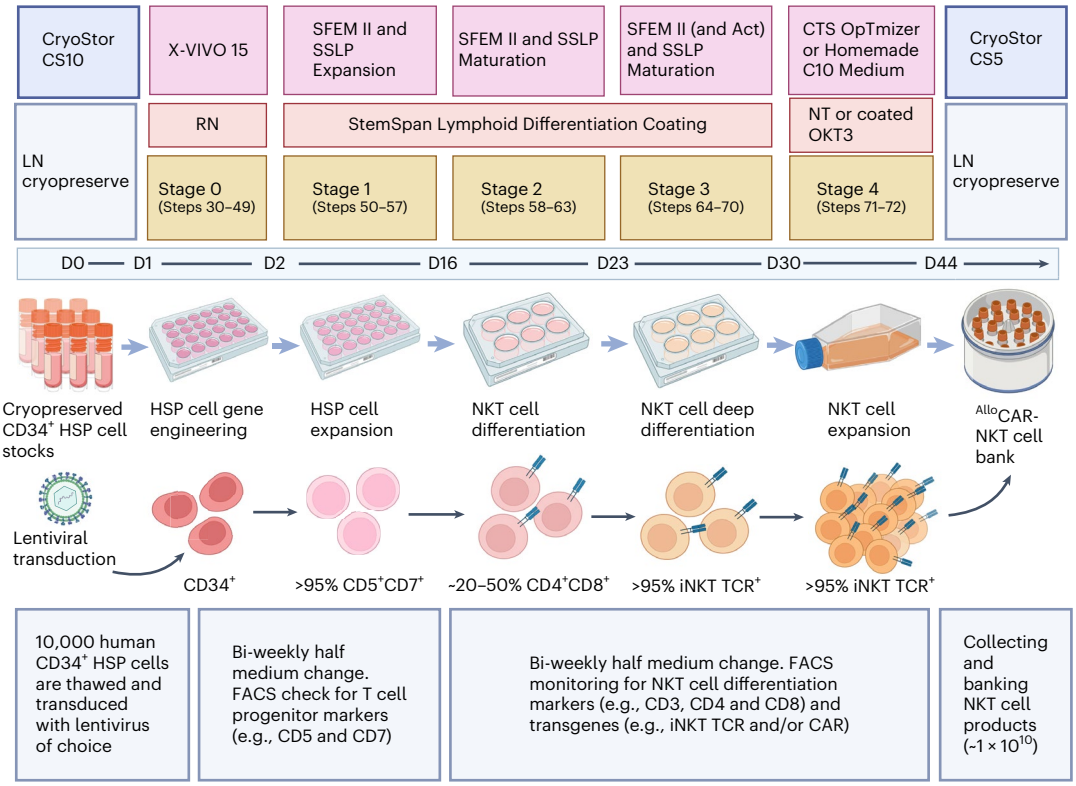


Fig. 1 | Generation of allogeneic CAR-NKT cells with genetically engineered HSP cells and feeder-free differentiation culture. A diagram showing the generation of allogeneic CAR-NKT cells using our technology. The culture media, coating materials and procedures are depicted. SFEM II, StemSpan SFEM II Medium; SSLP Expansion, StemSpan Lymphoid Progenitor Expansion Supplement; SSLP Maturation, StemSpan Lymphoid Progenitor Maturation Supplement; OKT3, Ultra-LEAF purified anti-human CD3 antibody (Clone OKT3); RN, RetroNectin; Act, ImmunoCult human CD3/CD28/CD2 T Cell Activator; LN, liquid nitrogen; NT, no treatment; D, day. The figure was created with BioRender.com.

adoption of our methods in other laboratories and biotech companies, here we describe detailed procedures for generating the ^{Allo}CAR-NKT cells with genetically engineered HSP cells and feeder-free differentiation culture (Fig. 1). We include optimized protocols for downstream preclinical analyses of the ^{Allo}CAR-NKT cells, encompassing evaluations of their pharmacology, efficacy, mechanism of action, pharmacokinetics/pharmacodynamics, safety and immunogenicity (Fig. 3).

Development of the protocol

In our approach, human cord blood (CB) CD34$^+$ HSP cells are transduced with a lentivector encoding a human NKT TCR gene together with a CAR gene, with an optional modulation-gene component (e.g., an immune-enhancement gene or/and a suicide gene), followed by a streamlined 6week culture to differentiate into ^{Allo}CAR-NKT cells[25]. This method is robust and of high yield; in our experiments, ~10^{10} ^{Allo}CAR-NKT cells were routinely generated from an initial input of 10,000 HSP cells (Table 2). Assuming linear extrapolation during scale-up production, we estimate that a single CB donor, containing ~5×10^6 CD34$^+$ HSP cells, can produce ~10^{12} ^{Allo}CAR-NKT cells, that can be formulated into ~1,000–10,000 doses (~10^8–10^9 cells per dose, based on approved CAR-T therapy doses[3,27]). The resulting ^{Allo}CAR-NKT cells are pure and clonal (all cells are NKT cells co-expressing a CAR), free of conventional αβ T cells and thereby are free of GvHD risk and can be directly cryopreserved as the final therapeutic cell product. Moreover, the cell culture is xenogeneic feeder free, making the method suitable for future clinical and commercial development.

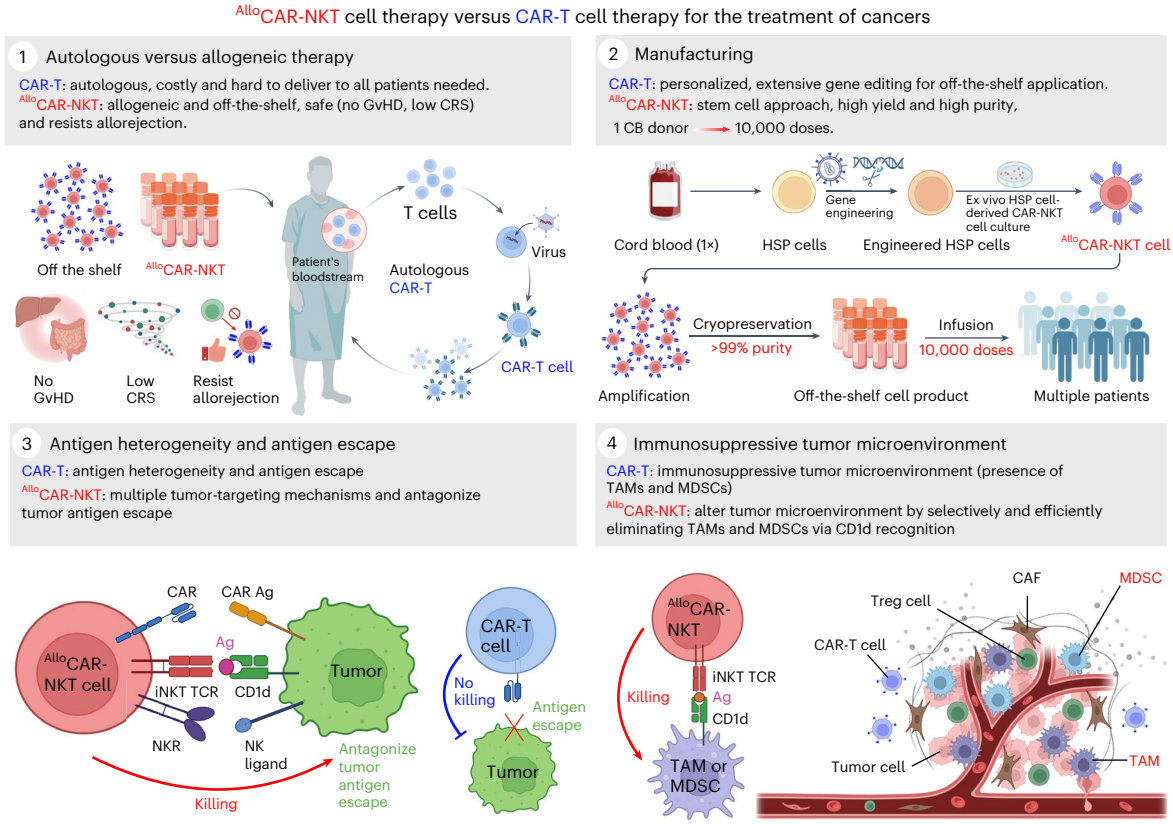


Fig. 2 | Comparison between our allogeneic HSP cell-derived CAR-NKT cell products and the conventional CAR-T cell products. A diagram illustrating the four major advantages of allogeneic CAR-NKT cells compared with conventional CAR-T cell products, including their allogeneic cell nature, high yield and purity in manufacturing, ability to counteract tumor antigen escape and capability to modify the immunosuppressive tumor microenvironment. CAF, cancer-associated fibroblast; Treg, regulatory T cell; CRS, cytokine release syndrome. The figure was created with BioRender.com.

Using this culture method, we have demonstrated the successful generation of HSP cell-engineered AlloCAR-NKT cell products for treating blood cancers and solid tumors[25]; those include CD19-targeting CAR-NKT (AlloCAR19-NKT) cell products for treating B cell malignancies[28], BCMA-targeting CAR-NKT (AlloBCAR-NKT) cell products for treating MM[29], GD2-targeting CAR-NKT (AlloGD2.CAR-NKT) cell products for treating GD2$^+$ solid tumors such as neuroblastoma[30], GPC3-targeting CAR-NKT (AlloGPC3.CAR-NKT) cell products for treating GPC3$^+$ solid tumors such as hepatocellular carcinoma[31] and EGFRvIII-targeting CAR-NKT (AlloECAR-NKT) cell products for treating EGFRvIII$^+$ solid tumors such as glioblastoma[32]. The AlloCAR-NKT cell production process is highly robust and versatile, spanning across diverse CB donors (over 15) and cargo genes[25]. These cargo genes may include CARs with different designs (CD28 or 4-1BB signaling domain), as well as additional genes such as IL-15. Remarkably, the variability in CBs and cargo genes had no discernible impact on the high yield and purity of the final AlloCAR-NKT cell products[25] (Table 2). Notably, incorporation of an IL-15 immune-enhancement gene in the AlloCAR-NKT cell product can enhance its in vivo persistence, clonal expansion and antitumor efficacy, while maintaining a high safety profile, consistent with previous autologous CAR-NKT cell clinical trials[8,24].

Application of the method

The development of AlloCAR-NKT cell products marks a substantial advancement in cancer immunotherapy, particularly for solid tumors, which have traditionally posed considerable challenges for CAR-T cell therapies. Unlike conventional autologous CAR-T approaches that

Table 1 | Summary of allogeneic HSP cell-derived NKT cell products

Cell product	Lentivector(s)	Description	References
^{Allo}NKT	Lenti/iNKT	Allogeneic HSP cell-derived NKT cells	25
^{Allo}BCAR-NKT	Lenti/iNKT-BCAR	Allogeneic HSP cell-derived BCMA-targeting CAR-engineered NKT cells	25
^{Allo15}BCAR-NKT	Lenti/iNKT-BCAR-IL15	Allogeneic HSP cell-derived IL-15-enhanced BCMA-targeting CAR-engineered NKT cells	25,26
^{Allo}BCAR-NKT/FG	Lenti/iNKT-BCAR and Lenti/FG	Allogeneic HSP cell-derived BCMA-targeting CAR-engineered NKT cells labeled with enhanced green fluorescence protein (FG)	25
^{Allo15}BCAR-NKT/FG	Lenti/iNKT-BCAR-IL15 and Lenti/FG	Allogeneic HSP cell-derived IL-15-enhanced BCMA-targeting CAR-engineered NKT cells labeled with FG	25
^{Allo}CAR19-NKT	Lenti/iNKT-CAR19	Allogeneic HSP cell-derived CD19-targeting CAR-engineered NKT cells	25
^{Allo15}CAR19-NKT	Lenti/iNKT-CAR19-IL15	Allogeneic HSP cell-derived IL-15-enhanced CD19-targeting CAR-engineered NKT cells	25
^{Allo}GD2.CAR-NKT	Lenti/iNKT-GD2.CAR	Allogeneic HSP cell-derived GD2-targeting CAR-engineered NKT cells	25
^{Allo15}GD2.CAR-NKT	Lenti/iNKT-GD2.CAR-IL15	Allogeneic HSP cell-derived IL-15-enhanced GD2-targeting CAR-engineered NKT cells	25
^{Allo}GPC3.CAR-NKT	Lenti/iNKT-GPC3.CAR	Allogeneic HSP cell-derived GPC3-targeting CAR-engineered NKT cells	25
^{Allo15}GPC3.CAR-NKT	Lenti/iNKT-GPC3.CAR-IL15	Allogeneic HSP cell-derived IL-15-enhanced GPC3-targeting CAR-engineered NKT cells	25
^{Allo}ECAR-NKT	Lenti/iNKT-ECAR	Allogeneic HSP cell-derived EGFRvIII-targeting CAR-engineered NKT cells	25
^{Allo15}ECAR-NKT	Lenti/iNKT-ECAR-IL15	Allogeneic HSP cell-derived IL-15-enhanced EGFRvIII-targeting CAR-engineered NKT cells	25
^{Allo15}NKT-TK	Lenti/iNKT-IL15-sr39TK	Allogeneic HSP cell-derived IL-15-enhanced sr39TK-engineered NKT cells	25
^{Allo15}NKT-TK/FG	Lenti/iNKT-IL15-sr39TK and Lenti/FG	Allogeneic HSP cell-derived IL-15-enhanced sr39TK-engineered NKT cells labeled with FG	25

necessitate individualized manufacturing from the patient's own cells, ^{Allo}CAR-NKT cells are allogeneic, derived from CB HSP cells[25]. This allogeneic nature ensures a ready-to-use and scalable cell product, effectively bypassing the lengthy and costly production processes associated with autologous therapies. The high purity, yield and robustness of ^{Allo}CAR-NKT cells, along with their enhanced NK cell-killing capacity, make them an ideal candidate for off-the-shelf immunotherapy[25]. The incorporation of CAR technology into these cells greatly enhances their specificity and efficacy, enabling them to target tumors through a triple-targeting mechanism that includes NKR cytotoxicity, iNKT TCR-dependent CD1d killing and CAR-mediated antigen recognition[25]. Beyond the ^{Allo}CAR-NKT cells we already generated, additional CARs can be engineered onto our HSP cell-derived NKT cells, including mesothelin -targeting CARs for ovarian cancer[33], malignant pleural mesothelioma[34], breast cancer[35] and pancreatic cancer[36]; HER2-targeting CARs for sarcoma[37], pediatric central nervous system tumors[38] and glioblastoma[39]; IL-13Rα2-targeting CARs for glioblastoma[40]; prostate-specific membrane antigen-targeting CARs for prostate cancer[41]; and CD70-targeting CARs for acute myeloid leukemia[42]. This innovative ^{Allo}CAR-NKT cell platform holds promise for improving patient outcomes by providing a readily available, cost-effective and potent therapeutic option for a wide range of cancers, overcoming the limitations of current CAR-T therapies while maintaining a high safety profile devoid of GvHD complications[25].

In recent years, CAR-T cell therapy has expanded beyond oncology to address various diseases, including GvHD, viral infections and autoimmune disorders[43]. Clinical successes with CD19-targeting CAR-T cells have been documented in systemic lupus erythematosus[44,45], idiopathic inflammatory myositis[44], systemic sclerosis[44], antisynthetase syndrome[46] and multiple sclerosis[47], while preclinical studies have shown promising results in treating encephalomyelitis[48] and rheumatoid arthritis[49]. Our ^{Allo}CAR-NKT cell products offer a promising source for allogeneic cell therapy for autoimmune diseases, given their high robustness and safety profile. Furthermore, our previous studies have demonstrated the potential of allogeneic NKT cells in controlling severe acute respiratory syndrome coronavirus 2 viral infections[50] and ameliorating GvHD symptoms following allogeneic hematopoietic stem cell transplantation[51]. These findings highlight the versatility and therapeutic potential of ^{Allo}CAR-NKT cells in addressing a wide array of diseases beyond cancer.

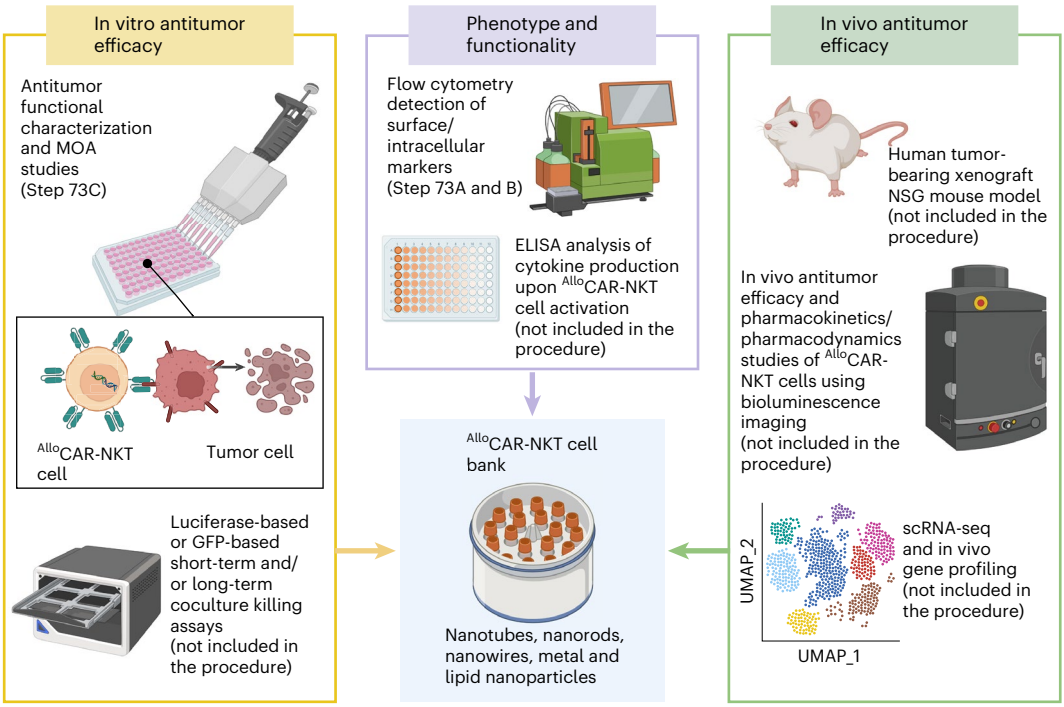


Fig. 3 | Downstream assays to evaluate the allogeneic CAR-NKT cells. A diagram showing the downstream assays to evaluate the pharmacology, efficacy, mechanism of action and pharmacokinetics/pharmacodynamics of allogeneic CAR-NKT cells. NSG, NOD scid gamma. The figure was created with BioRender.com.

Comparison with other methods

The conventional method for generating CAR-engineered cell products typically involves an autologous approach, wherein immune cells are isolated from patients, engineered and re-infused back into the patient[52–55]. Although an effective strategy, there are many logistical limitations including prolonged manufacturing processes, a lack of standardization, substantial costs and restrictions on patient selection that make allogeneic CAR-engineered strategies more attractive[56–58]. Various forms of allogeneic CAR-engineered cell products employ T cells with *TRAC* knockout[5,59,60], NK cells[7,61,62], innate T cells such as gamma delta (γδ T) and NKT cells[19,63], along with HSP cell-derived NKT cells. While all these approaches encompass allogeneic cell therapy, each strategy come with their own advantages and disadvantages.

Table 2 | Representative yield of allogeneic HSP cell-derived NKT cell products

NKT cell product	Input HSP cell number	Final cell yield (one representative data shown)
^{Allo}NKT	10,000	2.3935×10^{10}
^{Allo}BCAR-NKT	10,000	2.3782×10^{10}
^{Allo15}BCAR-NKT	10,000	3.2379×10^{10}
^{Allo}CAR19-NKT	10,000	1.9577×10^{10}
^{Allo15}CAR19-NKT	10,000	1.8937×10^{10}
^{Allo}GD2.CAR-NKT	10,000	2.1094×10^{10}
^{Allo15}GD2.CAR-NKT	10,000	3.2843×10^{10}
^{Allo}GPC3.CAR-NKT	10,000	2.9673×10^{10}
^{Allo15}GPC3.CAR-NKT	10,000	3.1947×10^{10}
^{Allo}ECAR-NKT	10,000	2.9018×10^{10}
^{Allo15}ECAR-NKT	10,000	2.9814×10^{10}

The approach involving genetically modified αβ T cells utilizes conventional αβ T cells for CAR antigen engineering and serves as an ideal cell type given their intrinsic cytotoxicity, persistence and their ability to clonally expand. To mitigate the risk of GvHD and allo-rejection, CRISPR–Cas9 is used to knockout the TRAC locus of the TCR[5,59,60,64], as well as components of antigen-presenting molecules such as B2M and CIITA[65,66]. Although successful in reducing GvHD, Stenger et al. demonstrated that disrupting the endogenous TCR compromises the cells' longevity and consequently the antitumor efficacy[67]. In contrast, our approach involves engineering HSP cell-derived CAR-NKT cells to express the iNKT TCR, enhancing their longevity and antitumor capacity and endowing them with the ability to alter the tumor microenvironment by targeting TAMs and MDSCs via TCR-dependent mechanisms[67,68]. This enhancement provides our cells with additional functionality compared with allogeneic T cell approaches.

Similar to HSP cell-derived CAR-NKT cells, innate-like T cells including PBM cell-derived endogenous γδ T and NKT cells, are utilized for allogeneic CAR-cell therapy. Innate T cells express invariant TCRs that are MHC-I independent and subsequently have reduced risk of GvHD compared with their T cell counterparts[69,70]. Despite their ideal characteristics, the utilization of innate cells for cell-based therapy is limited by their low abundance in peripheral blood. γδ T and NKT cells constitute only 1–5% and 0.01–0.2% of circulating T cells, respectively[71,72]. Another major problem intrinsic to PBM cell-derived cell products include donor–donor variations that may lead to uncontrollable differences between batches of cell products[63]. In contrast, our HSP cell-derived CAR-NKT cells overcome these challenges. Our protocol enables ~10^{12} CAR-NKT cells to be produces from a single CB donor (typically comprising ~1–10 × 10^6 $CD34^+$ HSP cells), allowing for efficient and scalable generation of allogeneic CAR-NKT cells. Additionally, by using HSP cells as the starting material instead of isolating the cells from peripheral blood, we diminish the effects of donor–donor variation, standardizing the manufacturing process to consistently produce the same cell product each time. Overall, this protocol provides a feasibility advantage over innate cell-based allogeneic cell platforms, providing a more robust and reproducible platform for allogeneic cell therapies.

NK cells provide an optimal cell type for allogeneic cell therapy because of their innate-like properties that confer MHC-I independent tumor-killing mechanisms and robust antitumor response[61,73–77]. Additionally, their MHC independence also eliminates the risk of GvHD[68,69]. The various starting materials that have been used to generate NK cells for allogeneic cell therapy include PBM cells, CB and induced pluripotent stem (iPS) cells. iPS cells, in particular, offer a versatile platform to generate an unlimited number of NK cells, can be efficiently engineered with CAR and armoring molecules (e.g. IL-15) and are susceptible to editing with CRISPR–Cas9 (refs. 61,73–77). iPS cell-derived NK cell-based platforms have the advantage in that they can be efficiently engineered at the iPS cell stage of NK cell development, ensuring an endless supply of engineered NK cells for allogeneic cell therapy[73,76]. Nevertheless, the expansion and effectiveness against tumors in vivo of NK cells might be more restricted compared with conventional αβ T cells[7,61,62]. Overall, while various allogeneic cell-based therapies have their unique advantages and limitations, the CAR-NKT cell-based platform effectively address some of the challenges faced by others and advance the field of stem cell-derived allogeneic cell therapy.

Limitations

While our HSP cell-derived CAR-NKT cell culture presents advantages over autologous and certain allogeneic cell-based therapies, our protocol encounters its own challenges as well. These challenges include the potential risk of allorejection, the absence of CD4 CAR-NKT cells and limitations in engineering approaches.

The risk of allorejection persists over allogeneic cell-based therapies, including our HSP cell-derived CAR-NKT cells, wherein the host's immune system recognizes the therapeutic cells as foreign and consequently rejects their engraftment[57,58,78]. This rejection diminishes their persistence within the host and compromises their effectiveness against tumors[57,58,78–80]. To mitigate the immunogenicity of our cells and minimize the risk of allorejection, genetic

engineering strategies can be incorporated into our HSP cell-derived CAR-NKT cell protocol. At the HSP cell stage, CRISPR–Cas9 can be employed to knockout *B2M* and *CIITA* thereby preventing HLA-I and HLA-II mismatch[57]. Additionally, enhancing HLA-E or CD47 expression can be used to reduce innate cell-mediated allorejection[81,82]. Multiple approaches exist to combat the challenge of allorejection and can enhance the persistence and overall efficacy of our HSP cell-derived CAR-NKT cells.

Within our ex vivo HSP cell-derived CAR-NKT cell culture, a limitation is the absence of single positive CD4 helper CAR-NKT cells. Our protocol generates primarily CD8 single-positive (SP) and CD4/CD8 double-negative (DN) CAR-NKT cells, a characteristic shared with many in vitro maturation methods favoring the production of CD8 SP and DN T cells[83]. Notably, one organoid-based T cell differentiation method has so far been demonstrated to generate CD4 SP T cells[84]. CD4 helper T cells play a pivotal clinical role and are an important cell subtype that have been implicated in enhancing the antitumor response. Studies in glioblastoma models have underscored the superiority of CD4 CAR-T cells in their antitumor response compared with CD8 CAR-T cells, demonstrating their cytotoxic capabilities[85,86]. Moreover, a balanced ratio of CD4 and CD8 CAR-T cells has exhibited synergistic effects with enhanced antitumor efficacy compared with individual CD4 and CD8 CAR-T cells in the in vivo tumor models[87]. The critical role of CD4 helper T cells in the antitumor response perpetuates the potential benefit of generating CD4 CAR-NKT cells. To improve our in vitro maturation protocol and facilitate the production of HSP cell-derived CD4 CAR-NKT cells, further optimization is necessary. A deeper comprehension of T cell lineage commitment is essential to address this gap, potentially leading to a more robust antitumor response.

Lastly, the use of HSP cells as our starting material is costly, finite and presents multiple constraints that limits our capacity for additional genetic engineering strategies alongside the CAR engineering. The finite nature of HSP cells limits our ability to efficiently apply genetic modification using CRISPR–Cas9 to enhance the final product's functionality. These modifications could include knocking out immunosuppressive signaling receptors including receptors for TGF-β, adenosine and PD-L1 to increase persistence[88–90] or targeting *B2M* and *CIITA* molecules to mitigate the risk of allorejection[19]. Therefore, using iPS cells as a starting material emerges as a promising avenue. iPS cells not only offer an unlimited source for generating CAR-NKT cells, but also circumvents the limitation intrinsic to using HSP cells as the starting material[63]. By leveraging stem cells, we can potentially overcome the inherent constraints associated with HSP cells and expand our genetic engineering capabilities to generate enhanced and cost-effective iPS cell-derived CAR-NKT cells.

Expertise needed to implement the protocol

The methodology outlined provides a well-defined and efficient protocol for reliably generating CAR-NKT cells from gene-engineered HSP cells. The protocol draws parallels with fundamental stem cell and T cell culturing methods and emphasizes the use of flow cytometry for effective cell monitoring. Expertise in human stem and T cell culture, along with proficiency in gene engineering and flow cytometry, is required to implement our protocol. We are confident that individuals who adhere to our protocol and have access to necessary materials can robustly and reliably generate substantial quantities of HSP cell-engineered CAR-NKT cells.

Experimental design

Overview

This protocol comprises two parts: the first delineates the generation of ^{Allo}CAR-NKT cells from HSP cell gene engineering and feeder-free culture, encompassing the steps of lentivirus generation (Steps 1–27), lentivirus titration (Steps 28–29), HSP cell engineering (Steps 30–49), HSP cell expansion (Steps 50–57), NKT cell differentiation (Steps 58–63), NKT cell deep differentiation (Steps 64–70) and NKT cell expansion (Steps 71–72). Note that NKT cell differentiation refers to the stage during which NKT cells progress from the CD4/CD8 DN stage to the CD4/CD8 double-positive stage and NKT cell deep differentiation refers to the stage during which NKT cells progress from the CD4/CD8 double-positive stage to the CD4/CD8 DN or CD8 SP stage. The second part outlines downstream assays (Step 73), highlighting the analysis

Table 3 | Virus titer of the representative lentivectors

Lentivector	Titer (IFU/mL)
Lenti/iNKT	$1.734 \times 10^8 \pm 7.47 \times 10^7$
Lenti/iNKT-BCAR	$1.628 \times 10^8 \pm 1.86 \times 10^7$
Lenti/iNKT-BCAR-IL15	$1.784 \times 10^8 \pm 5.61 \times 10^7$
Lenti/iNKT-CAR19	$1.473 \times 10^8 \pm 3.05 \times 10^7$
Lenti/iNKT-CAR19-IL15	$1.592 \times 10^8 \pm 4.29 \times 10^7$
Lenti/iNKT-GD2.CAR-IL15	$1.535 \times 10^8 \pm 2.53 \times 10^7$
Lenti/iNKT-GPC3.CAR-IL15	$1.603 \times 10^8 \pm 5.32 \times 10^7$
Lenti/iNKT-ECAR-IL15	$1.673 \times 10^8 \pm 3.63 \times 10^7$

of surface marker expression (option A), analysis of cytokine production (option B) and analysis of antitumor capacity (option C) of allogeneic CAR-NKT cells.

Lentivirus generation and titration

To optimize lentivirus generation for efficient transduction of $CD34^+$ HSP cells, several key points should be considered: (1) optimal vector design (Table 1). Ensure the iNKT TCR, CAR and additional genes are appropriately designed and inserted into the lentiviral vector, considering factors such as CAR structure, promoter strength and inclusion of necessary regulatory elements. (2) Packaging cell line. Select a suitable packaging cell line (e.g., HEK293T) for lentivirus production, ensuring high transfection efficiency and minimal cellular toxicity. (3) Transfection method (Steps 1–21). Employ a reliable transfection method (e.g., calcium phosphate precipitation, lipid-based transfection) to introduce the lentiviral vector into packaging cells. In our protocol, we adopted the Trans-IT-Lenti Transfection reagent (Mirus Bio) and a centrifugation concentration protocol using the Amicon Ultra Centrifugal Filter Units, according to the manufacturers' instructions (Millipore Sigma). (4) Purification of lentivirus (Steps 22–27). Utilize appropriate methods (e.g., ultracentrifugation, gradient centrifugation) to purify lentivirus particles from culture supernatants, ensuring high viral titer and purity. (5) Titration (Steps 28–29). Determine the viral titer accurately through titration assays (e.g., qPCR, fluorescent reporter assays) to establish the optimal multiplicity of infection for transduction (Tables 3 and 4). (6) Transduction protocol (Steps 30–49). Develop a robust transduction protocol, including the appropriate duration and conditions for lentiviral exposure to target cells, to achieve efficient HSP cell transduction while minimizing cellular stress or toxicity.

HSP cell-derived CAR-NKT cell culture method

This method contains a five-stage, 6 week process (Fig. 1). In this method, we use purified human CB-derived $CD34^+$ HSP cells acquired from commercial vendors such as HemaCare. Upon arrival, these HSP cells are cryopreserved and stored in liquid nitrogen for long-term preservation. At stage 0 (HSP cell engineering; Steps 30–49), the freeze-thawed $CD34^+$ HSP cells

Table 4 | Vector copy number of the representative lentivectors in allogeneic HSP cell-derived NKT cell products

Lentivector	NKT cell product	Vector copy number
Lenti/iNKT	^{Allo}NKT	3.39 ± 0.17
Lenti/iNKT-BCAR	^{Allo}BCAR-NKT	3.29 ± 0.10
Lenti/iNKT-BCAR-IL15	^{Allo15}BCAR-NKT	3.40 ± 0.05
Lenti/iNKT-CAR19	^{Allo}CAR19-NKT	3.43 ± 0.08
Lenti/iNKT-CAR19-IL15	^{Allo15}CAR19-NKT	3.52 ± 0.24
Lenti/iNKT-GD2.CAR-IL15	^{Allo15}GD2.CAR-NKT	3.26 ± 0.19
Lenti/iNKT-GPC3.CAR-IL15	^{Allo15}GPC3.CAR-NKT	3.19 ± 0.17
Lenti/iNKT-ECAR-IL15	^{Allo15}ECAR-NKT	3.51 ± 0.08

are transduced with a lentivector codelivering a human NKT TCR and a designated CAR, as well as additional genes, and cultured over 48 h in a classical X-VIVO 15-based HSP cell medium. At stage 1 (HSP cell expansion; Steps 50–57), gene engineered HSP cells are cultured in the stage 1 culture medium to support the HSP cell expansion[91,92]. At stage 2 (NKT cell differentiation; Steps 58–63), HSP cells are cultured in the stage 2 culture medium to support the NKT cell differentiation[93]. At stage 3 (NKT cell deep differentiation; Steps 64–70), generated NKT cells are cultured in the stage 3 culture medium to support the NKT cell deep differentiation[93]. At stage 4 (NKT cell expansion; Steps 71–72), three strategies are employed to support NKT cell expansion, including the usage of αCD3/αCD28 antibodies (Step 72A), α-galactosylceramide (αGC)-loaded healthy donor PBM cells (Step 72B), and K562-based artificial antigen-presenting cells (aAPCs; Step 72C). The entire five-stage culture (Steps 30–72) can be implemented in a feeder-free and serum-free manner, supporting clinical and commercial development[94]. Alternatively, the usage of feeder cell (αGC-loaded PBM cells or aAPCs)-dependent NKT cell expansion is also suitable for clinical and commercial development[24,94].

On the basis of our previous results, we did not observe notable differences in the allogeneic CAR-NKT cells generated using the three expansion methods (i.e., αCD3/αCD28 antibodies, αGC-loaded PBM cells and aAPCs) regarding their phenotype, functionality or tumor-killing efficacy[25,26]. However, the yield of allogeneic CAR-NKT cells generated using aAPCs was higher compared with the other two expansion methods[25,26]. Therefore, we recommend the aAPC expansion protocol when a higher yield of allogeneic CAR-NKT cells is desired. For implementing feeder-free culture, which supports the commercial development of cell products, the αCD3/αCD28 antibodies expansion protocol is recommended.

Downstream assays

After successfully generating allogeneic CAR-NKT cells from gene-engineered HSP cells using the provided protocol, the phenotype and functionality of these cells can be evaluated with downstream assays (Fig. 3). Comprehensive assessments of their pharmacology, efficacy, mechanism of action, pharmacokinetics/pharmacodynamics, safety and immunogenicity have been conducted in our previous publication[25,26]. Understanding the pharmacological properties of ^{Allo}CAR-NKT cells helps to elucidate their phenotype and functionality, which are essential for optimizing therapeutic efficacy. Efficacy analysis determines the ability of ^{Allo}CAR-NKT cells to effectively target and eliminate cancer cells, confirming the practical therapeutic benefits of the treatment. Studying the mechanism of action provides insights into how ^{Allo}CAR-NKT cells recognize and destroy cancer cells, which is vital for predicting responses and potential resistance mechanisms in patients. Pharmacokinetics/pharmacodynamics studies reveal how the ^{Allo}CAR-NKT cells distribute, metabolize and exert their effects over time within the body, influencing dosing schedules and predicting potential side effects. Safety evaluations are critical to identify any adverse effects or toxicity associated with ^{Allo}CAR-NKT cell therapies, ensuring that the treatment does not cause harm to patients. In addition, analyzing immunogenicity is important because it assesses the potential of ^{Allo}CAR-NKT cells to provoke an immune response, which can affect the persistence, efficacy and safety of the therapy, especially in an allogeneic setting where ^{Allo}CAR-NKT cells are derived from a donor other than the patient with cancer[25,26].

In this protocol, three key aspects are highlighted, including analysis of surface marker expression (Step 73A), analysis of cytokine production (Step 73B) and analysis of antitumor capacity (Step 73C) of ^{Allo}CAR-NKT cells. The analysis of surface marker expression and cytokine production in ^{Allo}CAR-NKT cells is performed using flow cytometry[25,26]. Their antitumor capabilities are evaluated through a series of in vitro tumor cell-killing assays. All utilized tumor cell lines have been engineered to express firefly luciferase and enhanced green fluorescence protein (Fluc-EGFP) dual-reporters, enabling luminescence detection[19,95]. In the in vitro assays, tumor cells and ^{Allo}CAR-NKT cells are cocultured for periods such as 24 or 48 h, after which the remaining tumor cells are analyzed via luminescence analyses. In certain assays, αGC is added to the coculture to assess the iNKT TCR-mediated tumor cell-killing mechanism[25,26]. Additionally, NKR antibodies, such as anti-NKG2D and anti-DNAM-1, are included in some cocultures to explore the NKR-mediated tumor cell-killing mechanism[25,26].

Materials

▲ **CRITICAL** The reagents and equipment listed in this section have been tested to ensure optimal performance for our protocols. Materials may be substituted for equivalents from other suppliers, but we cannot guarantee that they will perform equally.

Biological materials

Human CB-derived CD34$^+$ HSP cells

Purified CB-derived human CD34$^+$ HSP cells are purchased from commercial vendors such as HemaCare. Typically, one lot of CB samples, containing 1–2 × 10^6 CD34$^+$ HSP cells, is purchased. Upon receipt, the HSP cells are thawed, divided into aliquots of 50,000 cells per vial and cryopreserved in liquid nitrogen for long-term storage.

▲ **CRITICAL** Upon receipt from the commercial vendors, the CB HSP cells are tested for purity (identified as CD34$^+$ cells) and viability (identified as eFluor 506$^-$ cells) by flow cytometry. The purity should be greater than 98% and the viability should be greater than 95%. The cells should be promptly cryopreserved in CryoStor CS10 freeze medium using a CoolCell cell freezing container (BioCision) at a rate of −1 °C/min.

▲ **CRITICAL** In this study, all HSP cells used were cryopreserved, which are commonly utilized due to their availability, readiness and ability to ensure batch-to-batch consistency. The characteristics and capacity to differentiate into CAR-NKT cells of freshly isolated HSP cells were not tested.

▲ **CRITICAL** Long-term storage of HSP cells at −80 °C is not recommended. Once thawed, HSP cells should be used immediately.

▲ **CAUTION** In our study, the purified CB-derived human CD34$^+$ HSP cells are purchased from the HemaCare without identification information, under federal and state regulations. When using human HSP cells, it is crucial to adhere to appropriate national laws and institutional regulatory board guidelines, and to obtain informed consent.

Human PBM cells

Healthy donor PBM cells are obtained from the University of California, Los Angeles, Center for AIDS Research (UCLA/CFAR) Virology Core Laboratory without identification information, under federal and state regulations. Upon receipt, PBM cells are divided into aliquots of 1–5 × 10^7 cells per vial and cryopreserved in liquid nitrogen for long-term storage.

▲ **CRITICAL** Long-term storage of PBM cells at −80 °C is not recommended. PBM cells can be frozen and thawed multiple times, with up to three cycles generally considered acceptable.

▲ **CAUTION** In our study, the human PBM cells are obtained from the UCLA/CFAR Virology Core Laboratory without identification information, under federal and state regulations. When using human PBM cells, it is crucial to adhere to appropriate national laws and institutional regulatory board guidelines, and to obtain informed consent.

Tumor cell lines

Details about the tumor cell lines used in this study can be found in Table 5.

▲ **CRITICAL** The cell lines used in the research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

Packing cell line

The human embryonic kidney (HEK) 293T cell line is purchased from American Type Culture Collection (ATCC) (cat. no. CRL-3216, RRID: CVCL_0063), and used as the lentivirus packing cell line.

Reagents

Antibodies

Ultra-LEAF purified anti-human CD3 antibody (clone OKT3, BioLegend, cat. no. 317347; RRID: AB_2571994)

Table 5 | Information on the tumor cell lines used in this study

Cell line	Cancer type	Supplier	Catalog number	RRID
MM.1S	Human MM	ATCC	CRL-2974	CVCL_8792
K562	Human chronic myelogenous leukemia	ATCC	CCL-243	CVCL_0004
Raji	Human Burkitt's lymphoma	ATCC	CCL-86	CVCL_0511
NALM-6	Human acute lymphoblastic leukemia	ATCC	CRL-3273	CVCL_UJ05
A375	Human melanoma	ATCC	CRL-1619	CVCL_0132
U87MG	Human glioblastoma	ATCC	HTB-14	CVCL_0022
HEPG2	Human hepatocellular carcinoma	ATCC	HB-8065	CVCL_0027
HEP3B	Human hepatocellular carcinoma	ATCC	HB-8064	CVCL_0326
C3A	Human hepatocellular carcinoma	ATCC	CRL-3581	CVCL_1098
THP1	Human acute myeloid leukemia	ATCC	TIB-202	CVCL_0006
H226	Human lung cancer	ATCC	CRL-5826	CVCL_1544
A549	Human lung cancer	ATCC	CCL-185	CVCL_0023
H292	Human lung cancer	ATCC	CRL-1848	CVCL_0455
HCC1806	Human breast cancer	ATCC	CRL-2335	CVCL_1258
MDA-MB-231	Human breast cancer	ATCC	HTB-26	CVCL_0062
786O	Human pancreatic cancer	ATCC	CRL-1932	CVCL_1051
ACHN	Human pancreatic cancer	ATCC	CRL-1611	CVCL_1067
PC3	Human prostate cancer	ATCC	CRL-1435	CVCL_0035
OVCAR3	Human ovarian cancer	ATCC	HTB-161	CVCL_0465
SKOV3	Human ovarian cancer	ATCC	HTB-77	CVCL_0532
SW480	Human colon cancer	ATCC	CCL-228	CVCL_0546
OVCAR8	Human ovarian cancer	National Institutes of Health	NA	CVCL_1629

Ultra-LEAF purified anti-human CD28 antibody (clone CD28.2, BioLegend, cat. no. 302943; RRID: AB_2616667)

Anti-human CD45 (clone H130, BioLegend, cat. no. 304026, PerCP-conjugated, 1:500 dilution; RRID: AB_893337)

Anti-human TCRαβ (clone I26, BioLegend, cat. no. 306716, PE-Cy7-conjugated, 1:25 dilution; RRID: AB_1953257)

Anti-human CD3 (clone HIT3a, BioLegend, cat. no. 300330, PacBlue-conjugated, 1:500 dilution; RRID: AB_10551436)

Anti-human CD4 (clone OKT4, BioLegend, cat. no. 317414, PE-Cy7-conjugated, 1:400 dilution; RRID: AB_571959)

Anti-human CD8 (clone SK1, BioLegend, cat. no. 344708, PerCP-conjugated, 1:500 dilution; RRID: AB_1967149)

Anti-human CD8 (clone SK1, BioLegend, cat. no. 344714, APC-Cy7-conjugated, 1:500 dilution; RRID: AB_2044006)

Anti-human β2-microglobulin (B2M) (clone 2M2, BioLegend, cat. no. 316304, FITC-conjugated, 1:5,000 dilution; RRID: AB_492837)

Anti-human HLA-DR, DP, DQ (clone Tü 39, BioLegend, cat. no. 361707, PE-Cy7-conjugated, 1:250 dilution; RRID: AB_2564278)

Anti-human NKG2D (clone 1D11, BioLegend, cat. no. 320812, PE-Cy7-conjugated, 1:50 dilution; RRID: AB_2234394)

Anti-human DNAM-1 (clone 11A8, BioLegend, cat. no. 338312, APC-conjugated, 1:50 dilution; RRID: AB_2561952)

Anti-human Granzyme B (clone QA16A02, BioLegend, cat. no. 372204, APC-conjugated, 1:4,000 dilution; RRID: AB_2687028)

Anti-human Perforin (clone dG9, BioLegend, cat. no. 308126, PE-Cy7-conjugated, 1:50 dilution; RRID: AB_2572049)

Anti-human IFN-γ (clone B27, BioLegend, cat. no. 506518, PE-Cy7-conjugated, 1:50 dilution; RRID: AB_2123321)

Anti-human CD34 (clone 581, BD Biosciences, cat. no. 555822, PE-conjugated, 1:100 dilution; RRID: AB_396151)

Anti-human iNKT TCR Vα24^{-}Jβ18 (clone 6B11, BD Biosciences, cat. no. 552825, PE-conjugated, 1:10 dilution; RRID: AB_394478)

Goat anti-mouse IgG F(ab')2 secondary antibody (Thermo Fisher, cat. no. 31803, 1:50 dilution; RRID: AB_228311)

Anti-human IFN-γ (ELISA, capture, BD Biosciences, cat. no. 551221; RRID: AB_394099)

Anti-human IFN-γ (ELISA, detection, BD Biosciences, cat. no. 554550; RRID: AB_395472)

Anti-human TNF-α (ELISA, capture, BD Biosciences, cat. no. 551220; RRID: AB_394098)

Anti-human TNF-α (ELISA, detection, BD Biosciences, cat. no. 554511; RRID: AB_395442)

Anti-human IL-2 (ELISA, capture, BD Biosciences, cat. no. 554563; RRID: AB_398570)

Anti-human IL-2 (ELISA, detection, BD Biosciences, cat. no. 555040; RRID: AB_395666)

Anti-human IL-4 (ELISA, capture, BD Biosciences, cat. no. 554515; RRID: AB_398567)

Anti-human IL-4 (ELISA, detection, BD Biosciences, cat. no. 554483; RRID: AB_395422)

LEAF purified anti-human NKG2D antibody (clone 1D11, BioLegend, cat. no. 320810; RRID: AB_2133276)

LEAF purified anti-human DNAM-1 antibody (clone DX11, BioLegend, cat. no. 5597860; RRID: AB_397327)

LEAF purified mouse IgG2bk isotype control antibody (clone MG2b-57, BioLegend, cat. no. 401212)

Culture components

X-VIVO 15 serum-free hematopoietic stem cell medium (Lonza, cat. no. 02-060F)
StemSpan SFEM II Medium (Stemcell Technologies, cat. no. 09655)
StemSpan Lymphoid Progenitor Expansion Supplement (Stemcell Technologies, cat. no. 09915)
StemSpan Lymphoid Differentiation Coating Material (Stemcell Technologies, cat. no. 09925)

StemSpan Lymphoid Progenitor Maturation Supplement (10×) (Stemcell Technologies, cat. no. 09930)
ImmunoCult Human CD3/CD28/CD2 T Cell Activator (Stemcell Technologies, cat. no. 10970)
Cell Therapy Systems (CTS) OpTmizer T-Cell Expansion Serum Free Medium (SFM) (Thermo Fisher Scientific, cat. no. A1048501)
αGC (KRN7000) (Avanti Polar Lipids, cat. no. 867000P)
Dulbecco's modified Eagle's medium (DMEM) (Corning Cellgro, cat. no. 10-013-CV)
Penicillin–streptomycin–glutamine (100×), liquid (Life Technology, cat. no. 10378016)
Fetal bovine serum (FBS) (MilliporeSigma, cat. no. F2442)
RPMI 1640 (Corning cellgro, cat. no. 10-040-CV)
MEM non-essential amino acids (NEAA) solution (100×) (Gibco, cat. no. 11140050)
HEPES buffer solution (1 M) (Gibco, cat. no. 15630080)
Sodium pyruvate (100 mM) (Gibco, cat. no. 11360)
β-Mercaptoethanol (β-ME) (Millipore Sigma, cat. no. M7522)
Normocin (Invivogen, cat. no. ant-nr-2)
Recombinant human IL-2 (PeproTech, cat. no. 200-02)
Recombinant human IL-3 (PeproTech, cat. no. 200-03)
Recombinant human IL-7 (PeproTech, cat. no. 200-07)
Recombinant human IL-15 (PeproTech, cat. no. 200-15)
Recombinant human IFN-γ (PeproTech, cat. no. 300-02)
Recombinant human stem cell factor (PeproTech, cat. no. 300-07)
Recombinant human thrombopoietin (PeproTech, cat. no. 300-18)
Recombinant human Flt3 ligand (PeproTech, cat. no. 300-19)

Lentivirus packaging and envelope plasmids
pCMV-R8.9 gag/pol packaging plasmid (kindly provided by D. B. Kohn and C. S. Seet, UCLA[96])
pCAGGS-VSVG envelope plasmid (kindly provided by D. B. Kohn and C. S. Seet, UCLA[96])

Other reagents
Bovine serum albumin (Roche, cat. no. 10735078001)
RetroNectin (RN) recombinant human fibronectin fragment (Takara. cat. no. T100B)
CryoStor CS5 cell cryopreservation medium (Millipore Sigma, cat. no. C2874)
CryoStor CS5 and CS10 cell cryopreservation medium (Millipore Sigma, cat. no. C2999)
Dulbecco's phosphate-buffered saline D-PBS (Gibco, cat. no. 14190144)
PBS (1×) (Gibco, cat. no. 10010023)
PBS (10×) (Gibco, cat. no. 70011044)
Prostaglandin E2 (PGE2) (Cayman Chemical, cat. no. 14010)
Trizma base (Millipore Sigma, cat. no. T1503)
Sodium chloride (Amresco, cat. no. X190)
Tween-20 (Amresco, cat. no. M147)
DMEM (Corning, cat. no. 10-017)
UltraCULTURE serum free medium (Lonza, cat. no. 12-725F)
TransIT-293 transfection reagent (Mirus Bio, cat. no. MIR 2700)
Poly-L-lysine Solution (0.01%) (Millipore Sigma, cat. no. P4707)
Sodium butyrate (MilliporeSigma, cat. no. B5887)
Opti-MEM reduced serum medium (Gibco, cat. no. 31985070)
GlutaMAX supplement (100×) (Gibco, cat. no. 35050061)
HEPES buffer (1×) (Gibco, cat. no. 15630080)
Streptavidin–horseradish peroxidase conjugate (Invitrogen, cat. no. SA10001)
Trypsin–EDTA (0.25%), phenol red (Gibco, cat. no. 25-200-114)
Phorbol-12-myristate-13-acetate (PMA) (Calbiochem, cat. no. 524400)
Ionomycin calcium salt (*Streptomyces conglobatus*, Calbiochem, cat. no. 407952)
IFN-γ (ELISA, standard) (eBioscience, cat. no. 29-8319-65)
TNF-α (ELISA, standard) (eBioscience, cat. no. 29-8329-65)

IL-2 (ELISA, standard) (eBioscience, cat. no. 29-8029-65)
IL-4 (ELISA, standard) (eBioscience, cat. no. 29-8049-65)
Tetramethylbenzidine (KPL, cat. no. 5120-0053)
Human IL-15 Quantikine ELISA kit (R&D Systems, cat. no. D1500)
Human IL-17A ELISA Max Deluxe kit (BioLegend, cat. no. 433914)
Mouse SAA-3 ELISA kit (Millipore Sigma, cat. no. EZMSAA3-12K)
D-luciferin (Caliper Life Science, cat. no. 119222)
DNeasy Blood & Tissue kit (Qiagen, cat. no. 69506)
Fixable Viability Dye eFluor 506 (eBioscience, cat. no. 65-0866-18)
Synthesized guide RNAs (gRNAs) (Synthego)
Cas9-NLS purified protein (Berkeley Microlab)
P3 Primary Cell 4D-Nucleofector X kit S (Lonza, cat. no. V4XP-3032)
GolgiStop Protein Transport Inhibitor (BD Biosciences, cat. no. 554724)

Equipment

- 0.22 µm and 0.45 µm bottle top filters (Fisher Millipore, cat. nos. SCGPT05RE/ S2HVU05RE)
- 1 L corning bottle (Fisher Scientific, cat. nos. 06-414-1C/06-414-1D)
- 1-, 2-, 5-, 10-, 25- and 50-mL polystyrene serological pipettes (Thermo Fisher Scientific, cat. nos. 170353N/170354N/170355N/170356N/170357N/170358N)
- 1.5 mL microcentrifuge tubes (USA Scientific, cat. no. 1615-5500)
- 1,000 and 200 µL filter pipette tips (USA Scientific, cat. nos. 1126-7810/1120-8810)
- 10×, 20×, 40× and 63× objectives confocal microscope (Zeiss, model no. LSM 800)
- 10 kDa MWCO Amicon Ultra Centrifugal Filter (Millipore Sigma, cat. no. UFC910024)
- 15 and 50 mL conical tubes (Corning, cat. nos. 05-538-59A/05-526B)
- 150 mm cell culture dishes (Fisher Scientific, cat. no. 12-565-352)
- 30 mm conical tube rack (Fisher Scientific, cat. no. 14-809-44)
- 4D-Nucleofector X Unit (Lonza, cat. no. AAF-1003X)
- 500 mL disposable filter units with 0.2 µm polyethersulfone membrane (Thermo Fisher Scientific, cat. no. 566-0020)
- 6- and 24-well ultralow attachment culture plates (Corning, cat. nos. CLS3471/CLS3473)
- 6- and 24-well Corning Costar Tissue Culture (TC)-treated multiple well plates (Corning, 3516/3524)
- 6-, 12- and 24- well Falcon clear, flat-bottom not treated multiwell cell culture plates (Corning, cat. nos. 351146/351143/351147)
- Autoclave (Tuttnauer, model no. 5596 SP-1)
- Benchtop minicentrifuge (Benchmark Scientific, cat. no. C1012)
- Benchtop centrifuge (Fisher Scientific, model no. AccuSpin 400)
- Biological safety cabinet (Baker Company, Sterilgard Series)
- Branson M2800 mechanical ultrasonic cleaner (Marshall Scientific, cat. no. CPX-952-216R)
- Cell-counting slides (Invitrogen/Thermo Fisher Scientific, cat. no. C10228)
- CELLSTAR 24-well cell culture nontreated multiwell plates (VWR, cat. no. 82050-888)
- Charged microscope slides (Fisher Scientific, cat. no. 22-035813)
- CO_2 incubator (5% CO_2, 37 °C, 90% humidity; Thermo Fisher Scientific, cat. no. 3310)
- CoolCell cell-freezing container (Corning, cat. no. 432001)
- Countess II automated cell counter (Thermo Fisher Scientific, cat. no. AMQAX1000)
- ChemiDoc Touch Gel Imaging System (Bio-Rad, cat. no. 1708370)
- Chromium platform (10X Genomics, Chromium X series)
- Chromium next GEM single cell 3′ kit v3.1 and the Chromium next GEM chip G single cell kit (10X Genomics, cat. nos. PN-1000268/PN-1000120)
- CryoCube F740hi ULT Freezer (Eppendorf, cat. no. F740520035)
- Cryogenic vials (Thermo Fisher Scientific, cat. no. 12-567-501)
- Delicate task wipers (Fisher Scientific, cat. no. 34120)
- Desktop label maker (Brother, model no. PTD400RF)
- Eppendorf Mastercycler pro PCR System (Fisher Scientific, cat. no. E950040025)

- Fine-tip marking pens (Fisher Scientific, cat. no. 13-379-4)
- Hydrophobic barrier PAP pen (Vector Laboratories, cat. no. H-4000)
- Inverted microscope (Zeiss, model no. Axiovert 40 CFL)
- Infinite M1000 microplate reader (Tecan, cat. no. 30190085)
- Illumina NovaSeq S4 reagent kit (100 cycles) (Illumina, cat. no. 20028401)
- Locator cryogenic rack and box systems (Thermo Fisher, cat. no. CY50965)
- Microtube rack (Fisher Scientific, cat. no. 22-313630)
- MACSQuant analyzer 10 flow cytometer (Miltenyi Biotec, cat. no. 130-096-343)
- MilliporeSigma Milli-Q IQ 7000 ultrapure water system (Fisher Scientific, cat. no. ZIQ7000T0)
- Mettler Toledo FiveEasy F20 pH/mV meters (Fisher Scientific, cat. no. 01-912-345)
- Nontreated Falcon polystyrene 6-well microplates (Fisher Scientific, cat. no. 08-772-49)
- NanoDrop 2000/2000c spectrophotometers (Fisher Scientific, cat. no. ND2000CLAPTOP)
- Orbital shaker, CO_2 resistant (Thermo Fisher Scientific, cat. no. 88881102)
- P1000, P200, P20 and P2 Eppendorf variable adjustable-volume pipettes (Fisher Scientific, cat. nos.13-690-032/13-690-030/13-690-027/13-690-029)
- Razor blades (Fisher Scientific, cat. no. 12-640)
- Rectangle cover glasses (Fisher Scientific, cat. no. 12-545-F)
- Self-laminating polyester cryogenic laboratory labels (Fisher Scientific, cat. no. 19-102-744)
- Syringe filters with 0.2 µm polyethersulfone membrane (Thermo Fisher Scientific, cat. no. 42225-PS)
- Syringe filters with 0.2 µm nylon membrane (Thermo Fisher Scientific, cat. no. 42225-NN)
- Thermo Scientific value lab upright freezers (Fisher Scientific, cat. no. 04LFAETSA)
- Thermo Scientific Temperature Safety Valve (TSV) value undercounter refrigerator (Fisher Scientific, cat. no. FBV05RPSA)
- Untreated 6-well culture plates (Corning, cat. no. CLS3736)
- Ultra Bright Blue LED Transilluminator (BTLab SYSTEMS, cat. no. BT507)
- Vortex-Genie 2 (Scientific industries, Inc., cat. no. SI-0236)
- Weighing dishes (Sigma-Aldrich, cat. no. Z154873)

Software

- R package Seurat (v.4.0.0) (Seurat, https://satijalab.org/; RRID: SCR_016341)
- FlowJo software version 9 (BD Biosciences, https://www.flowjo.com/solutions/flowjo/downloads/previous-versions; RRID: SCR_008520)
- Photoshop CC (Adobe, https://www.adobe.com/products/photoshop.html; RRID: SCR_014199)
- Prism (GraphPad, https://www.graphpad.com/scientific-software/prism/; RRID: SCR_002798)
- Image J (National Institutes of Health, https://imagej.nih.gov/ij/; RRID: SCR_003070)

Reagent setup

▲ **CRITICAL** Use sterile technique throughout the procedure to maintain sterility and prevent contamination of the medium and buffer solution.

1× PBS buffer

Prepare 1× PBS buffer by combining 1 volume of 10× PBS with 9 volumes of Milli-Q H_2O in a sterile 4 L container. Adjust the pH to 7.4 and mix thoroughly. Attach a 0.22 µm filter top to a sterile bottle and filter the buffer solution to remove any particulate matter. Transfer the filtered buffer into sterile 1 L bottles and store at room temperature (20–22 °C) for up to 6 months.

Cytokine stocks

The cytokines are dissolved in 0.1% BSA in 1× PBS buffer (wt/vol) at a concentration of 20–50 ng/mL, vortexed well and aliquoted into 50–200 µL portions for future use. The cytokine stocks are stored at −20 °C for up to 1 year. The cytokines should only be thawed once. Once thawed, they can be stored at 4 °C for up to 2 weeks.

RN stock

The RN recombinant human fibronectin fragment is dissolved in 1× PBS buffer at a concentration of 1 µg/µL, vortexed well and aliquoted into 50–200 µL portions for future use. The RN stock is stored at −20 °C for up to 1 year.

PMA stock

Dissolve 1 mg of PMA in 400 µL of DMSO to prepare a 2.5 mg/mL PMA stock solution. Aliquot 10 µL of the PMA stock into each tube and store at −80 °C for up to 1 year.

Ionomycin stock

Dissolve 1 mg of ionomycin calcium salt in 400 µL of DMSO to prepare a 2.5 mg/mL ionomycin stock solution. Aliquot 10 µL of the ionomycin stock into each tube and store at −80 °C for up to 1 year.

Stage 0 HSP cell culture medium

Prepare the HSP cell culture medium for seeding human $CD34^+$ HSP cells by supplementing X-VIVO 15 serum-free hematopoietic stem cell medium with 50 ng/mL SCF, 50 ng/mL Flt3 ligand, 50 ng/mL thrombopoietin and 20 ng/mL IL-3. Attach a 0.22 µm filter top to the sterile bottle and filter the medium through. For every 300 µL of stem cell medium, 1×10^4 frozen-thawed human $CD34^+$ HSP cells should be resuspended. Freshly prepared medium remains viable at 4 °C for ~2 weeks. To ensure optimal quality and experimental integrity, discard any medium that exceeds this 2 week period.

▲ **CRITICAL** The X-VIVO 15 medium stock should be stored at 4 °C, protected from light.

Stage 1 HSP cell expansion medium

After transducing stage 0 human $CD34^+$ HSP cells, prepare medium for transduced human $CD34^+$ HSP cells for stage 1 culture. Prepare HSP cell expansion medium by supplementing StemSpan SFEM II Medium with 10× StemSpan Lymphoid Progenitor Expansion Supplement. Refer to the table below for the stock concentrations and volumes needed. Attach a 0.22 µm filter top to the sterile bottle and filter the medium through. For every 500 µL of HSP cell expansion medium, 2×10^4 transduced human $CD34^+$ HSP cells should be resuspended. Freshly prepared medium remains viable at 4 °C for ~2 weeks. To ensure optimal quality and experimental integrity, discard any medium that exceeds this 2 week period.

Component	Stock concentration	HSP cell expansion medium	
		Final concentration	Volume to add
StemSpan SFEM II serum-free medium	N/A	N/A	N/A
StemSpan Lymphoid Progenitor Expansion Supplement (10×)	10×	1×	100 µL/mL

Stage 2 NKT cell differentiation medium

Prepare stage 2 NKT cell differentiation medium by supplementing StemSpan SFEM II medium with 10× StemSpan Lymphoid Progenitor Maturation Supplement. Refer to the table below for the stock concentrations and volumes needed. Attach a 0.22 µm filter top to the sterile bottle and filter the medium through. For every 500 µL of NKT cell differentiation medium, 1×10^5 stage 2 NKT cells should be resuspended. Freshly prepared medium remains viable at 4 °C for ~2 weeks. To ensure optimal quality and experimental integrity, discard any medium that exceeds this 2 week period.

Component	Stock concentration	NKT cell differentiation medium	
		Final concentration	Volume to add
StemSpan SFEM II serum-free medium	N/A	N/A	N/A
StemSpan Lymphoid Progenitor Maturation Supplement (10×)	10×	1×	100 µL/mL

Stage 3 NKT cell deep differentiation medium

Prepare NKT cell deep differentiation medium by supplementing StemSpan SFEM II medium with 10× StemSpan Lymphoid Progenitor Maturation Supplement, and 10 ng/mL human recombinant IL-15. In addition, 12.5 µL/mL ImmunoCult human CD3/CD28/CD2 T Cell Activator is added at the beginning of the stage 3 culture. Refer to the table below for the stock concentrations and volumes needed. Attach a 0.22 µm filter top to the sterile bottle and filter the medium through. For every 1 mL of NKT cell deep differentiation medium, 5×10^5 to 1×10^6/mL stage 2 NKT cells should be resuspended. Freshly prepared medium remains viable at 4 °C for ~2 weeks. To ensure optimal quality and experimental integrity, discard any medium that exceeds this 2 week period.

Component	Stock concentration	NKT cell deep differentiation medium	
		Final concentration	Volume to add
StemSpan SFEM II serum-free medium	N/A	N/A	N/A
StemSpan Lymphoid Progenitor Maturation Supplement (10×)	10×	1×	100 µL/mL
Human recombinant IL-15	50 ng/µL	10 ng/mL	0.2 µL/mL

Stage 4 NKT cell expansion medium

Prepare NKT cell expansion medium for deeply differentiated NKT cells for stage 4 culture. Prepare NKT cell expansion medium using either (i) a feeder-free, serum-free CTS OpTmizer T cell expansion SFM or (ii) a homemade C10 medium, supplemented with 10 ng/mL IL-7 and 10 ng/mL IL-15. Attach a 0.22 µm filter top to the sterile bottle and filter the medium. The resuspension concentration of NKT cells varies depending on the specific expansion protocols employed. Detailed instructions can be found in the procedure section. Freshly prepared medium remains viable at 4 °C for ~2 weeks. To ensure optimal quality and experimental integrity, discard any medium that exceeds this 2 week period.

C10 medium

Prepare C10 medium for culturing T or NKT cells. Prepare 100 mL of FBS, 10 ml of penicillin–streptomycin–glutamine (100×), 10 mL of MEM NEAA, 10 mL of HEPES buffer solution (1 M), 10 mL of 100 mM sodium pyruvate, 10 mL of 5 mM β-ME and 2 mL of Normocin (500×) to make 1000 mL C10 medium. Prepare 848 mL of RPMI medium separately and combine with the above components. Refer to the table below for the volumes needed. Use an autoclaved 1 L bottle as the container for sterilized C10 medium. Attach a 0.22 µm filter top to the sterile bottle and filter the medium through. Store the C10 medium at 4 °C in the tissue culture fridge. It is suitable for storage for up to 1 month.

Component	Volume (mL)
RPMI	848
FBS	100
Penicillin–streptomycin–glutamine (100×)	10
MEM NEAA	10
HEPES buffer solution (1 M)	10
Sodium pyruvate (100 mM)	10
β-ME (5 mM)	10
Normocin (500×)	2

D10 medium

Prepare D10 medium for culturing adherent cells such as 293T, A375 or MDA-MB-231 cells. Thaw 100 mL of FBS, 10 mL of penicillin–streptomycin–glutamine (100×) and 2 mL of Normocin (500×) in a 37 °C water bath to make 1000 mL D10 medium. Prepare 888 mL of DMEM medium separately and combine with the thawed components. Refer to the table

below for the volumes needed. Use an autoclaved 1 L bottle as the container for sterilized D10 medium. Attach a 0.22 µm filter top to the sterile bottle and filter the medium through. Store the D10 medium at 4 °C in the tissue culture fridge. It is suitable for storage for up to 1 month.

Component	Volume (mL)
DMEM	888
FBS	100
Penicillin–streptomycin–glutamine (100×)	10
Normocin (500×)	2

D10-VP medium

Prepare D10-VP medium for culturing adherent cells 293T for lentivirus production. Thaw 50 mL of FBS in a 37 °C water bath to make 500 mL D10 medium. Prepare 450 mL of DMEM medium without pyruvate separately and combine with the thawed FBS. Refer to the table below for the volumes needed. Use an autoclaved 500 mL bottle as the container for sterilized D10-VP medium. Attach a 0.22 µm filter top to the sterile bottle and filter the medium through. Store the D10-VP medium at 4 °C in the tissue culture fridge. It is suitable for storage for up to 1 month.

Component	Volume (mL)
DMEM (without pyruvate)	450
FBS	50

Ultra-GH medium

Prepare Ultra-GH medium for virus concentration for lentivirus production. For every 15 mL of Ultra-GH medium, mix 14.4 mL DMEM/F12 advanced medium with 150 µL Gibco GlutaMAX supplement (100×), 150 µL thawed FBS and 300 µL Gibco HEPES buffer (1 M). Refer to the table below for the volumes needed. Use a sterile container of appropriate volume for the Ultra-GH medium. Attach a 0.22 µm filter top to the sterile bottle and filter the medium through. Store the Ultra-GH medium at 4 °C in the tissue culture fridge. The medium can be prepared when needed and can be stored for up to 24 h.

Component	Volume (mL)
DMEM/F12 advanced medium	14.4
FBS	0.15
Gibco GlutaMAX supplement (100×)	0.15
Gibco HEPES buffer (1 M)	0.3

ELISA wash buffer

Weigh 121.14 g of Trizma base and dissolve it in 1 L of Milli-Q water. Mix the solution with a stir bar and adjust the pH to 8, creating 1 L of 1 M Tris solution. Transfer the 1 M Tris to a 4 L bucket, add 163.5 g of sodium chloride while stirring with a stir bar. Once the sodium chloride dissolves, add 10 mL of Tween-20 and 3 L of Milli-Q water to achieve a total volume of 4 L, constituting 5× concentrated ELISA wash buffer. For a 1× working concentration, dilute 4 L of Milli-Q water with 1 L of 5× concentrated ELISA wash buffer. Store the wash buffer at room temperature for up to 6 months.

1× MACSQuant running buffer

The 1× MACSQuant running buffer is used for FACS. Prepare 1× MACSQuant running buffer by combining 1 volume of 16× MACSQuant running buffer with 15 volumes of Mili-Q H_2O in a 4 L bucket and store at room temperature for up to 6 months.

▲ **CRITICAL** When mixing the 16× MACSQuant running buffer with Milli-Q H_2O, add the H_2O first, followed by the MACSQuant buffer to avoid bubble formation.

Ethanol mixture (70% (vol/vol))
Add 737 mL of 190 proof ethanol to a 1 L graduated cylinder. Fill the graduated cylinder to 1 L with ultrapure water. Store at room temperature in a sealed container or spray bottle for up to 6 months.
▲ **CAUTION** Ethanol is highly flammable and can cause irritation when inhaled; keep away from flames and use in well-ventilated areas.

Engineered tumor cell lines
To make stable tumor cell lines overexpressing firefly luciferase and enhanced green fluorescence protein dual reporters, transduce the parental tumor cell lines (Table 5) with lentiviral vectors encoding the intended gene(s). At 72 h post lentivector transduction, subject cells to flow cytometry sorting to isolate gene-engineered cells for making stable cell lines. To generate human CD3-overexpressing HEK 293T (293T-CD3) cells, transduce the parental HEK 293T cell line with a lentiviral vector encoding human CD3. At 72 h post lentivector transduction, subject cells to flow cytometry sorting to isolate $CD3^+$ cells for making a stable cell line.

aAPC lines
The aAPC cell line is generated by engineering the K562 cell line (Table 5) to overexpress human CD83/CD86/4-1BBL costimulatory receptors[96]. Generate the aAPC-BCMA, aAPC-CD19, aAPC-GD2, aAPC-GPC3 and aAPC-EGFRvIII cell lines by further engineering the parental aAPC line to overexpress human BCMA, CD19, GD2, GPC3 and EGFRvIII, respectively. The engineered aAPC cell lines are employed to selectively stimulate ^{Allo}CAR-NKT cells expressing their corresponding CAR(s). For example, the aAPC-BCMA cell line is used to stimulate ^{Allo}BCAR-NKT cells. The engineered aAPC cell lines can be utilized either directly after thawing from liquid nitrogen or following a period of culturing. For expansion and culturing, grow aAPCs in C10 medium at a recommended cell density ranging from 0.5 to 2×10^6 cells/mL.

Procedure

Generation of lentivirus
● **TIMING** 5 d
▲ **CAUTION** When handling lentiviruses, it is crucial to follow strict safety precautions to minimize the risk of exposure and ensure laboratory safety. These include working within a biosafety cabinet to contain aerosols and prevent airborne transmission. All personnel should wear appropriate personal protective equipment, such as gloves, laboratory coats and eye protection, and be trained in the safe handling of viral vectors. It is important to decontaminate all surfaces and equipment with appropriate disinfectants after use, and any spills should be promptly and safely cleaned up according to institutional guidelines.
▲ **CRITICAL** In this protocol, all referenced day numbers (e.g., day 1) denote the specific days within that procedure. Each section outlines day numbers starting from day 0.
▲ **CRITICAL** Using DMEM medium without sodium pyruvate for virus generation is often preferred because sodium pyruvate can serve as an antioxidant and a source of energy for cells, potentially interfering with viral replication or affecting virus production. Therefore, omitting sodium pyruvate from the medium helps to create conditions more conducive to efficient viral replication and production.
▲ **CRITICAL** Omit penicillin–streptomycin addition, as it may disrupt the lipofection process.
▲ **CRITICAL** Filter all media with a 0.2 µm filter; syringe or 500 mL filter systems are suitable.
1. On day 1, seed HEK293T cells for virus production. First, collect 293T cells and suspend them at 5×10^5 cells/mL in D10-VP medium. For each dish/flask, 293T cells are added in accordance with the following cell numbers:
   - 10 cm dish: $5 \times 1\,0^6$ cells in 10 mL medium
   - T75 flask: 6.25×10^6 cells in 12.5 mL medium
   - T150 flask: 12.5×10^6 cells in 25 mL medium.

2. Coat cell culture dishes with a 0.01% poly-L-lysine solution.
   - 10 cm dish: 3 mL 0.01% poly-L-lysine solution
   - T75 flask: 4 mL 0.01% poly-L-lysine solution
   - T150 flask: 7.5 mL 0.01% poly-L-lysine solution

   ▲ **CRITICAL STEP** The 0.01% poly-L-lysine solution can be reused to coat up to ten dishes or flasks.

   ▲ **CRITICAL STEP** The function of poly-L-lysine solution in culturing 293T cells for virus generation is to enhance cell adherence to the culture vessel surface. This promotes cell attachment and spreading, facilitating efficient cell growth and virus production.
3. Ensure even coverage by swirling the plate to coat the entire surface; transfer any remaining solution to the next plate. Discard excess solution after coating all plates.
4. Seed the precise volume of 293T cells into the designated flasks. Incubate cells overnight at 5% CO2, 37 °C, maintaining the plating volumes exactly, as subsequent transfection steps rely on the initial plating media.

   ▲ **CRITICAL STEP** Maintain precise plating volumes as subsequent transfection steps rely on the initial plating media and necessitate exactness in volume allocation.

   ◆ **TROUBLESHOOTING**
5. On day 2, prewarm the required volume of Opti-MEM for activating the lipofection reagent as outlined in the table in Step 7.
6. Before usage, gently flick the Mirus lipofection reagent to ensure proper mixing.
7. Prepare solution A as specified in the following table.

Solution A	Per 10 cm dish	Per T75 flask	Per T150 flask
Opti-MEM	500 µL	625 µL	1,250 µL
TransIT 293T (Mirus)	35 µL	43.75 µL	87.5 µL
Total volume	535 µL	668.75 µL	1,337.5 µL

8. Thoroughly mix the solution by inverting the tube multiple times or employing brief vortexing (pulse/gentle agitation).
9. Incubate the mixture at room temperature for 20 min.

   ▲ **CRITICAL STEP** Do not combine solution A and B until the completion of the 20 min incubation period for solution A.
10. As solution A undergoes the 20 min incubation, prepare solution B as specified in the following table.

Solution B	Per 10 cm dish	Per T75 flask	Per T150 flask
Lentivirus plasmid	5 µg	6.25 µg	12.5 µg
Packaging plasmid (gag/pol)	5 µg	6.25 µg	12.5 µg
Envelope plasmid (VSV-G)	1 µg	1.25 µg	2.5 µg
TE (or PBS)	To 50 µL	To 62.5 µL	To 125 µL
Total volume	50 µL	62.5 µL	125 µL

    ▲ **CRITICAL STEP** In this study, we utilize the pCMV-R8.9 gag/pol packaging plasmid and the pCAGGS-VSVG envelope plasmid[96]. Alternative packaging plasmids could also be utilized; however, they may result in different viral titers.
11. Thoroughly mix solution B and set it aside at room temperature for subsequent steps.
12. Once the incubation of solution A is finished, combine solution A with solution B. Gently invert the tube ~5 times to ensure thorough mixing of A and B. Incubate the combined solution for 15–30 min at room temperature.
13. Following incubation, add the lipofection mix drop-wise directly onto the cells using a P1000 pipette, employing the per-plate volume of A + B as indicated below.

	Per 10 cm dish	Per T75 flask	Per T150 flask
Total volume (A + B)	585 µL	731.25 µL	1,462.5 µL

14. Gently rock the plate to ensure even distribution of the components. Place the plate back into the incubator and incubate for 16–18 h.
15. On day 3, after 16-18 h posttransfection, directly add 0.5 M sodium butyrate (NaBu) and 1 M HEPES to each dish/flask, achieving a final concentration of 10 mM NaBu and 20 mM HEPES.

	Per 10 cm dish	Per T75 flask	Per T150 flask
10 mM NaBu (0.5 M stock, −80 °C)	200 µL	250 µL	500 µL
20 mM HEPES (1 M stock, 4 °C)	200 µL	250 µL	500 µL

16. Gently distribute the solution by rocking the plate or flask to ensure even distribution.
17. Place the plates and flasks back into the incubator to continue incubating for an additional 8 h.
18. After the 8 h incubation period, carefully remove all the media and perform a gentle wash with 1× PBS using the following volumes:
    - 10 cm dish: 10 mL 1× PBS
    - T75 flask: 12.5 mL 1× PBS
    - T150 flask: 25 mL 1× PBS

    To minimize liquid flow during pipetting and avoid washing off 293T cells, crucial for maximizing final yield, follow these guidelines:
    - Pipetting technique: use a slow and gentle pipetting technique to prevent excessive liquid movement within the culture vessel
    - Pipette positioning: ensure the pipette tip is positioned close to the surface of the media to minimize disruption to the cell layer
    - Slow dispensing: dispense liquids slowly against the wall of the vessel to reduce disturbance to the cells at the bottom
    - Avoid touching cell layer: be cautious not to touch the cell layer at the bottom of the vessel while pipetting
19. Substitute the washed media with Ultra-GH medium (UltraCULTURE supplemented with 1× GlutaMAX and 20 mM HEPES) according to the following volumes:
    - 10 cm dish: 6 mL Ultra-GH medium
    - T75 flask: 7.5 mL Ultra-GH medium
    - T150 flask: 15 mL Ultra-GH medium
20. Return the plates and flasks to the incubator for an incubation period of 48 h.
    ▲ **CRITICAL STEP** All following steps to be completed on ice if otherwise stated.
21. On day 5, following 48 h of incubation, gently swirl plates to ensure homogenization and wash virus particles off cell surfaces.
    ◆ **TROUBLESHOOTING**
22. Collect the supernatant and filter it through a 0.45 µm filter.
    ▲ **CRITICAL STEP** Avoid using a 0.2 µm filter as this may damage the titer. Lentivirus, ~0.25 µm, could be compromised if filtered through a 0.2 µm filter, potentially damaging the packing envelopes.
23. Concentrate the virus by loading up to 15 mL of supernatant into the top of an Amicon Ultra-15 100K device (a single device can sequentially concentrate a total of 30 mL).
24. Centrifuge using the TC swinging bucket centrifuge from Beckman Coulter at 4,000*g*, 4 °C for 40 min. The supernatant should typically be concentrated ~100-fold (e.g., 150 µL from 15 mL) at the conclusion of the spin.
    ▲ **CRITICAL STEP** If the concentrate volume exceeds 300 µL after the initial spin, add an extra 20 min to the centrifugation.
    ◆ **TROUBLESHOOTING**
25. Load more supernatant on top and repeat the concentration process if required.
26. Upon completion, use a P200 pipette to collect the concentrated supernatant from the top chamber. Gently pipette to ensure thorough mixing of the viral concentrate. Combine

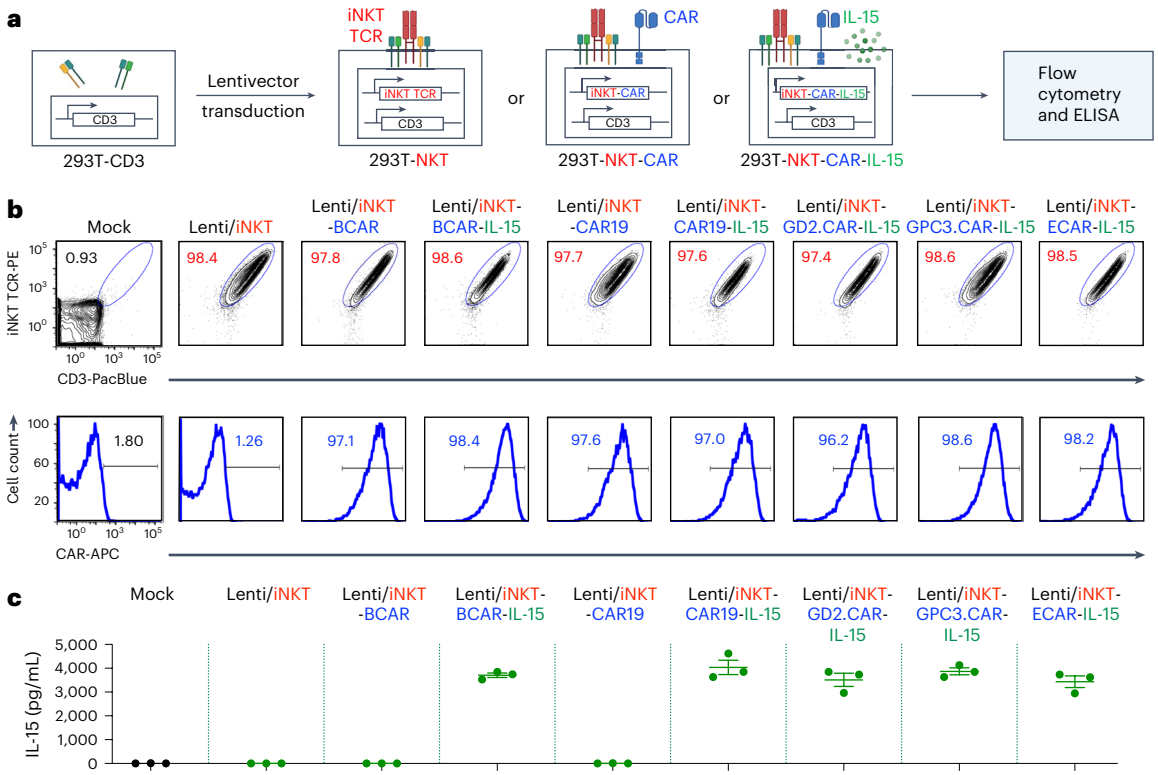


Fig. 4 | All lentivectors show successful gene delivery on CD3-overexpressing 293T (293T-CD3) cells. a, Experimental design. Concentrated lentivirus was added to 293T-CD3 cell cultures. Three days later, a portion of 293T-CD3 cells were collected and analyzed for surface expression of iNKT TCR and CAR using flow cytometry, and production of IL-15 using ELISA. **b**, FACS detection of iNKT TCR and CAR on 293T-CD3 cells transduced with the indicated lentivectors. iNKT TCR was stained using a 6B11 monoclonal antibody and CAR was stained using an anti-mouse IgG F(ab')2 antibody. **c**, ELISA analyses of IL-15 production by 293T-CD3 cells transduced with the indicated lentivectors ($n = 3$). Representative of three experiments.

all aliquots into a separate tube and gently mix to homogenize the viral concentrate. Divide the concentrate into 20 µL aliquots in 1.5 mL Eppendorf tubes and store them at −80 °C for cryopreservation.

■ **PAUSE POINT** Cryopreserved lentivirus aliquots can be stored at −80 °C for up to 6 months.

27. (Optional) Test the generated lentiviruses using a human CD3-overexpressing 293T (293T-CD3) cell line (Fig. 4a). This cell line is utilized to study virus transduction of lentivectors encoding human TCR (i.e., iNKT TCR), as TCR expression requires CD3 presence on the cell surface for detection using flow cytometry. Evaluate lentivectors for encoding genes such as iNKT TCR, CARs or human IL-15, utilizing flow cytometry or ELISA assays following standard procedures (Fig. 4b,c). This step is designed to verify that the transgenes in the lentivectors are expressed successfully and function normally in human 293T-CD3 cells. Subsequent steps (Steps 28–29) are designed to calculate the lentivirus titer.

◆ **TROUBLESHOOTING**

Titration of lentivirus

● **TIMING 7 d**

▲ **CRITICAL** Follow these steps for titering a single virus. Adjust procedures accordingly when titering multiple viruses. Use the concentrated, aliquoted virus from −80 °C for titration, as virus titer diminishes after freeze–thaw cycles, necessitating titering after one freeze–thaw cycle.

28. Titer the generated lentivirus. Follow option A if using 293T-CD3 cells and option B if using healthy donor PBM cell-derived T cells. The selection between these two options should be based on the availability of materials to the users.
▲ **CRITICAL STEP** This step is crucial for assessing the success of transduction and the proliferation of modified cells.

(A) **Virus titration using 293T-CD3 cells**

(i) On day 0, seed 50,000 293T-CD3 cells in 0.5 mL D10 medium per well in multiple wells of a 24-well tissue culture-treated plate. Additionally, seed an extra three wells for cell counting purposes.
(ii) On day 1, count the cell numbers in the seeded 293T-CD3 cells from three wells and determine their average. These cell numbers are essential for calculating the virus titer.
(iii) Set up six sterile Eppendorf tubes in the tissue culture hood for a 1:10 serial dilution of the virus stock.
(iv) Add 998 µL of D10 to the first tube and 900 µL of D10 medium to the remaining five tubes.
(v) Add 2 µL of concentrated virus to the first tube and mix thoroughly.
(vi) Transfer 100 µL from the first tube to the second tube, mix well. Then, transfer 100 µL from the second tube to the third tube and mix thoroughly.
(vii) Repeat the same procedure for the remaining tubes, excluding the last tube (no virus control).
(viii) Gently add 500 µL from each tube to the previously seeded 293T-CD3 cells in separate wells. (first tube is 1:1,000 dilution, second tube is 1:10,000 dilution and so forth).
(ix) Incubate the plate at 37 °C for 3 d.
(x) On day 4, collect the cells by trypsinization and transfer them to FACS tubes. Wash the cells with PBS. Proceed with the general FACS staining procedure to detect human iNKT TCR. Cells positive for iNKT TCR are identified as transduced cells.

(B) **Virus titration using healthy donor PBM cell-derived T cells**

(i) On day 0, add 300 µL of αCD3 Ab (1 µg/mL in PBS) per well in a 24-well tissue culture-treated plate.
(ii) Incubate for 90 min at 37 °C.
(iii) After coating, wash the wells with PBS.
(iv) Thaw healthy donor PBM cells for T cell stimulation.
(v) Add 1×10^6 cells/mL at 2 mL per well along with 1 µg/mL αCD28 Ab and 30 ng/mL human IL-2.
(vi) Incubate at 37 °C for 48 h.
(vii) On day 2, resuspend activated T cells in the antibody-coated plate by gently pipetting up and down and gently scratching using a syringe tip.
(viii) Count the cells, centrifuge the cell pellets and resuspend them.
(ix) Add 1×10^6 cells/mL at 0.5 mL per well, supplemented with 30 ng/mL IL-2 and 20 µL of concentrated lentivirus.
(x) Incubate at 37 °C for transduced T cell expansion.
(xi) On day 3, add 0.5 mL of C10 medium supplemented with 30 ng/mL IL-2 to each well.
(xii) On day 4, centrifuge the cell pellet.
(xiii) Remove the old medium and add 1 mL of new C10 medium supplemented with 30 ng/mL IL-2.
(xiv) On day 5–7, perform staining of the transduced T cells with antibodies for flow cytometry analysis to detect human iNKT TCR. Cells positive for iNKT TCR are identified as transduced cells.

29. Calculate the virus titer (Table 3). Use the following formula to determine the infectious units per milliliter (IFU/ml):

Seeded cells (50,000) × % positive cells/volume of virus used = IFU/mL

▲ **CRITICAL STEP** When calculating the virus titer, consider only wells with >2% and <30% fluorescent-positive cells. Methods for titering assume one integration event per cell. If the percentage exceeds 30%, there is a risk of counting cells with multiple integration events, potentially underestimating the true titer.

HSP cell engineering

● **TIMING 2 d**

▲ **CRITICAL** During HSP cell culture, as no antibiotics are added to the medium, maintain sterile conditions in the incubator to prevent cell contamination. Perform daily checks on cell condition to ensure optimal growth density and verify the absence of contamination.

30. Thaw the RN stock stored at −20 °C as a 1 µg/µL solution.
31. Dilute RN to 20 µg/mL in PBS and add 1 mL to each well in a 6-well nontissue culture-treated plate. Incubate at room temperature for 2 h.
32. Aspirate the RN and replace it with 1 mL of 2% BSA in PBS (wt/vol). Incubate at room temperature for 30 min.
33. Aspirate and replace with 2 mL 1× PBS. Use RN coated plates on the same day.
34. Thaw $CD34^+$ HSP cells in a 37 °C water bath, add 500 µL X-VIVO 15 medium dropwise to dilute the thawed HSP cells and transfer the cells to a 15 mL conical tube. Centrifuge the cells at 300*g* for 10 min.
35. Aspirate the supernatant, resuspend in 10 mL of stage 0 HSP cell culture medium and count the cells.
36. Centrifuge at 300*g* for 10 min and aspirate the supernatant.
37. Resuspend $CD34^+$ HSP cells up to 1×10^6 cells/mL in stage 0 HSP cell culture medium.
38. Aspirate 1× PBS from the RN-coated wells and add cells at 1 mL per well.
    ◆ **TROUBLESHOOTING**
39. Incubate at 37 °C, 5% CO_2 for 12–18 h.
40. Thaw concentrated virus supernatant (from Step 26), calculating the volume sufficient for multiplicity of infection of 100 or for a final concentration of 1×10^8 IFU/mL.
41. Gently pipette the thawed supernatant to mix (avoid vortexing) and add it directly to the wells. Gently rock the plate to ensure mixing. Incubate cells at 37 °C, 5% CO_2 for 24 h.
42. (Optional) To generate allogeneic CAR-NKT cells with knockout of genes of interest, such as *B2M* and *CIITA*[26], CRISPR–Cas9 gene editing can be applied to HSP cells at this step. Follow the detailed protocol provided in Box 1.
43. Collect cells by gentle pipetting to remove them from the plate and transfer to a conical tube.
    ▲ **CRITICAL STEP** In all subsequent steps, collect the HSP cells and their derivatives by gently pipetting up and down five times.
44. Wash wells with an equal volume of cold stage 0 HSP cell culture medium to remove any cells still adhering to the plate and transfer to the conical tube.
45. Check under a microscope and perform additional washes with cold stage 0 HSP cell culture medium as necessary to collect all cells from the plate.
    ▲ **CRITICAL STEP** RN is adhesive and some HSP cells may attach to the bottom of the plate. Ensure thorough washing and pipetting multiple times to collect all attached HSP cells.
46. Centrifuge at 300*g* for 10 min and aspirate the medium.
47. Resuspend cells in 10 mL of stage 0 HSP cell culture medium and count.
48. (Optional) Continue culturing 100,000 HSP cells in the 1 mL of HSP cell culture medium for 3 d and perform flow cytometry. This step is designed to analyze the HSP cell transduction. Intracellular human iNKT TCR Vβ11 is detected, and cells positive for Vβ11 are identified as transduced cells.
    ◆ **TROUBLESHOOTING**
49. Centrifuge at 300*g* for 10 min, aspirate medium and resuspend cells in the HSP cell expansion medium at a concentration of 2×10^4 cells per 500 µL for use in the next stage.

BOX 1

CRISPR–Cas9 gene editing on HSP cells

● **TIMING 1 d**

Procedure

1. Set up the electroporation device (e.g., 4D-Nucleofector X Unit; Lonza) following the manufacturer's instructions and previous studies[19,26].
   ▲ **CRITICAL STEP** Different electroporation devices require different protocols and the optimal protocol should be established based on the manufacturer's instructions.
2. Prepare master ribonucleoprotein (RNP) mixes in 1.5 mL sterile, RNAse-free Eppendorf tubes. Add gRNAs (1 µL of each gRNA at 100 µM) to the P3 solution (P3 Primary Cell 4D-Nucleofector X Kit S, Lonza), then add Cas9 (4 µL at 6.5 mg/mL) to achieve a final mix volume of 20 µL. Mix well and incubate for 15 min at room temperature to allow complex formation.
   ▲ **CRITICAL STEP** Add Cas9 to the gRNA to minimize precipitation during RNP complex formation.
   ▲ **CRITICAL STEP** Place the RNP complexes on ice until ready to conduct electroporation.
3. Collect $CD34^+$ HSP cells, count, and centrifuge 2×10^5 HSP cells per condition at 90*g* for 10 min at room temperature.
4. Aspirate the medium and resuspend HSP cells in the premade 20 µL RNP mixes. Pipette gently to mix.
5. Transfer the HSP cells to a cuvette and set up the electroporation procedure (pulse once at 250 V for 5 ms).
6. Rest the cells at room temperature for 10 min.
7. Recover HSP cells from the cuvettes by adding 80 µL of HSP cell culture medium.
8. Transfer all HSP cells to 900 µL of HSP cell culture medium and add the cells to each well of a 6-well non-tissue culture-treated plate. Incubate the cells at 37 °C, 5% CO_2 for 24 h. After this incubation, proceed to Step 42 in the protocol for the next steps.
9. To analyze the HSP cell gene editing efficacy, continue culturing 100,000 cells in the 1 mL of HSP cell culture medium for 3 more days and perform flow cytometry. Stain the cells with HLA molecule antibodies, including anti-B2M and anti-HLA-II (HLA-DR, DP, DQ) antibodies and the cell viability dye e506. The expected gene editing efficacy for HSP cells, identified as $B2M^-HLA\text{-}II^-$ cells, should exceed 50%. The viability of these HSP cells, marked as $e506^-$ cells, should be above 90%.

HSP cell expansion

● **TIMING 2 weeks**

▲ **CRITICAL** During HSP cell culture at any stage, utilize flow cytometry to assess cell viability, NKT cell percentage and the expression of other necessary markers.

50. Thaw the StemSpan Lymphoid Differentiation Coating Material at room temperature.
51. Add 500 µL StemSpan Lymphoid Differentiation Coating Material into one well of the CELLSTAR 24-well cell culture nontreated multiwell plate. Coat the plate for 2 h at room temperature or, alternatively, overnight at 4 °C.
    ◆ **TROUBLESHOOTING**
    ▲ **CRITICAL STEP** If the coating material is sticky, make sure to pipette multiple times to cover the whole well completely.
52. Remove coating material, and add 1 mL of D-PBS buffer to rinse away any residual coating material.
53. Seed the cells (from Step 49) into the coated wells of a 24-well plate at 500 µL per well and culture for 3 d.
54. On day 4, supplement the culture with an additional 500 µL of HSP cell expansion medium in each well and continue culturing the cells for an additional 4 d.
    ▲ **CRITICAL STEP** At this stage, HSP cells typically have a low cell number and do not tend to overgrow. However, if overgrowth occurs, indicated by yellowing medium or >70% confluence observed under a microscope, split the cells by collecting, centrifuging at 300*g* for 10 min and then resuspending HSP cells in fresh HSP cell expansion medium before seeding them into two wells.
55. On day 7, remove half of the old medium and replace it with 500 µL of fresh HSP cell expansion medium. Continue culturing the cells for an additional 4 d.
56. On day 11, remove half of the old medium and replace it with 500 µL of fresh HSP cell expansion medium. Continue culturing the cells for an additional 3 d.
57. On day 14, collect HSP cells, count cells, centrifuge them at 300*g* for 10 min and resuspend them in NKT cell differentiation medium at a concentration of 1×10^5 cells per 1 mL for use in the next stage. Owing to the constrained cell culture capacity in the research laboratory,

a culture containing 1–2 × 10^5 cells should be processed to the next steps, while any remaining cells should be cryopreserved at a concentration of 2 × 10^5 cells in 500 µL of freezing medium

■ **PAUSE POINT** Cryopreserved cells can be stored in liquid nitrogen for up to 6 months. The cells can be thawed and the protocol can be resumed from this point.

▲ **CRITICAL STEP** CryoStor CS5 or CS10 cell cryopreservation media are recommended to use for cell cryopreservation.

▲ **CRITICAL STEP** Throughout the generation of NKT cells, cells can be collected weekly and subjected to analysis using flow cytometry and single-cell RNA sequencing (scRNA-seq). These techniques enable monitoring of the differentiation and developmental processes of the cells, providing valuable insights into their phenotype and gene expression profiles at various stages of maturation (Fig. 5a–e).

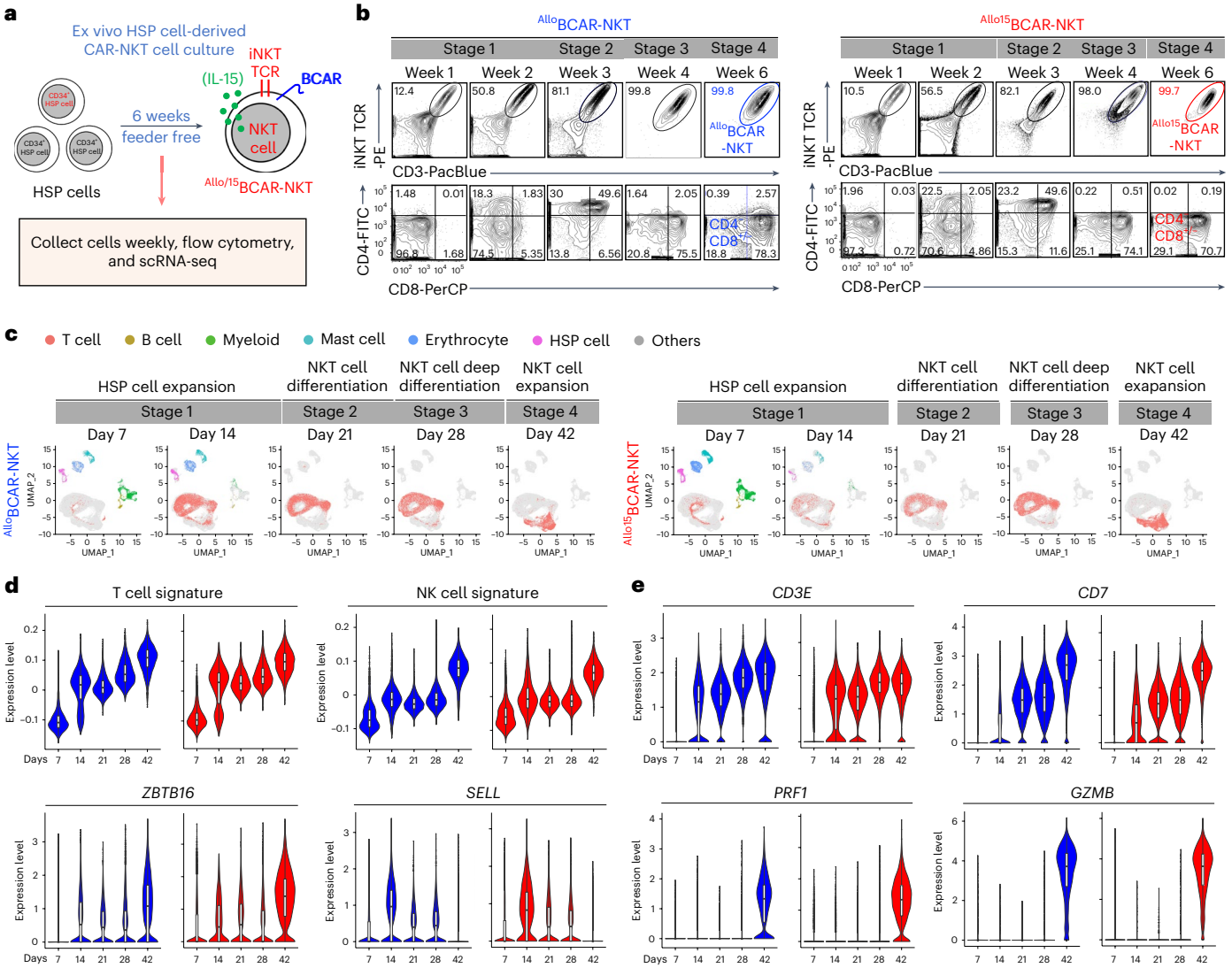


Fig. 5 | Ex vivo development of $^{Allo/15}$BCAR-NKT cells. a, Experimental design. $^{Allo/15}$BCAR-NKT cells were generated from the gene-engineered HSP cells in an ex vivo culture. The cells were collected weekly for analysis using flow cytometry and scRNA-seq. **b**, FACS monitoring of the generation of $^{Allo/15}$BCAR-NKT cells. NKT cells were identified as iNKT TCR$^+$CD3$^+$ cells, and the expression of their CD4 and CD8 coreceptors was monitored. iNKT TCR was stained using a 6B11 monoclonal antibody. **c**, Uniform manifold approximation and projection (UMAP) visualization of $^{Allo/15}$BCAR-NKT cell development. scRNA-seq data are presented. Each dot represents a single cell and is colored according to its cell cluster assignment. **d**, Violin plots showing the expression distribution of T cell and NK cell signature genes in the $^{Allo/15}$BCAR-NKT cell samples. **e**, Violin plots showing the expression distribution of the indicated genes in the $^{Allo/15}$BCAR-NKT cell samples. Representative of 1 (**c**–**e**) and >10 (**a** and **b**) experiments. Flow cytometry and scRNA-seq analyses for several datasets can be found in Fig. 1e,g of ref. 25. Images in **b** and **c** adapted from ref. 25 under a Creative Commons license CC BY 4.0.

NKT cell differentiation

● **TIMING** 1 week

▲ **CRITICAL** Freshly cultured or thawed HSP cells can be utilized for NKT cell differentiation at this stage. Throughout the NKT cell differentiation culture, conduct daily checks on cell condition to ensure optimal growth density and confirm the absence of contamination.

58. Thaw the StemSpan Lymphoid Differentiation Coating Material at room temperature.
59. Add 1 mL StemSpan Lymphoid Differentiation Coating Material into one well of the nontreated Falcon polystyrene 6-well microplates. Coat the plate for 2 h at room temperature or, alternatively, overnight at 4 °C.
    ▲ **CRITICAL STEP** If the coating material is sticky, make sure to pipette multiple times to cover the whole well completely.
60. Remove coating material and 1 mL of D-PBS buffer to rinse away any residual coating material.
61. Seed the cells (from Step 57) into the coated wells of a 6-well plate at 2 mL per well and culture for 1 week.
62. During this week, passage cells two to three times to maintain a cell density at $0.5–1 \times 10^6$ cells/mL; use NKT cell differentiation medium to resuspend cells at every passage. Passaging NKT cells involves collecting the cells by gently pipetting up and down five times, centrifuging at 300*g* for 10 min and then resuspending them in fresh NKT cell differentiation medium at a concentration of 1×10^5 cells per mL.
    ▲ **CRITICAL STEP** During this stage, cells from a single well could be ultimately distributed across two to four wells of the 6-well plate.
    ▲ **CRITICAL STEP** Other types of nontreated plates, such as nontreated Falcon polystyrene 12-well microplates, can also be utilized. Adjust the cell number and culture medium volume accordingly.
63. At the end of this stage, use flow cytometry to detect the NKT cell differentiation. Collect a small portion of NKT cells (~1×10^5 cells) and stain with NKT cell markers, including NKT TCR (6B11), CD3, TCRαβ, CD4, CD8 and the cell viability dye e506. The expected percentage of NKT cells (identified as NKT $TCR^+CD3^+TCR\alpha\beta^+$ cells) is greater than 70%. These NKT cells should progress to the $CD4^+CD8^+$ stage, with ~20–50% of NKT cells being $CD4^+CD8^+$. The viability of NKT cells (identified as $e506^-$ cells) should be greater than 90%.
    ◆ **TROUBLESHOOTING**

NKT cell deep differentiation

● **TIMING** 1 week

64. Thaw the StemSpan Lymphoid Differentiation Coating Material at room temperature.
65. Add 1 mL StemSpan Lymphoid Differentiation Coating Material into one well of the nontreated Falcon polystyrene 6-well microplates (Thermo Fisher Scientific). Coat the plate for 2 h at room temperature or, alternatively, overnight at 4 °C.
    ▲ **CRITICAL STEP** If the coating material is sticky, make sure to pipette multiple times to cover the whole well completely.
66. Remove coating material and add 1 mL of D-PBS buffer to rinse away any residual coating material.
67. One week after the start of NKT cell differentiation, collect cells from the previous stage (Step 62), count them, centrifuge at 300*g* for 10 min and resuspend them in NKT cell deep differentiation medium at a concentration of 5×10^5 cells per 1 mL. Add ImmunoCult human CD3/CD28/CD2 T cell activator to the culture medium to achieve a final concentration of 12.5 µL/mL.
68. Seed the cells into the coated wells of a 6-well plate at 2 mL per well and culture for 1 week.
69. During this week, passage cells two to three times to maintain a cell density at $0.5–1 \times 10^6$ cells/mL; use NKT deep differentiation medium to resuspend cells at every passage. Passaging NKT cells involves collecting the cells by gently pipetting up and down five times, centrifuging at 300*g* for 10 min and then resuspending them in fresh NKT cell differentiation medium at a concentration of 5×10^5 cells per mL.
    ▲ **CRITICAL STEP** During this stage, cells from a single well could be ultimately distributed across four to eight wells of the 6-well plate.

▲ **CRITICAL STEP** ImmunoCult human CD3/CD28/CD2 T cell activator should not be added at every cell passage to avoid overstimulation of NKT cells.

▲ **CRITICAL STEP** Other types of nontreated plates, such as nontreated Falcon polystyrene 12-well microplates, can also be utilized. Adjust the cell number and culture media volume accordingly.

70. At the end of this stage, use flow cytometry to detect the NKT cell deep differentiation. Collect a small portion of NKT cells (~1×10^5 cells) and stain with NKT cell markers, including NKT TCR (6B11), CD3, TCRαβ, CD4, CD8 and the cell viability dye e506. The expected percentage of NKT cells (identified as NKT $TCR^+CD3^+TCR\alpha\beta^+$ cells) is greater than 95%. These NKT cells should progress to the $CD4^-CD8^-$ or $CD4^-CD8^+$ stage. The viability of NKT cells (identified as $e506^-$ cells) should be greater than 90%.

NKT cell expansion

● **TIMING 2 weeks**

71. Before proceeding with NKT cell expansion, collected NKT cells from the previous stage and count.

▲ **CRITICAL STEP** NKT cell counts are essential for determining the appropriate culture plate size, cell numbers of healthy donor PBM cells or aAPCs, and the required expansion medium and cytokine concentrations.

72. Mature NKT cells can be expanded using three expansion approaches: for an αCD3/αCD28 expansion approach follow option A, for an αGC/PBM cell expansion approach follow option B or for an aAPC expansion approach follow option C. During this stage, NKT cells or their derivatives could be cultured in nontreated Falcon polystyrene 6-well microplates, 150 mm cell culture dishes or G-Rex 6M well plate. The NKT cell expansion medium can be a feeder-free, serum-free CTS OpTmizer T-Cell Expansion SFM or a homemade C10 medium.

(A) **αCD3/αCD28 expansion**

(i) Dilute Ultra-LEAF purified anti-human CD3 antibody to a concentration of 1 μg/mL using D-PBS.

(ii) Place 1 mL of the diluted anti-human CD3 antibody solution into one well of nontreated Falcon polystyrene 6-well microplates and incubate for 2 h at room temperature or, alternatively, overnight at 4 °C.

(iii) Remove the antibody solution and add 1 mL of D-PBS buffer to rinse away any residual antibody solution.

(iv) Resuspend mature NKT cells in the NKT cell expansion medium supplemented with 10 ng/mL human IL-7, 10 ng/mL human IL-15 and 1 μg/mL Ultra-LEAF purified anti-human CD28 antibody at 5×10^5 cells/mL.

(v) Add 2 mL of NKT cell suspension to each precoated well and culture for 3 d.

(vi) Collect NKT cells, count and resuspend in fresh NKT cell expansion medium supplemented with 10 ng/mL human IL-7 and IL-15, at $0.5–1 \times 10^6$ cells/mL.

(vii) Add 2 mL of NKT cell suspension into one well of non-precoated nontreated Falcon polystyrene 6-well microplates and culture for 2 weeks.

(viii) During the NKT cell expansion, passage NKT cells two to three times per week to maintain a cell density at $0.5–1 \times 10^6$ cells/mL; add NKT cell expansion medium supplemented with 10 ng/mL human IL-7 and IL-15 at every passage. Passaging NKT cells involves collecting the cells by gently pipetting up and down five times, centrifuging at 300*g* for 10 min and then resuspending them in fresh NKT cell expansion medium at a concentration of 5×10^5 cells per mL.

▲ **CRITICAL STEP** As the NKT cell population reaches a high number during the later stages, these cells can be transferred and cultured in 150 mm cell culture dishes at a cell density of $0.5–1 \times 10^6$ cells/mL.

(ix) After 2 weeks of culture, cryopreserve the NKT cells for downstream assays. Freeze the NKT cells in CS10 freeze medium at a concentration of 1×10^7 cells per mL.

■ **PAUSE POINT** Cryopreserved NKT cells can be stored in liquid nitrogen for up to 1 year.

(B) **αGC/PBM cell expansion**

(i) Calculate the cell number for healthy donor PBM cells based on the NKT cell count. Given an NKT:PBM cell ratio of 1:2–1:5, if 1×10^7 NKT cells are generated from the last step, thaw and use $2–5 \times 10^7$ healthy donor PBM cells for this step.
▲ **CRITICAL STEP** To account for potential loss and death of PBM cells during incubation, it is recommended to use a higher quantity of PBM cells.

(ii) Thaw healthy donor PBM cells, resuspend them in C10 medium at a cell density of $0.5–1 \times 10^6$ cells/mL and maintain the cells on ice.
▲ **CRITICAL STEP** Avoid leaving the cells on ice for an extended period (no longer than 1 h) and ensure not to resuspend the cells at too high a density (no higher than 5×10^6 cells/mL) to prevent PBM cell aggregation and cell death.
◆ **TROUBLESHOOTING**

(iii) Determine the quantity of αGC needed, with 1 μg of αGC per 1×10^6 PBM cells. Retrieve the αGC stock from the −20 °C freezer and place it in an 80 °C water bath for 5 min. Subsequently, transfer the glass vial to a 50 °C sonicator and sonicate for 5 min. Vortex the aliquot for 1 min and add 200 μL of prewarmed C10 medium to dissolve the αGC. Perform an additional 5 min sonication and vortex for 1 min. Adjust the final concentration to 5 μg/mL αGC-C10 medium by adding prewarmed C10 medium.
▲ **CRITICAL STEP** This step is designed to ensure complete dissolution of αGC in C10 medium, and extending both vortex and sonication times can prove beneficial in achieving this goal.
▲ **CRITICAL STEP** αGC aliquots from −20 °C are for single use only. Refreezing after dilution with media is not recommended.
▲ **CRITICAL STEP** Maintain αGC-C10 medium in a 37 °C water bath as αGC is prone to precipitation at lower temperatures.
◆ **TROUBLESHOOTING**

(iv) Centrifuge the PBM cells and resuspend them in αGC-C10 medium at a cell density of $1–5 \times 10^6$ cells/mL. Seed the cells into 6-well microplates or 100 mm cell culture dishes and incubate in the 37 °C incubator for 1 h.
▲ **CRITICAL STEP** Avoid leaving the cells in the incubator for an extended period (less than 1 h), as prolonged incubation may lead to cell death.
◆ **TROUBLESHOOTING**

(v) Collect the resulting αGC-loaded PBM cells (αGC/PBM cells), transfer to a 50 mL conical tube, place on ice and irradiate at 6,000 rads.
▲ **CAUTION** Different types of irradiators can be used for this purpose, including X-ray machines, gamma irradiators or linear accelerators. It is crucial to follow appropriate safety protocols when working with irradiation equipment to ensure the safety of researchers and maintain the integrity of the experiment.
▲ **CRITICAL STEP** Place cells on ice when irradiating them to achieve temperature control, slow down cellular processes and enhance resistance to radiation-induced damage. Cooling on ice is particularly useful in minimizing DNA repair processes, reducing cellular stress and ensuring uniform exposure to radiation.

(vi) Postirradiation, transfer the PBM cells to a biological safety hood. Filter the PBM cells using 70 μm sterile cell strainers to eliminate aggregates and count the PBM cells.

(vii) Centrifuge the PBM cells and then combine them with NKT cells collected from the previous stage at an NKT:PBM cell ratio of 1:2–1:5. Resuspend the cell mixture in NKT cell expansion medium supplemented with 10 ng/mL human IL-7 and IL-15 at a concentration of $0.5–1 \times 10^6$ cells/mL.

(viii) Add 2 mL of NKT cell suspension into one well of a non-precoated nontreated Falcon polystyrene 6-well microplate and culture for 2 weeks.

(ix) During the NKT cell expansion, passage NKT cells two to three times per week to maintain a cell density at $0.5–1 \times 10^6$ cells/mL; supplement NKT cell expansion medium with 10 ng/mL human IL-7 and IL-15 is added at every passage.

(x) After 2 weeks of culture, cryopreserve the NKT cells for downstream assays. Freeze the NKT cells in CS10 freeze medium at a concentration of 1×10^7 cells per mL.
■ **PAUSE POINT** Cryopreserved NKT cells can be stored in liquid nitrogen for up to 1 year.

(C) **aAPC expansion**

(i) Calculate the cell number for aAPCs based on the NKT cell count. Given an NKT:aAPC ratio of 1:1–1:2, if 1×10^7 NKT cells are generated from the last step, thaw and use $1–2 \times 10^7$ aAPCs for this step.
▲ **CRITICAL STEP** In contrast to healthy donor PBM cells, aAPCs exhibit higher robustness and consistently maintain high cell viability postirradiation. Consequently, the preparation of an additional number of aAPCs is considered optional.

(ii) Collect aAPCs, resuspend them in C10 medium at a cell density $1–5 \times 10^6$ cells/mL, transfer to a 50 mL conical tube, place on ice and irradiate at 10,000 rads.
▲ **CAUTION** Different types of irradiators can be used for this purpose, including X-ray machines, gamma irradiators or linear accelerators. It is crucial to follow appropriate safety protocols when working with irradiation equipment to ensure the safety of researchers and maintain the integrity of the experiment.

(iii) Postirradiation, transfer the aAPCs to a biological safety hood. Filter the aAPCs using 70 µm sterile cell strainers to eliminate aggregates and count the aAPCs.
◆ **TROUBLESHOOTING**

(iv) Centrifuge the aAPCs and then combine them with NKT cells obtained from the previous step at an NKT:aAPC ratio of 1:1–1:2. Resuspend the cell mixture in NKT cell expansion medium supplemented with 10 ng/mL human IL-7 and IL-15 at a concentration of 0.5–1 × 106 cells/mL.

(v) Add 2 mL of NKT cell suspension into one well of a non-precoated nontreated Falcon polystyrene 6-well microplates and culture for 2 weeks.

(vi) During the NKT cell expansion, passage NKT cells two to three times per week to maintain a cell density at 0.5–1 × 106 cells/mL; supplement NKT cell expansion medium 10 ng/mL human IL-7 and IL-15 is added at every passage.

(vii) After 2 weeks of culture, cryopreserve the NKT cells for downstream assays. Freeze the NKT cells in CS10 freeze medium at a concentration of 1 × 107 cells per mL.
■ **PAUSE POINT** Cryopreserved NKT cells can be stored in liquid nitrogen for up to 1 year.

Downstream assays

● **TIMING** variable

73. The functionality of ^{Allo}NKT or ^{Allo}CAR-NKT cells can be evaluated with downstream assays: for analysis of surface marker expression follow option A, for analysis of cytokines released by activated cells follow option B and for analysis of antitumor capacity follow option C.

(A) **Analysis of surface marker expression of ^{Allo}NKT or ^{Allo}CAR-NKT cells using flow cytometry**

(i) Thaw the cryopreserved NKT cells, count them and prepare a minimum of 1×10^5 cells from each sample for staining. Put each sample in a FACS tube in the original culture medium or PBS. Add another 2 mL of PBS if the volume in the FACS tube is low.

(ii) Make panels with fluorochrome-conjugated antibodies and PBS based on targeting surface markers. For each sample in a FACS tube, the total volume of the panel is 50 µL.
▲ **CRITICAL STEP** The choice of antibodies in the panel varies based on the experimental design and the specific objectives of each experiment. For instance, to assess the NK phenotype of ^{Allo}NKT or ^{Allo}CAR-NKT cells, NK receptors such as NKG2D and DNAM-1 are stained. Similarly, T cell markers such as CD3, CD4 and CD8 are used to analyze the T cell characteristics of ^{Allo}NKT or ^{Allo}CAR-NKT cells.

(iii) Spin down the cells at 300*g* for 5 min to pellet cells. Aspirate the supernatant.
(iv) Resuspend each pellet in a panel. Incubate at 4 °C for 15 min in the dark to prevent photobleaching.
(v) Add 2 mL of 1× PBS and mix. Then spin down the cells at 300*g* to wash out unbound antibodies. Aspirate the supernatant.
(vi) Resuspend the pellet in 1× PBS. The resuspension volume depends on the number of cells in a sample. The resulting solution should be relatively clear.
(vii) Run samples on a flow cytometer. Analyze the results using the FlowJo software.

(B) **Analysis of cytokines released by activated ^{Allo}NKT or ^{Allo}CAR-NKT cells using PMA/ionomycin stimulation and flow cytometry**

(i) Take one aliquot of the PMA stock and add 490 µL of C10 medium to achieve a 50 µg/mL working solution sufficient for a single day's use. To stimulate NKT cells, add this solution to C10 at 1 µL/mL, resulting in a final concentration of 50 ng/mL.
(ii) Take one aliquot of the Ionomycin stock and add 40 µL of C10 medium to create a 500 µg/mL working solution for a single day's usage. For NKT cell stimulation, add this solution to C10 at 1 µL/mL, resulting in a final concentration of 500 ng/mL.
(iii) Resuspend cells at a concentration of 1×10^6 cells/mL in C10 medium with both PMA and ionomycin.
(iv) For NKT cells stimulated with PMA and ionomycin, add 5 ng/mL IL-2 to prevent activation-induced cell death.
(v) Seed 1 mL of cells in capped FACS tubes, leaving the caps loose.
(vi) Incubate the cells at 37 °C, 5% CO_2 for 1 h.
(vii) Add GolgiStop to the cells at 4 µL/6 mL. Gently agitate to mix without disturbing the cells.
(viii) Incubate further at 37 °C, 5% CO_2 for 4–6 h.
(ix) Tighten the caps on the FACS tubes.
(x) Analyze the cells directly using intracellular staining or store them at 4 °C overnight and perform intracellular staining the next day.

▲ **CRITICAL STEP** The choice of antibodies in the panel varies based on the experimental design and the specific objectives of each experiment. For instance, to evaluate the cytokine production by ^{Allo}NKT or ^{Allo}CAR-NKT cells, Intracellular cytokines such as human IL-2, IL-4, IFN-γ and TNF-α are stained[25,26].

(C) **Analysis of antitumor capacity of ^{Allo}NKT or ^{Allo}CAR-NKT cells using in vitro tumor cell-killing assays**

(i) Collect and count effector (E) and tumor (T) cells. Effector cells refer to therapeutic cells such as ^{Allo}NKT or ^{Allo}CAR-NKT cells, while tumor cells denote the targeted tumor cell lines (Table 5). Centrifuge both types of cells separately at 300*g* for 5 minutes and resuspend the pellets in C10 medium.
(ii) Coculture the effector and tumor cells at designated E:T ratios (e.g., E:T = 1:1, E:T = 2:1) with or without the addition of αGC (100 ng/mL) in a Corning 96-well clear-bottom black plate. Usually, put 10,000 tumor cells in each well. Optionally, add 10 µg/mL LEAF purified anti-NKG2D, anti-DNAM-1 antibody, or LEAF purified mouse IgG2bk isotype control antibody to mixed cells for studying the tumor cell-killing mechanism mediated by the NK-activating receptors.

▲ **CRITICAL STEP** For the 96-well cell culture plate, the maximum capacity of each well for cells is 200 µL.

▲ **CRITICAL STEP** Black plates for enhancing the sensitivity of detection by minimizing the background interference are essential for the following plate-reading step. Use black plates to block background light to prevent photobleaching. A dark condition can also maintain the reactivity and stability of D-luciferin.

(iii) Incubate at 37 °C, 5% CO_2 for 8–24 h.
(iv) Add 50 µL 150 µg/mL D-luciferin to each well.
(v) Incubate for 5 min in the dark.

(vi) Read out the luciferase activities with an Infinite M1000 microplate reader. The remaining tumor cells are detected through luminescence readings. The tumor cell-killing capacity of ^{Allo}CAR-NKT cells is assessed by comparing the reduction in tumor cell numbers without ^{Allo}CAR-NKT cells to the numbers observed with ^{Allo}CAR-NKT cells present.

Troubleshooting

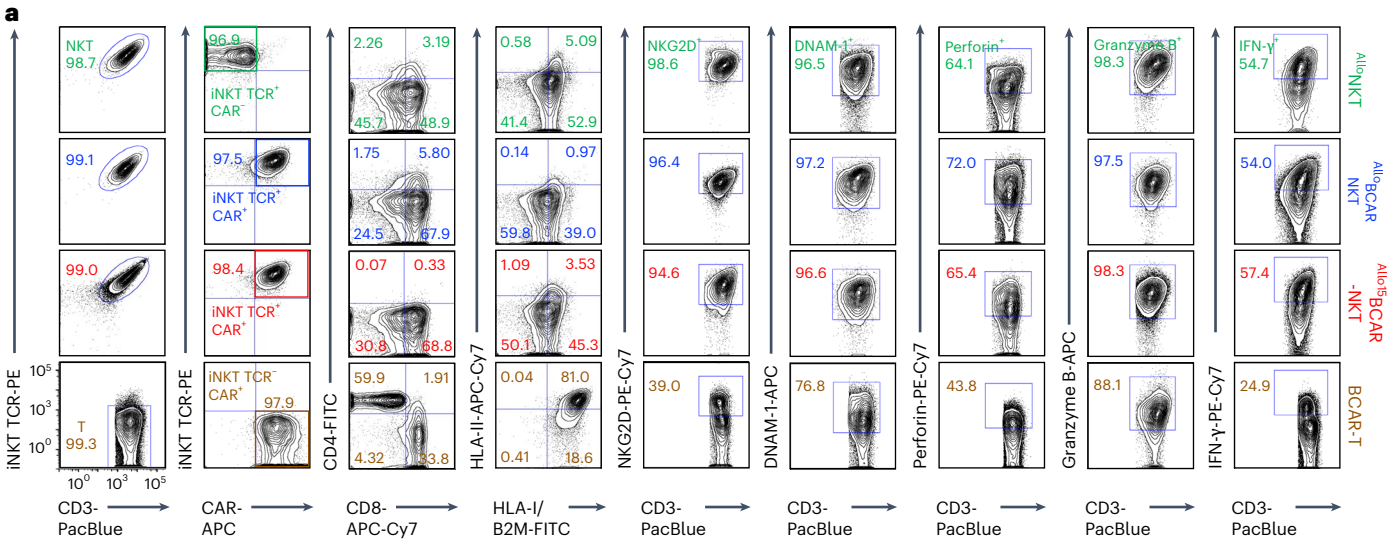
Troubleshooting advice can be found in Table 6.

Table 6 | Troubleshooting table

Step	Problem	Possible reason	Solution
4	293T cells are too confluent or too sparse	The timing of seeding 293T cells is either too early or too late	Adjust the seeding number or the seeding time of 293T cells for optimal virus production
21	Some 293T cells are detached	An excessive number of 293T cells are seeded, or they are cultured exceeding 48 h	Adjust the seeding number or the seeding time of 293T cells for optimal virus production
24	Concentrated virus has more volume than expected	Debris from 293T cells remains in the supernatant, blocking the Amicon device	Refilter the supernatant and centrifuge it for an additional 20 min
27	Virus transduction is not successful	Virus quality is low	Test virus transduction on 293T-CD3 cells, avoid repeated freeze and thaw of viruses, avoid leaving viruses at room temperature and avoid storing viruses at 4 °C
38	No or few HSP cells are found under microscope	HSP cell number is low at engineering stage	Continue with the cell culture, and HSP cells should be observed under the microscope ~4–5 d after HSP cell expansion
48	No TCR detection on HSP cells using flow cytometry	HSP cells have no CD3 expression and cannot display surface TCR	Use intracellular staining antibody for iNKT TCR detection or use anti-CAR antibody for CAR detection
51	The coating solution evaporates	Plates are left in 4 °C for an extended period	Cover the plates with Parafilm or plastic wrap
63	No NKT cells detected	Incorrect flow antibodies	Ensure the use of the correct antibody to detect NKT cells
		The efficiency of lentivirus transduction is insufficient	Increase the lentivirus quantity or regenerate new lentivirus with a higher titer
		Reagents, cytokines or medium have expired, or have been stored at 4 °C for an extended period	Use new reagents and cytokines, and make fresh cell culture medium
72B(ii)	Observe numerous PBM cell aggregates	PBM cells have low cell viability and quality	Collect a new batch of healthy donor derived PBM cells
72B(iii)	Observe the presence of crystals in the αGC-C10 medium	αGC precipitates due to low temperature	Sonicate and vortex the αGC-C10 medium for an extended duration, ensuring the maintenance of the αGC-C10 medium at a 37 °C water bath
72B(iv)	PBM cells adhering to the cell plate pose challenges in collection	The monocytes are more prone to adherence	Use nonadherent culture plates, such as those coated with hydrogels or treated to resist cell adhesion, or gentle wash during incubation to minimize cell adherence
72C(iii)	The cell number of aAPCs is low	aAPCs die and form aggregates postirradiation	Collect additional aAPCs and repeat the process, being careful not to leave aAPCs on ice for an extended duration

Timing

Steps 1–27, lentivirus generation: 5 d
Steps 28–29, lentivirus titration: 7 d
Steps 30–49, HSP cell engineering: 2 d
Steps 50–57, HSP cell expansion: 2 weeks
Steps 58–63, NKT cell differentiation: 1 week
Steps 64–70, NKT cell deep differentiation: 1 week
Steps 71–72, NKT cell expansion: 2 weeks
Step 73, downstream assays: variable
Box 1, CRISPR–Cas9 gene editing on HSP cells: 1 d



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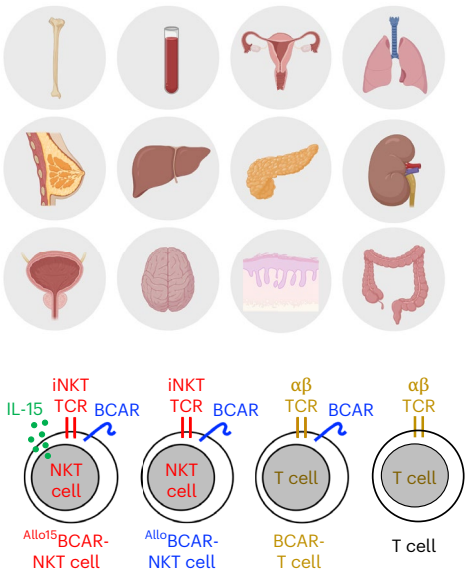

c

Cancer types	Cell line models
Multiple myeloma	MM.1S-FG
Chronic myeloid leukemia	K562-FG
Acute lymphoblastic leukemia	NALM6-FG
Acute myeloid leukemia	THP1-FG
Burkitt's lymphoma	RAJI-FG
Ovarian cancer	OVCAR3-FG
	OVCAR8-FG
	SKOV3-FG
Lung cancer	H226-FG
	A549-FG
	H292-FG

Cancer types	Cell line models
Breast cancer	HCC1806-FG
	MDA-MB-231-FG
Hepatocellular carcinoma	HEPG2-FG
	HEP3B-FG
	C3A-FG
	SNU432-FG
Pancreatic cancer	ASPC1-FG
	CAPAN2-FG
Renal cell carcinoma	786O-FG
	ACHN-FG
Prostate cancer	PC3-FG
Glioblastoma	U87-FG
Melanoma	A375-FG
Colon cancer	SW480-FG

d

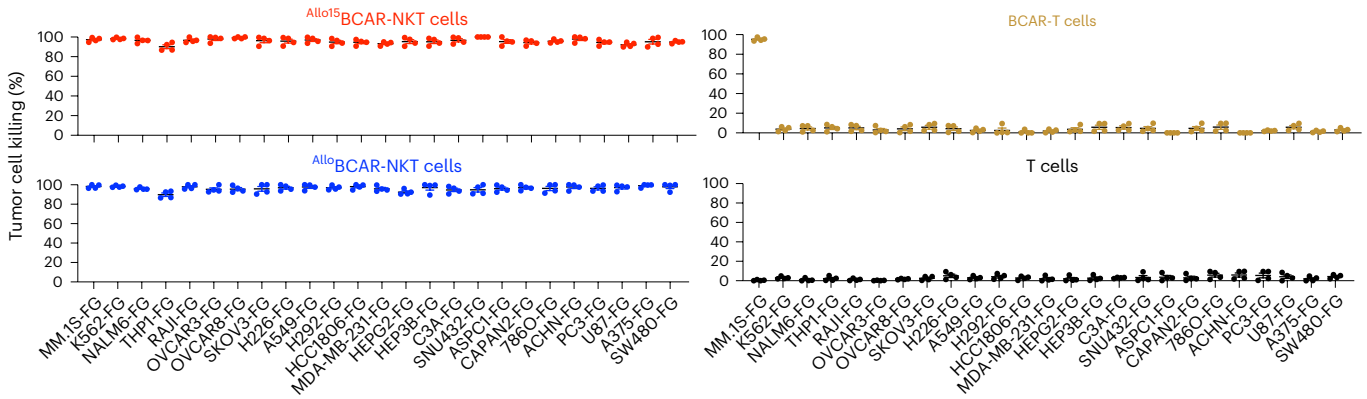

Fig. 6 | Phenotype, functionality and antitumor efficacy of allogeneic CAR-NKT cells. a, FACS detection of surface markers and intracellular cytotoxic molecules in allogeneic CAR-NKT cells. PBM cell-derived conventional T cells engineered with the same BCAR (denoted as BCAR-T cells) were included as a control. **b**–**d**, Studying the antitumor efficacy of $^{Allo/15}$BCAR-NKT cells against multiple tumor cell lines, which were engineered to overexpress the firefly luciferase and FG dual reporters: the multiple cancer cell types that can be targeted by allogeneic CAR-NKT cells, and the used therapeutic cells, with BCAR-T cells and PBM cell-derived conventional T cells included as control cells (**b**); the tumor cell lines used in the in vitro tumor cell-killing assays and their cancer types (**c**) and the tumor cell-killing data at 24 h (E:T ratio = 5:1, $n = 4$ from three different cell product donors) (**d**). A series of in vitro tumor cell-killing assays were conducted, utilizing various tumor cell lines and four types of therapeutic cell products. Representative of three experiments. Data are presented as the mean ± s.e.m.

Anticipated results

The protocol described here enables the user to generate HSP cell-derived NKT or CAR-NKT cells with high yield and purity (Figs. 1 and 2). We have successfully generated over 15 different NKT cell products expressing diverse CARs (i.e., CARs targeting BCMA, CD19, GD2, GPC3 and EGFRvIII) with various designs (i.e., CD28 or 4-1BB signaling domain) (Table 1 and Figs. 4 and 5). Besides CARs, other genetic elements can also be incorporated into the allogeneic CAR-NKT cell products, such as those encoding the immune-enhancement molecules (e.g., IL-15), safety controls (e.g., sr39TK) and reporters (e.g., Fluc and EGFP) (Table 1 and Fig. 4b,c). Impressively, all evaluated cargo genes exhibited no apparent disruption in the manufacture, yield and quality of the allogeneic CAR-NKT cell products[25] (Table 2 and Fig. 5). In addition, the pharmacology, efficacy, mechanism of action, pharmacokinetics/pharmacodynamics, safety and immunogenicity should also be evaluated (Fig. 3).

The 293T-CD3 cell line is used to assess the successful transduction of lentivectors and the expression of transgenes (Fig. 4a). Without lentivector transduction, 293T-CD3 cells do not exhibit surface CD3 expression (Fig. 4b). Three days after transduction with lentivectors encoding iNKT TCR, 293T-CD3 cells show high levels of iNKT TCR and CD3, detectable by flow cytometry (Fig. 4b). Similarly, 3 d posttransduction with lentivectors encoding a CAR, the cells express high levels of CAR, also detectable by flow cytometry (Fig. 4b). Additionally, 3 d after transduction with lentivectors encoding human IL-15, 293T-CD3 cells produce considerable levels of human IL-15, measurable by ELISA (Fig. 4c).

During the ex vivo development of ^{Allo}CAR-NKT cells, these cells are collected weekly for analysis using methods such as flow cytometry and sRNA-seq (Fig. 5a). Flow cytometry tracks the increasing percentage of NKT cells in the culture over successive weeks. By the end of week 6, the ^{Allo}CAR-NKT cells achieve high purity, with over 99% of cells identified as NKT cells (Fig. 5b). Throughout the culture, these ^{Allo}CAR-NKT cells exhibit a typical NKT cell developmental trajectory marked by changes in CD4/CD8 coreceptor expression (Fig. 5b). These cells transition from CD4/CD8 DN to CD4/CD8 double-positive, and finally to CD4/CD8 DN or CD8 SP. By week 6, the majority of ^{Allo}CAR-NKT cells (>99%) display either a CD4/CD8 DN or CD8 SP phenotype (Fig. 5b). Additionally, scRNA-seq analyses reveal that during the differentiation, ^{Allo}CAR-NKT cells follow a typical T cell lineage commitment and NKT cell development pathway, progressively upregulating genes associated with T and NK cell features, and NKT cell characteristics including transcription factor (i.e., PLZF encoded by *ZBTB16*) and cytotoxic markers (Perforin encoded by *PRF1*, and Granzyme B encoded by *GZMB*) (Fig. 5c–e).

These allogeneic CAR-NKT cells demonstrate potent antitumor efficacy and employ multiple mechanisms for tumor killing, including CAR/TCR/NKR-mediated triple tumor targeting[25] (Figs. 2 and 6). They efficiently eradicate a wide range of tumor cells, spanning both blood cancers and solid tumors[25] (Fig. 6b–d). Notably, even in the absence of CAR target expression on tumor cells, allogeneic CAR-NKT cells can still effectively kill tumor cells due to their high expression of NKRs such as NKG2D and DNAM-1 (Fig. 6a). Blocking these NKRs reduces the tumor cell-killing capacity, confirming the presence of an NKR-mediated tumor cell-killing mechanism (Supplementary Fig. 1).

Data availability

All associated data are presented in the protocol paper or Supplementary Information. The genomics data were reanalyzed from the public repository Gene Expression Omnibus database: GSE241996 (scRNA-seq, related to Fig. 5c–e). Additional information and materials will be made available upon reasonable request. Source data are provided with this paper.

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Author contributions

Y.-R.L., K.Z., D.L. and L.Y. designed the experiments, analyzed the data and wrote the manuscript. L.Y. conceived and oversaw the study, with assistance from Y.-R.L., K.Z. and D.L. Y.-R.L., K.Z. and D.L. performed all experiments, with assistance from Y. Zhu, T.H., J.Y., Y. Zhou, Y.F., Z.L., Y.C. and S.S.

Competing interests

Y.-R.L., D.L., J.Y., Y. Zhou and L.Y. are inventors on patents relating to this study filed by UCLA. J.Y. is currently an employee of Appia Bio. Y. Zhou is currently an employee of Amberstone Biosciences. L.Y. is a scientific advisor to AlzChem and Amberstone Biosciences, and a cofounder, stockholder and advisory board member of Appia Bio. Appia Bio licensed some patents relating to this study from UCLA. None of the declared companies contributed to or directed any of the research reported in this article. The other authors declare no competing interests.

Additional information

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