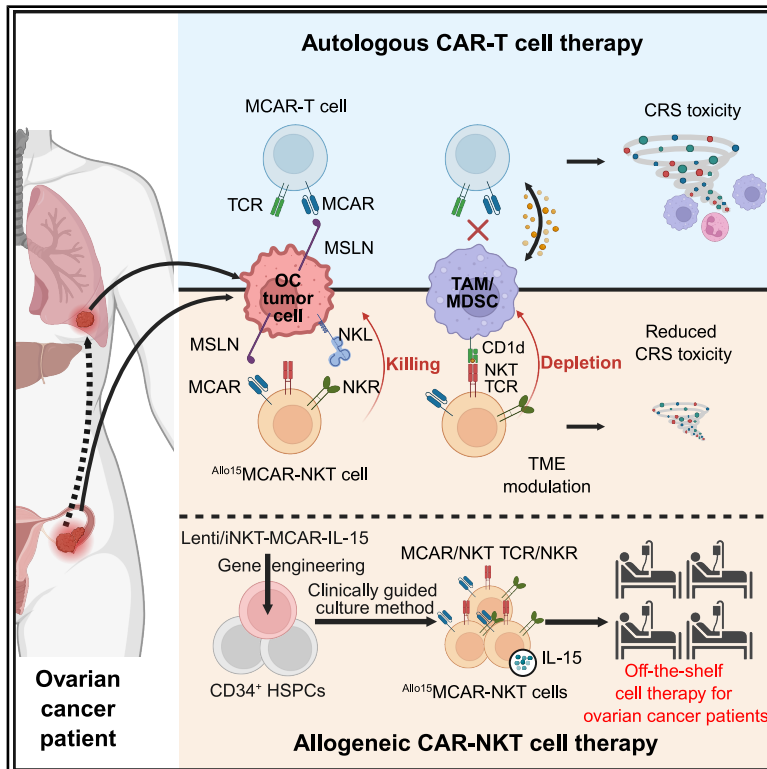


Overcoming ovarian cancer resistance and evasion to CAR-T cell therapy by harnessing allogeneic CAR-NKT cells

Graphical abstract



Authors

Yan-Ruide Li, Zhe Li, Yichen Zhu, ..., Pin Wang, Sanaz Memarzadeh, Lili Yang

Correspondence

smemarzadeh@mednet.ucla.edu (S.M.),
liliyang@ucla.edu (L.Y.)

In brief

Li et al. overcame ovarian cancer resistance and evasion to conventional CAR-T therapy by generating allogeneic CAR-NKT cells from hematopoietic stem and progenitor cells. These CAR-NKT cells showed superior antitumor efficacy, a favorable safety profile, and resistance to allorejection, highlighting their unique advantages and off-the-shelf potential for treating ovarian cancer.

Highlights

- Allo¹⁵CAR-NKT cells overcome OC resistance and evasion hindering CAR-T cells
- Allo¹⁵CAR-NKT cells exhibit potent efficacy with tumor homing and TME modulation
- Allo¹⁵CAR-NKT cells minimize cytokine release syndrome toxicity
- Allo¹⁵CAR-NKT cells induce minimal GvHD and resist host cell-mediated allorejection

Pre-clinical Research

Li et al., 2025, Med 6, 100804
October 10, 2025 © 2025 The Authors. Published by Elsevier Inc.
<https://doi.org/10.1016/j.medj.2025.100804>



Article

Overcoming ovarian cancer resistance and evasion to CAR-T cell therapy by harnessing allogeneic CAR-NKT cells

Yan-Ruide Li,^{1,2} Zhe Li,^{1,2} Yichen Zhu,^{1,2} Miao Li,^{1,2} Yuning Chen,^{1,2} Derek Lee,^{1,2} Christopher J. Ochoa,^{3,4,5} Tanya Singh,^{3,5} Gabriella DiBernardo,^{3,5} Wenbin Guo,⁶ Shuo Li,⁷ Yang Zhou,^{1,2} Yanqi Yu,^{1,2} Adam Kramer,^{1,2} Matthew Wilson,^{1,2} Kuangyi Zhou,^{1,2} Zachary Spencer Dunn,^{1,2,8} Ying Fang,^{1,2} Jiaji Yu,^{1,2} Yu Jeong Kim,^{1,2} Jie Huang,^{1,2} Xinyuan Shen,^{1,2} Yu-Chen Wang,⁹ Ryan Hon,^{1,2} Taylor Basso,^{1,2} Evelyn Chen,^{1,2} Enbo Zhu,^{1,2} Siyu Lin,^{10,11,12} Adam Neal,^{3,5} Neda A. Moatamed,⁷ Scott S. Oh,¹³ Saeed Sadeghi,¹⁴ Calvin Pan,⁹ Aijun Wang,^{10,11,12} Aldons J. Lusis,^{1,9,15} Xianghong Jasmine Zhou,⁷ Jin J. Zhou,¹⁶ Matteo Pellegrini,^{6,17,18} Pin Wang,⁸ Sanaz Memarzadeh,^{3,4,5,19,20,21,*} and Lili Yang^{1,2,4,5,19,22,23,24,*}

¹Department of Microbiology, Immunology & Molecular Genetics, University of California, Los Angeles (UCLA), Los Angeles, CA 90095, USA

²Department of Bioengineering, UCLA, Los Angeles, CA 90095, USA

³Department of Obstetrics and Gynecology, UCLA, Los Angeles, CA 90095, USA

⁴Molecular Biology Institute, UCLA, Los Angeles, CA 90095, USA

⁵Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, UCLA, Los Angeles, CA 90095, USA

⁶Bioinformatics Interdepartmental Program, UCLA, Los Angeles, CA 90095, USA

⁷Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, UCLA, Los Angeles, CA 90095, USA

⁸Mork Family Department of Chemical Engineering and Materials Science, University of Southern California (USC), Los Angeles, CA 90089, USA

⁹Division of Cardiology, Department of Medicine, UCLA, Los Angeles, CA 90095, USA

¹⁰Department of Biomedical Engineering, University of California, Davis (UCD), Davis, CA 95616, USA

¹¹Department of Surgery, School of Medicine, UCD, Sacramento, CA 95817, USA

¹²Institute for Pediatric Regenerative Medicine, Shriners Hospitals for Children, Sacramento, CA 95817, USA

¹³Section of Interventional Pulmonology, Keck School of Medicine, USC, Los Angeles, CA 90089, USA

¹⁴Division of Hematology-Oncology, Department of Medicine, David Geffen School of Medicine, UCLA, Los Angeles, CA 90095, USA

¹⁵Human Genetics, UCLA, Los Angeles, CA 90095, USA

¹⁶Department of Biostatistics, Fielding School of Public Health, UCLA, Los Angeles, CA 90095, USA

¹⁷Institute for Quantitative and Computational Biosciences—The Collaboratory, UCLA, Los Angeles, CA 90095, USA

¹⁸Department of Molecular Cell and Developmental Biology, UCLA, Los Angeles, CA 90095, USA

¹⁹Jonsson Comprehensive Cancer Center, David Geffen School of Medicine, UCLA, Los Angeles, CA 90095, USA

²⁰The VA Greater Los Angeles Healthcare System, Los Angeles, CA 90073, USA

²¹Department of Molecular and Medical Pharmacology, UCLA, Los Angeles, CA 90095, USA

²²Parker Institute for Cancer Immunotherapy, UCLA, Los Angeles, CA 90095, USA

²³Goodman-Luskin Microbiome Center, UCLA, Los Angeles, CA 90095, USA

²⁴Lead contact

*Correspondence: smemarzadeh@mednet.ucla.edu (S.M.), lilijiang@ucla.edu (L.Y.)

<https://doi.org/10.1016/j.medj.2025.100804>

CONTEXT AND SIGNIFICANCE Ovarian cancer remains one of the most lethal gynecologic malignancies, often recurring due to treatment resistance and immune evasion. Current chimeric antigen receptor-engineered T (CAR-T) cell therapies have shown limited success against ovarian cancer, largely due to tumor heterogeneity and an immunosuppressive tumor environment. This study explores an alternative approach using allogeneic CAR-engineered invariant natural killer T (^{Allo}CAR-NKT) cells derived from blood stem cells. By applying a clinically guided culture method, the researchers generated ^{Allo}CAR-NKT cells with high efficiency, purity, and potent antitumor activity. These engineered cells not only directly target tumor cells but also remodel the tumor environment and display a favorable safety profile. The findings suggest that ^{Allo}CAR-NKT cells offer a safer, more effective, and readily available immunotherapy for patients with ovarian cancer.

SUMMARY

Background: Ovarian cancer (OC) poses a significant challenge for conventional chimeric antigen receptor-engineered T (CAR-T) cell therapy, due to frequent recurrence linked to tumor heterogeneity, platinum resistance, immune evasion, and an immunosuppressive tumor microenvironment (TME).

Methods: Here, we analyze primary OC patient samples and identify a unique opportunity for allogeneic CAR-NKT (^{Allo}CAR-NKT) cells to concurrently attack OC tumor cells and their TME. Leveraging stem cell gene engineering and a clinically guided culture method, we achieve robust generation of ^{Allo}CAR-NKT cells at high yield and purity.

Findings: Compared to conventional CAR-T cells, ^{Allo}CAR-NKT cells demonstrate superior anti-OC efficacy, showcasing multiple OC-targeting mechanisms, focused tumor homing, and pronounced TME modulation. ^{Allo}CAR-NKT cells also exhibit a high safety profile with reduced cytokine release syndrome. Additionally, these cells do not induce graft-versus-host disease and resist host immune-cell-mediated allorejection.

Conclusions: These findings underscore the unique efficacy and safety advantages, as well as the off-the-shelf potential of ^{Allo}CAR-NKT cell therapy for OC.

Funding: Major funding was provided by the California Institute for Regenerative Medicine (CIRM).

INTRODUCTION

Ovarian cancer (OC) is the leading cause of death among women with gynecological malignancies.^{1,2} High-grade serous carcinomas arising from the ovary, fallopian tube, or endometrium are among the most aggressive subtypes of gynecologic cancers.^{1,2} The heightened lethality of OC results from late-stage diagnoses, primarily due to nonspecific symptoms and the absence of effective screening methods.^{3,4} Consequently, a substantial proportion of patients present with disseminated disease beyond the pelvic region, characterized by metastatic involvement in the pulmonary, hepatic, splenic, and other extrapelvic organs.^{5,6} The standard therapeutic regimen post diagnosis entails a multi-faceted approach involving surgical excision and chemotherapy.⁷ For OC, platinum-based agents, such as carboplatin, cisplatin, or nedaplatin, combined with a taxane like paclitaxel, constitute the most frequently administered chemotherapeutic interventions.⁸ While initial treatment elicits positive responses with marked reduction in tumor burden, a significant cohort of patients experiences relapse characterized by more aggressive disease progression.⁸ A pivotal factor in this recurrence is the emergence of resistance to platinum-based therapies.⁸ Consequently, addressing the challenge of platinum-resistant, recurrent OC remains an unmet medical need.

Immunotherapy has emerged as an attractive approach for the treatment of recurrent OC, encompassing the application of immunomodulators such as checkpoint inhibitors and monoclonal antibodies, as well as the utilization of immune-cell-based products, notably chimeric antigen receptor (CAR)-engineered T (CAR-T) cells.^{9–16} Several clinical trials are presently under way to assess the safety and therapeutic potential of various conventional CAR-T cell products that target diverse tumor antigens in OC, including mesothelin (MSLN; NCT01583686), mucin-16 (MUC16; NCT02498912), and claudin-6 (CLDN6; NCT04503278).^{10,17–19} These early clinical trials demonstrated feasibility, general safety, and some (but limited) efficacy, encouraging further development of cell-based immunotherapy for OC.^{10,17,18} However, signifi-

cant challenges persist in current conventional CAR-T cell-based immunotherapy for OC. These challenges encompass the inherent heterogeneity of OC tumor cells and their variable antigen expression,²⁰ the presence of therapy-resistant and immuno-evasive cancer stem cells (CSCs),^{21–23} and an extremely unfavorable peritoneal tumor microenvironment (TME) comprising a substantial population of immunosuppressive cells, especially tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs).^{24–27} Notably, a reciprocal interplay exists between CSCs and TAMs/MDSCs in OC, exacerbating tumor progression and significantly impeding therapeutic responses.^{6,28–30} In addition, cytokine release syndrome (CRS) represents an important safety concern for conventional CAR-T cells, particularly in the context of peritoneal OC,^{12,31} while the substantial presence of peritoneal macrophages interacts with CAR-T cells, potentially intensifying the severity of CRS.^{32,33} Therefore, advanced therapies are urgently needed to effectively and concurrently target OC tumors and their TME, overcoming OC resistance and immune evasion while minimizing CRS-related toxicity.

Several studies have investigated the OC TME to identify targetable biomarkers.^{34–38} Our recent research revealed the high and specific expression of CD1d on TAMs and MDSCs within the human OC TME.²¹ Interestingly, CD1d is the natural ligand for the unique T cell receptor (TCR) (invariant V α 24-J α 18 TCR α chain paired with a limited selection of β chains, dominantly V β 11) expressed by invariant natural killer T (NKT) cells, rendering CD1d^{hi} TAMs/MDSCs susceptible to targeting by NKT cells.^{21,39–41} NKT cells, also known for their powerful tumor cell killing capacity,^{42–46} hold promise for OC treatment by concurrently targeting both OC tumor and its TME. However, the extremely low numbers of human NKT cells in blood (0.001%–0.1%) present challenges in generating sufficient quantities for cancer therapy.⁴⁷ New approaches are therefore required to advance the development and application of NKT cell-based therapy for OC.

Recently, we reported a technology to robustly generate therapeutic levels of allogeneic CAR-engineered NKT (CAR-NKT)

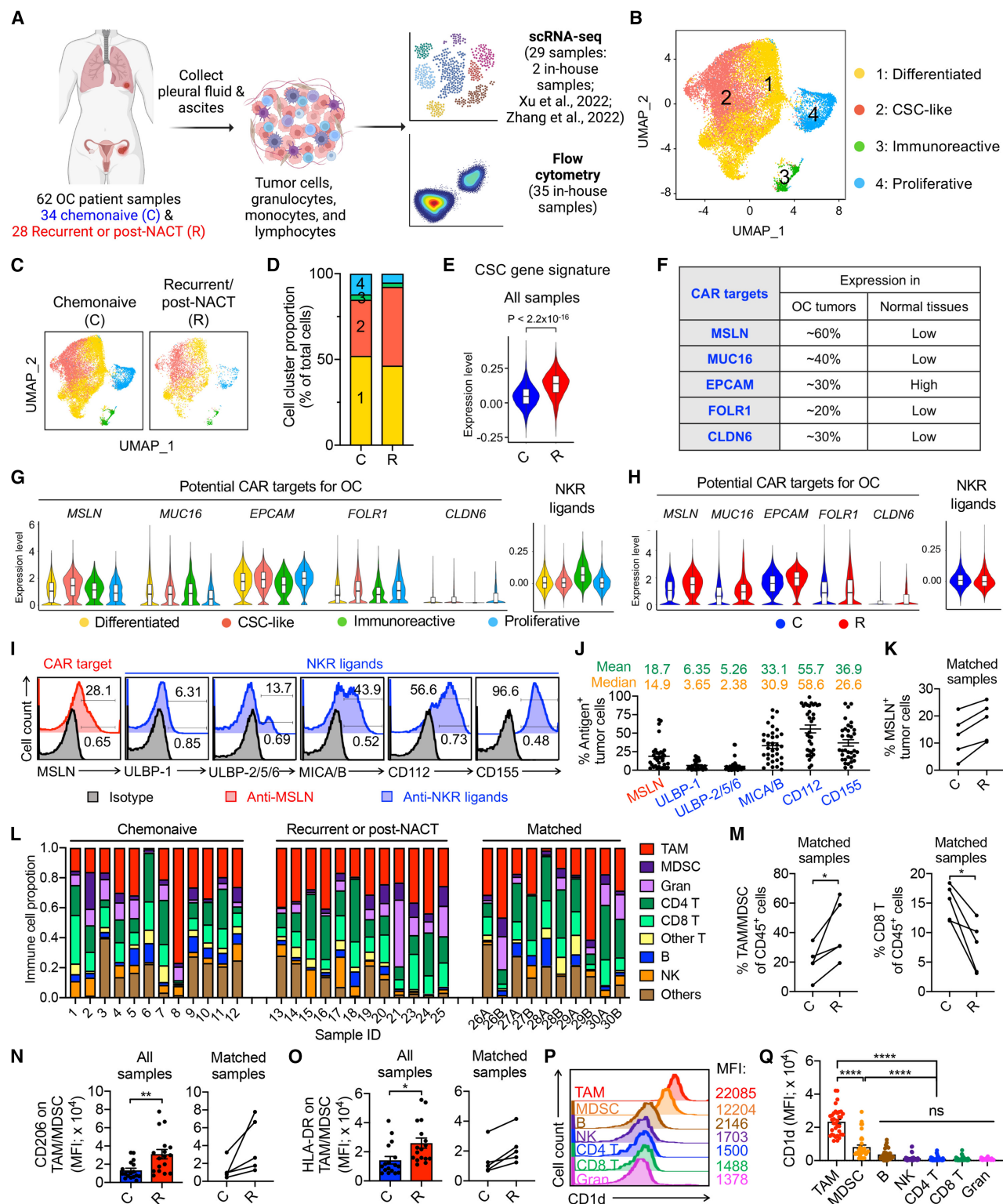


Figure 1. Transcriptome and biomarker profiling of primary OC tumor cells and their TME identifies opportunities for CAR-NKT cell therapy
(A) Experimental design to profile OC tumor cells and their TME using scRNA-seq and flow cytometry. A total of 62 primary OC patient samples were included, comprising 34 platinum chemo-naive samples (C) and 28 recurrent or post-neoadjuvant chemotherapy (NACT) samples (R).

(legend continued on next page)

cells through hematopoietic stem and progenitor cell (HSPC) gene engineering and a clinically guided culture method, demonstrating their therapeutic potential for treating blood cancers.⁴⁸ Here, we build on this technology to tackle solid tumors, with a special focus on OC. The study incorporates a substantial collection of primary OC patient samples representing various disease states, a diverse array of *in vitro* assays and *in vivo* humanized mouse models, and a large set of advanced techniques including transcriptome profiling, epigenomic mapping, and live animal imaging. We conducted comprehensive preclinical evaluations comparing allogeneic CAR-NKT cells to conventional CAR-T cells, encompassing their pharmacology, efficacy, mechanism of action (MOA), pharmacokinetics/pharmacodynamics (PK/PD), safety, and immunogenicity.

RESULTS

Transcriptome and biomarker profiling of primary OC tumor cells and their TME identify opportunities for CAR-NKT cell therapy

We performed a comprehensive analysis of primary OC tumor cells and their TME using single-cell RNA sequencing (scRNA-seq) and flow cytometry (Figure 1A). Our study included 35 in-house collected OC patient samples, consisting of 17 chemo-naïve (C) samples and 18 recurrent or post-neoadjuvant chemotherapy (R) samples, with five paired C/R samples from the same patients (Table S1). Additionally, we incorporated two public scRNA-seq datasets,^{49,50} which contained 27 OC patient samples, consisting of 17 C and 10 R samples, with 10 paired C/R samples from the same patients (Figure S1A). The availability of paired samples allowed for direct study of chemoresistance-associated changes in OC tumor cells.

scRNA-seq analysis revealed four distinct clusters within OC tumor cells, corresponding to differentiated, CSC-like, immunoreactive, and proliferative populations (Figure 1B).⁵¹ Notably, R samples exhibited an enrichment of CSC-like cells compared to C samples (Figures 1C–1E, S1B, and S1C), confirming CSCs' potential association with platinum resistance.²² A survey of five potential CAR antigens that have been explored for targeting OC (i.e., MSLN, MUC16, EPCAM, FOLR1, and CLDN6) showed diverse expression patterns (Figures 1F–1H). Notably, MSLN was ranked among the top, showing overall high expression levels in OC tumor cells, low or intermediate expression

levels in normal tissues, and particularly enhanced expression in CSCs and R samples (Figures 1F–1H and Table S2).⁵² Interestingly, high and stable expression of a large set of activating natural killer receptor (NKR) ligands was detected for all OC samples and across all four tumor cell populations and C/R stages, suggesting an opportunity for NKR-directed targeting of OC (Figures 1G and 1H).²¹ In addition, another scRNA-seq study analyzing OC patient samples collected at various tumor sites (i.e., primary tumors, omentum metastases, and ascites) indicated that metastatic OC tumor cells in ascites exhibited an enrichment of CSC-like cells, along with the highest levels of MSLN and similar levels of NKR ligands, compared to tumor cells in primary tumor sites and omentum metastases (Figures S1D–S1I).³⁶ The expressions of MSLN and NKR ligands on OC tumor cells were further validated using flow cytometry (Figures 1J–1K).

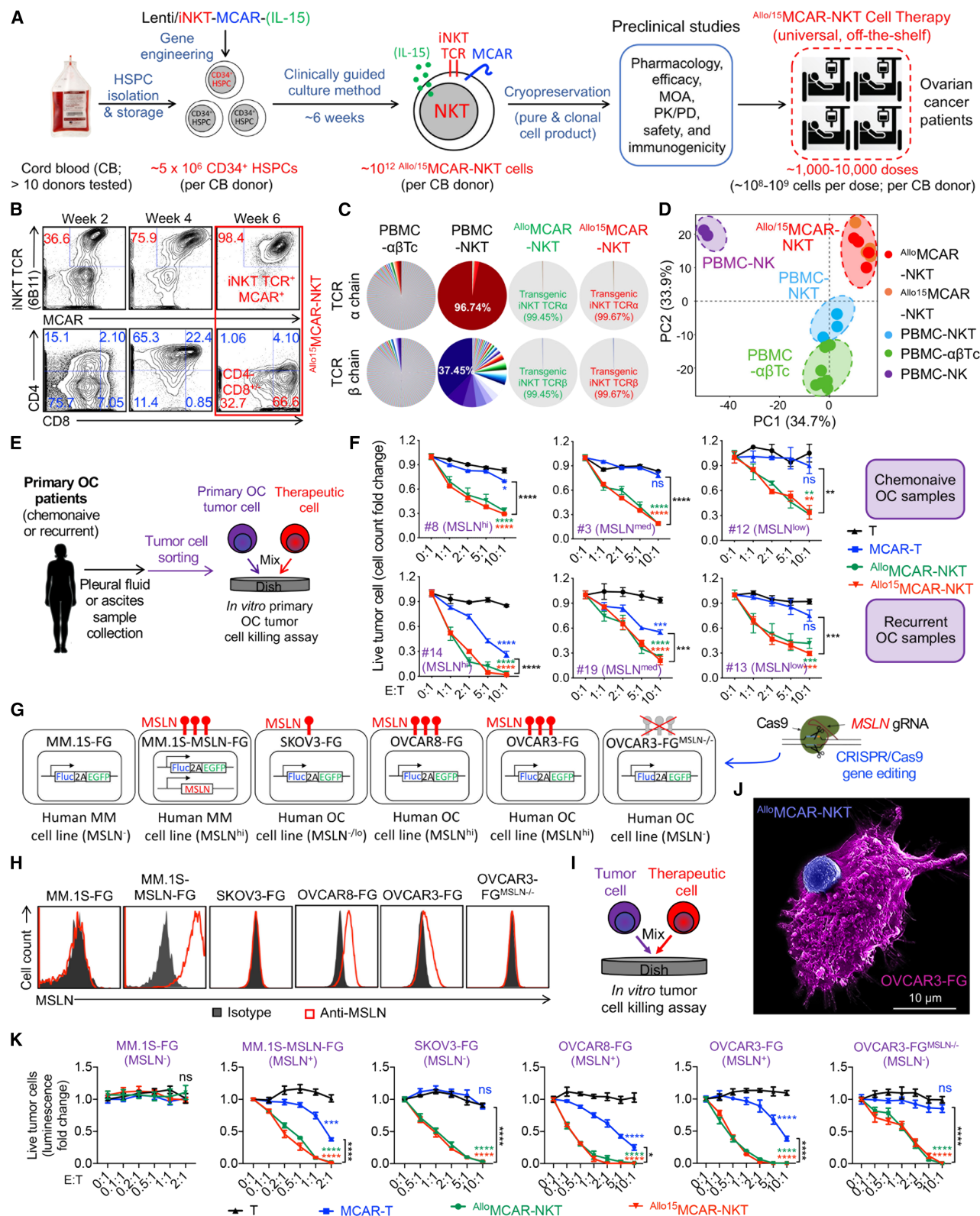
Flow-cytometry analysis of the TME of OC samples detected significant proportions of TAMs and MDSCs, as well as the presence of other immune-cell types (e.g., T cells, B cells, NK cells, and granulocytes; Figures 1L and S1J). Comparison of paired C/R samples showed in the R samples a decrease of tumor-infiltrating CD8 T cells along with an increase of TAMs and MDSCs displaying the immunosuppressive phenotype (i.e., CD206^{hi} and HLA-DR^{hi}), which may have contributed to OC recurrence (Figures 1M–1O).²¹ A biomarker screening identified CD1d, a nonpolymorphic MHC class I-like molecule, to be highly expressed on TAMs and MDSCs but not on other TME cells (Figures 1P and 1Q). Intriguingly, CD1d is the natural ligand of the NKT TCR, making the CD1d^{hi} TAMs and MDSCs vulnerable targets of NKT cells.^{21,39,40}

Besides their invariant TCR that targets OC TME, NKT cells also co-express many NKRs that target OC tumor cells.⁵³ NKT cells can certainly be engineered with additional CARs, such as an MSLN-targeting CAR (MCAR), to enhance their OC-targeting capacity, particularly against the CSC-enriched populations associated with recurrent OC (Figures 1G and 1H). Therefore, the transcriptome and biomarker profiling of primary OC samples identified the vulnerability of OC to CAR-NKT cell therapy that can leverage CAR/TCR/NKR multi-targeting mechanisms to simultaneously target OC tumor cells and their immunosuppressive TME, thereby offering a promising new therapeutic opportunity to combat OC. Utilizing HSPC gene engineering and a clinically guided culture method,⁴⁸ we successfully generated allogeneic HSPC-engineered MSLN-targeting CAR-NKT cells

(B–H) Profiling primary OC tumor cells using scRNA-seq. (B) Combined UMAP plot showing the formation of four major cell clusters. CSC, cancer stem cell. (C) Individual UMAP plots of the C and R samples. (D) Bar graphs showing the cell-cluster proportions of the indicated samples. (E) Violin plots showing the expression distribution of CSC gene signature in C and R samples. (F) Table showing the potential CAR targets for OC and their expression in OC tumors and normal tissues. Data are summarized from The Human Protein Atlas (<https://www.proteinatlas.org/>). The percentage of OC patients exhibiting high expression levels of the indicated genes in tumor cells was calculated relative to the total number of patients. (G and H) Violin plots showing the expression distribution of genes encoding the indicated CAR targets and activating natural killer receptor (NKR) ligands, compared among the four cell clusters (G) or between the C and R samples (H).

(I–Q) Profiling OC tumor cells (I–K) and their TME (L–Q) using flow cytometry. (I) Representative fluorescence-activated cell sorting (FACS) plots showing the detection of CAR target (i.e., MSLN) and NKR ligands on OC tumor cells. (J) Quantification of (I) ($n = 34$). (K) Quantification of MSLN⁺ tumor cells in matched C and R sample pairs ($n = 5$). (L) TME immune-cell composition of primary OC samples ($n = 34$). (M) Quantification of tumor-associated macrophage and myeloid-derived suppressor cell (TAM/MDSC) and CD8 T cell proportions in the matched C and R sample pairs ($n = 5$). (N and O) Quantification of CD206 (N) and HLA-DR (O) expression on TAM/MDSCs, compared between a total of 34 C and R samples and five matched sample pairs. (P and Q) FACS measurements of CD1d expression on the indicated immune cells in the OC TME. Gran, granulocyte. (P) Representative FACS plots. (Q) Quantification of (P) ($n = 34$).

Data are presented as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ by Student's *t* test (M, N, and O) or one-way ANOVA (Q). *p* values of violin plots were determined by Wilcoxon rank-sum test (E). See also Figure S1; Tables S1 and S2.



(legend on next page)

for OC treatment. Upon establishment of the CAR-NKT cell therapy platform, additional CARs (e.g., CARs targeting MUC16 and CLDN6) can be seamlessly incorporated to target a broader spectrum of OC antigens and effectively address the tumor heterogeneity within OC.^{17,20,37,54}

Allogeneic CAR-NKT cells targeting OC can be generated using HSPC engineering and a clinically guided culture method of high yield, purity, and robustness

Human cord blood (CB) CD34⁺ HSPCs were transduced with a single lentivector co-delivering genes encoding a pair of iNKT TCR (α and β chains) and an MCAR, with or without an additional human interleukin-15 (IL-15) (denoted as Lenti/iNKT-MCAR-IL-15 or Lenti/iNKT-MCAR, respectively) (Figures 2A and S2A). Both Lenti/iNKT-MCAR-IL-15 and Lenti/iNKT-MCAR lentivectors efficiently transduced HSPCs (Figure S2B). The iNKT TCR has been previously used to develop both autologous and allogeneic HSPC-engineered NKT cell therapies^{48,53,55}; the MCAR is adapted from a clinically proven design that has been confirmed for safety and MSLN-targeting function^{10,56}; and the secreting form of human IL-15 has been utilized to improve *in vivo* NKT cell performance without causing evident toxicity.^{57–59}

Lentivector-transduced HSPCs were then cultured using a clinically guided culture method⁴⁸ over 6 weeks to generate allogeneic MCAR-NKT (^{Allo}MCAR-NKT) cells or their IL-15-enhanced derivatives (denoted as ^{Allo}IL-15 MCAR-NKT cells) (Figures 2B and S2C). The differentiation of ^{Allo}IL-15 MCAR-NKT cells followed a typical NKT cell developmental path, from CD4⁺CD8[−] double-negative (DN) to CD4⁺CD8⁺ double-positive (DP), and eventually to CD8⁺ single-positive (SP) or DN stages (Figures 2B and S2C). Note that the final ^{Allo}IL-15 MCAR-NKT cell product did not contain a CD4⁺ SP population that is typically present in native human NKT cells (Figures 2B and S2C).^{60–62} In general, CD8 SP/DN human NKT cells are regarded as possessing pro-inflammatory characteristics and high cytotoxicity, making these cells attractive for cancer therapy.^{63–66} Importantly, the co-delivery of iNKT TCR and CAR genes using a single lentivector resulted in a pure NKT cell product with nearly 100% CAR co-expression, owing to the positive selection and

allelic exclusion mechanisms inherent to the culture method (Figures 2B and S2C).⁶⁷ These mechanisms rendered the allogeneic MCAR-NKT cell products pure and “clonal,” obviating the need for further enrichment or purification procedures.

The manufacture was highly robust, yielding ^{Allo}IL-15 MCAR-NKT cells of high purity and yield (Figure S2D and Table S3). It was estimated that from a single CB donor (comprising $\sim 5 \times 10^6$ HSPCs), $\sim 10^{12}$ ^{Allo}IL-15 MCAR-NKT cells could be generated that can potentially be formulated into $\sim 1,000$ – $10,000$ doses for allogeneic CAR-NKT cell therapy (Figure S2D).⁶⁸ The resulting CAR-NKT cell product contained nearly undetectable bystander conventional $\alpha\beta$ T cells that thereby are free of graft-versus-host disease (GvHD) risk and can be directly cryopreserved as the final therapeutic cell product (Figure 2C and Table S3).⁴⁵

Notably, the incorporation of an IL-15 gene did not interfere with the differentiation and production of allogeneic MCAR-NKT cells, yielding allogeneic MCAR-NKT cells co-expressing IL-15 alongside the NKT TCR and MCAR (denoted as ^{Allo}IL-15 MCAR-NKT cells; Figures 2B, S2C, and S2D). In this study, we conducted comprehensive assessments of allogeneic MCAR-NKT cells in comparison with conventional MCAR-T cells for treating OC. The conventional MCAR-T cells were generated using a standard method by engineering healthy donor peripheral blood mononuclear cell (PBMC)-derived T cells to express the same MCAR.^{10,56}

^{Allo}MCAR-NKT cells display a typical NKT cell phenotype and a Th1-prone cytotoxic functionality

A gene-profiling study using bulk RNA-seq revealed that ^{Allo}IL-15 MCAR-NKT cells closely resembled endogenous human PBMC-derived NKT (PBMC-NKT) cells, ranked second to PBMC-derived conventional $\alpha\beta$ T (PBMC- $\alpha\beta$ Tc) cells, and were largely different from PBMC-derived NK (PBMC-NK) cells (Figure 2D). Gene-expression analysis further revealed the distinct molecular signatures of ^{Allo}IL-15 MCAR-NKT cells. Overall, ^{Allo}IL-15 MCAR-NKT cells displayed an NKT-typical and T helper 1 (Th1)-prone phenotype based on their transcription factor expression profiles (Figure S2E). These cells exhibited high expression of *ZBTB16* (encoding PLZF, a signature transcription factor of NKT and NK cells)⁶⁹ and *TBX21* (encoding T-bet, a key

Figure 2. Allogeneic CAR-NKT cells targeting OC can be generated combining HSPC engineering with a clinically guided culture method, achieving high yield, purity, and potency

(A) Schematics showing the development of a human HSPC-engineered allogeneic MSLN-targeting CAR-armed NKT (^{Allo}MCAR-NKT) cell product, as well as their IL-15-enhanced derivative (denoted as ^{Allo}IL-15 MCAR-NKT cells). HSPC, CD34⁺ hematopoietic stem and progenitor cell; MOA, mechanism of action; PK/PD, pharmacokinetics/pharmacodynamics.

(B) FACS monitoring of the generation of ^{Allo}IL-15 MCAR-NKT cells over the 6-week culture.

(C) Single-cell TCR sequencing analysis of ^{Allo}IL-15 MCAR-NKT cell products. Healthy donor PBMC-derived conventional $\alpha\beta$ T (PBMC- $\alpha\beta$ Tc) and NKT (PBMC-NKT) cells were included as controls.

(D) Bulk RNA sequencing analysis of ^{Allo}MCAR-NKT ($n = 4$) and ^{Allo}IL-15 MCAR-NKT ($n = 4$) cell products. PBMC- $\alpha\beta$ Tc ($n = 7$), PBMC-NKT ($n = 3$), and PBMC-NK ($n = 2$) cells were included as controls (n indicates different donors). Principal-component analysis (PCA) plot is presented showing the ordination of all five cell types. (E and F) Studying the OC tumor cell killing capacity of ^{Allo}IL-15 MCAR-NKT cells against primary OC patient samples. PBMC-derived conventional T cells and their MCAR-armed derivatives (denoted as T and MCAR-T cells, respectively) were included as therapeutic cell controls. (E) Experimental design. (F) Tumor cell killing data at 24 h ($n = 3$ – 4).

(G–K) Dissecting the tumor cell killing mechanisms of ^{Allo}IL-15 MCAR-NKT cells using a collection of human tumor cell lines. (G) Schematics showing the six human tumor cell lines utilized in this study. All were engineered to express the firefly luciferase (Fluc) and enhanced green fluorescent protein (EGFP) dual reporters (FG). (H) FACS detection of MSLN expression on the indicated tumor cells. (I) Experimental design. (J) Scanning electron microscope visualization of an ^{Allo}MCAR-NKT cell attacking an OVCAR3-FG cell. (K) Tumor cell killing data at 24 h ($n = 4$).

Representative of one (C and D), three (E–K), and more than ten experiments (A and B). Data are presented as the mean $\pm$ SEM. ns, not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by one-way ANOVA (F and K). See also Figures S2, S3, S8, and S9; Table S3.

regulator of Th1 commitment in T cells),⁷⁰ while displaying low expression of *GATA3* (encoding *GATA3*, a transcription factor involved in Th2 polarization) (Figure S2E).^{70,71}

Notably, in comparison to PBMC-NKT and even PBMC-NK cells, ^{Allo/15}MCAR-NKT cells expressed exceedingly higher levels of NK-activating receptor genes (e.g., *NCR1*, *NCR2*, *NCAM1*, and *KLRK1*) but significantly lower levels of NK-inhibitory receptor genes (e.g., *KIR3DL1*, *KIR3DL2*, *KIR3DL3*, and *KLRG1*), suggesting their superior NK-related antitumor function (Figure S2E).⁵³ Furthermore, compared to endogenous PBMC-NKT and PBMC- $\alpha\beta$ Tc cells, ^{Allo/15}MCAR-NKT cells expressed significantly lower levels of exhaustion marker genes (e.g., *PDCD1*, *LAG3*, *CTLA4*, and *TIGIT*), indicating a less-exhaustion feature of ^{Allo/15}MCAR-NKT cells (Figure S2E).^{72–74} Interestingly, compared to all PBMC-derived cells, ^{Allo/15}MCAR-NKT cells expressed much lower levels of HLA-related genes, which could potentially enable their resistance to host T cell-mediated allo-rejection (Figure S2E).^{53,75}

Flow-cytometry analysis of ^{Allo/15}MCAR-NKT cells further validated their NKT cell phenotype. Compared to conventional MCAR-T cells (Figures S2F–S2I), ^{Allo/15}MCAR-NKT cells expressed higher levels of NK receptors (NKR; e.g., CD161, NKG2D, and DNAM-1) and T cell memory markers (e.g., CD45RO), and produced exceedingly higher levels of effector cytokines (e.g., interferon- γ [IFN- γ], IL-2, and tumor necrosis factor α [TNF- α]) and cytotoxic molecules (e.g., granzyme B and perforin), agreeing with their CD8 SP/DN phenotype and favoring their cancer therapy application (Figures 2B, S2C, and S2J).^{76,77}

To validate the NKT TCR function, ^{Allo/15}MCAR-NKT cells were stimulated with an agonist glycolipid antigen, α -galactosylceramide (α GC) (Figure S2K).^{78,79} ^{Allo/15}MCAR-NKT cells demonstrated robust proliferation; compared to ^{Allo}MCAR-NKT cells, ^{Allo/15}MCAR-NKT cells exhibited slightly greater proliferating capacity, accompanied by substantial IL-15 production (Figures S2L and S2M). Meanwhile, ^{Allo/15}MCAR-NKT cells secreted high levels of Th1 cytokines (i.e., IFN- γ , TNF- α , and IL-2) and low levels of Th2/Th17 cytokines (i.e., IL-4 and IL-17a) (Figure S2N), confirming a Th1-prone functionality of ^{Allo/15}MCAR-NKT cells aligning with their CD8 SP/DN phenotype (Figures 2B and S2C).^{64,80}

^{Allo}MCAR-NKT cells directly kill OC tumor cells at high efficacy and use multiple tumor-targeting mechanisms, outperforming conventional CAR-T cells

^{Allo/15}MCAR-NKT cells are expected to target OC tumor cells through multiple surface receptors, involving CAR recognition of the MSLN CAR antigen and NKR recognition of a broad array (more than 30) of NK ligands expressed on OC tumor cells (Figures 1G–1J).^{42,43,58,81} These multiple tumor-targeting mechanisms may enable ^{Allo/15}MCAR-NKT cells to counteract OC tumor immune evasion in the context of immunotherapy.^{82–84}

Twenty primary OC samples were used to evaluate the tumor cell killing capacity of ^{Allo/15}MCAR-NKT cells (Figure 2E). Variable expressions of MSLN and NKR ligands were detected on tumor cells from all OC samples (Figures 1I, 1J, and S3A; Table S2). In an *in vitro* tumor cell killing assay, ^{Allo/15}MCAR-NKT cells demonstrated superior effectiveness in killing primary OC tumor cells, including the recurrent ones, compared to conventional

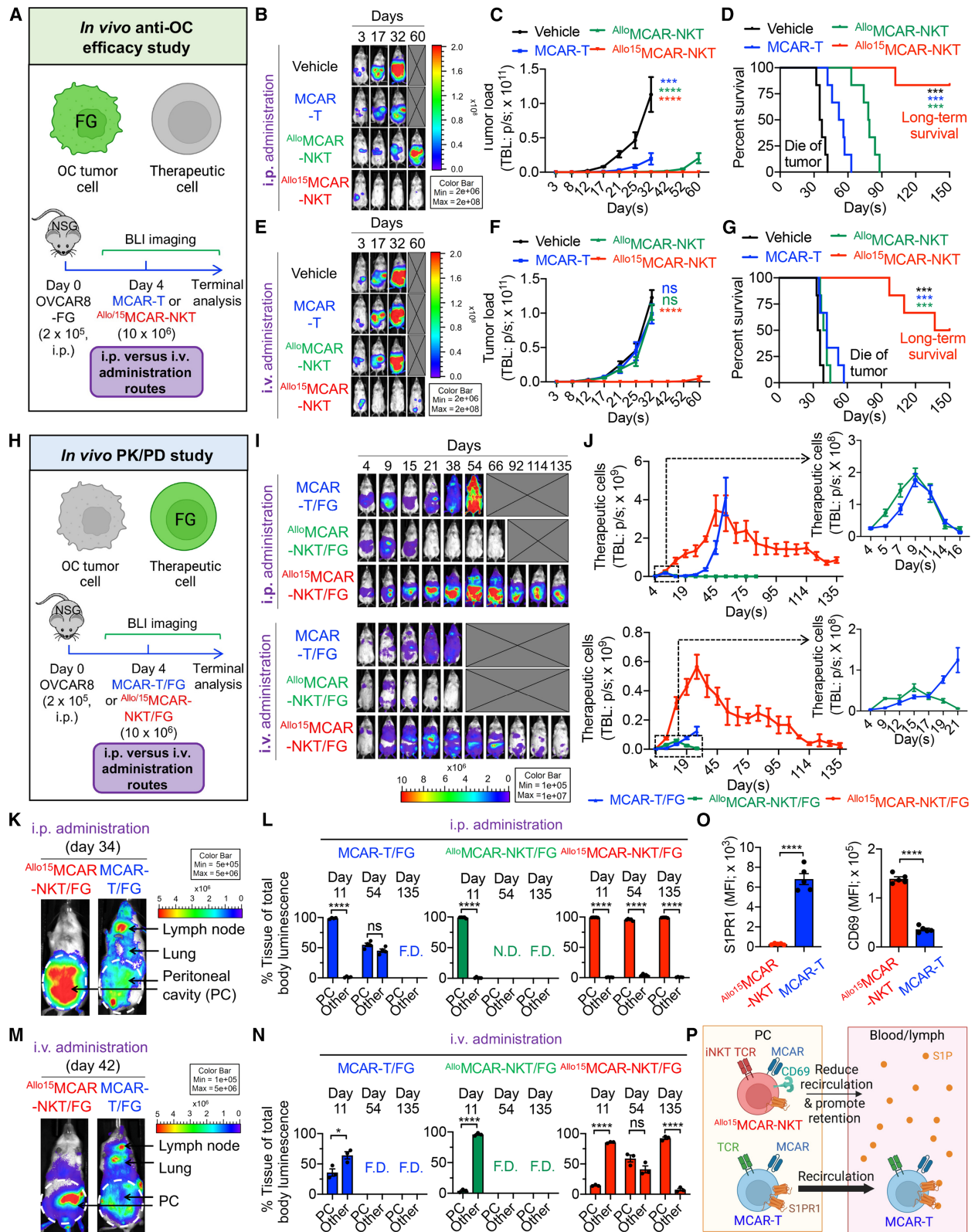
MCAR-T cells (Figure 2F). Interestingly, even with no or low MSLN expression, ^{Allo/15}MCAR-NKT cells exhibited a considerable tumor killing capability (Figure 2F), confirming a CAR-independent tumor killing potential.

Next, we performed a series of *in vitro* tumor cell killing assays, involving six tumor cell lines: two human multiple myeloma cell lines (i.e., MM.1S-FG and MSLN-overexpressing MM.1S-MSLN-FG), and four human OC cell lines (SKOV3-FG, OVCAR8-FG, OVCAR3-FG, and MSLN-knockout OVCAR3-FG^{MSLN-/-}; Figures 2G–2I). These cell lines were gene engineered to overexpress the firefly luciferase and enhanced green fluorescence protein dual reporters (FG), allowing for sensitive monitoring using both flow cytometry and bioluminescence assays (Figure 2G). Of note, the two MM.1S cell lines are resistant to NK-mediated killing whereas the other four OC cell lines are susceptible, providing us an opportunity to examine NKR-mediated tumor killing of ^{Allo/15}MCAR-NKT cells (Figure S3B).⁵³ Additionally, these six tumor cells expressed varying levels of MSLN, allowing us to study CAR-mediated tumor killing of ^{Allo/15}MCAR-NKT cells (Figure 2H).

Using scanning electron microscopy, we visualized the direct attack of OC tumor cells by allogeneic MCAR-NKT cells (Figure 2J). Using *in vitro* bioluminescence assay, flow cytometry, and ELISA, we decoded the multi-pronged MOA of allogeneic MCAR-NKT cells targeting OC (Figures 2K and S3C–S3J). MCAR-T cells solely relied on the MCAR-MSLN recognition for tumor cell targeting, as evidenced by their selective killing of MSLN⁺ tumor cells (Figure 2K). In contrast, ^{Allo/15}MCAR-NKT cells targeted both MSLN⁺ and MSLN⁻ tumor cells, indicating a CAR-independent targeting capacity (Figure 2K). Indeed, ^{Allo/15}MCAR-NKT cells killed MSLN⁻ tumor cells through NKR (i.e., NKG2D and DNAM-1) recognition (Figures S3C and S3D). Overall, compared to conventional MCAR-T cells, ^{Allo/15}MCAR-NKT cells showcased superior tumor cell killing capacity (Figure 2K), corresponding with their more pronounced upregulation of activation and degranulation markers (i.e., CD69 and CD107a) as well as production of cytotoxic molecules (i.e., perforin and granzyme B) and pro-inflammatory cytokines (i.e., IFN- γ ; Figures S3E–S3G).

In addition, an *in vitro* serial tumor cell killing assay was implemented to assess the long-term OC killing capacity of allogeneic MCAR-NKT cells (Figure S3H). In the presence of CAR antigen, ^{Allo/15}MCAR-NKT cells outperformed conventional MCAR-T cells (Figure S3I). In a scenario of CAR antigen loss, ^{Allo/15}MCAR-NKT cells still exhibited impressive long-term tumor killing, while conventional MCAR-T cells totally failed to kill OC tumor cells (Figure S3J). Under both conditions, ^{Allo/15}MCAR-NKT cells stood out as the best performer (Figures S3I and S3J), agreeing with an antitumor enhancement function of IL-15 for human NKT cell long-term performance that has been reported in previous pre-clinical studies and clinical trials.^{48,59,85}

Of note, the stability of the allogeneic MCAR-NKT cell products is appealing. ^{Allo/15}MCAR-NKT cells were cryopreserved as aliquots (10×10^6 cells per mL per aliquot), followed by thawing aliquots over time and testing the viability and cytotoxicity of the recovered ^{Allo/15}MCAR-NKT cells. Over 2 years, the viability of the recovered ^{Allo/15}MCAR-NKT cells dropped only slightly (from ~90% to ~80%; Figure S3K), and the cytotoxicity of the



(legend on next page)

recovered live $Allo^{15}$ MCAR-NKT cells was largely preserved (Figures S3L and S3M).

$Allo^{15}$ MCAR-NKT cells showcase superior *in vivo* anti-OC efficacy associated with focused OC tumor homing compared to conventional CAR-T cells

As a peritoneal disease, OC is treated systemically via intravenous (i.v.) administration or locally via intraperitoneal (i.p.) administration, aiming for targeted delivery and enhanced efficacy.^{86,87} We therefore studied both i.v. and i.p. administration routes of the therapeutic cells (i.e., $Allo^{15}$ MCAR-NKT and conventional MCAR-T cells) (Figure 3A). A mirror-imaging system was utilized to evaluate in parallel the *in vivo* anti-OC efficacy and PK/PD of the therapeutic cells in a collection of human OC xenograft mouse models. In the efficacy studies, OVCAR8 tumor cells but not therapeutic cells were labeled with FG to visualize tumor loads (Figures 3A–3G), whereas in the PK/PD studies, the therapeutic cells but not tumor cells were labeled with FG to assess the kinetics and dynamics of the therapeutic cells (Figures 3H–3N).

When delivered via i.p. administration, $Allo^{15}$ MCAR-NKT cells led to tumor eradication and long-term survival (> 150 days) in the majority (five out of six) of experimental mice, while MCAR-T and $Allo^{15}$ MCAR-NKT cells led to only partial tumor growth suppression and limited survival benefit (Figures 3B–3D). Mice treated with MCAR-T cells succumbed due to a combination of progressive tumor growth and GvHD (Figures 3B–3D). When delivered via i.v. administration, neither MCAR-T nor $Allo^{15}$ MCAR-NKT cells effectively suppressed tumor growth, resulting in early mortality, while $Allo^{15}$ MCAR-NKT cells suppressed tumor growth and prolonged survival, albeit with reduced efficacy compared to the i.p. administration route (Figures 3B–3G). These findings highlight that $Allo^{15}$ MCAR-NKT cells possess superior anti-OC activity compared to MCAR-T and $Allo^{15}$ MCAR-NKT cells, with i.p. administration providing the greatest therapeutic benefit.

In the PK/PD study, when delivered via i.p. administration, $Allo^{15}$ MCAR-NKT/FG cells mounted a remarkable expansion of over 100-fold, reaching its peak around week 8, followed by a gradual decline and a long-term persistence exceeding

130 days (Figures 3I and 3J). In the absence of IL-15 enhancement, the $Allo^{15}$ MCAR-NKT/FG cells showed a moderate 5-fold expansion, peaking around week 1, followed by a rapid decline and a significantly shortened persistence of approximately 2 weeks (Figures 3I and 3J). In contrast, conventional MCAR-T/FG cells displayed a moderate expansion and a decline in the first 2 weeks, which is likely attributed to the stimulation driven by tumor antigens,⁸⁸ followed by an extended expansion after 4 weeks associated with GvHD (Figures 3I and 3J).^{53,89,90} Importantly, $Allo^{15}$ MCAR-NKT/FG and conventional MCAR-T/FG cells demonstrated dramatically different biodistribution *in vivo*. Throughout the duration of the *in vivo* antitumor response, $Allo^{15}$ MCAR-NKT/FG cells exhibited highly focused retention in the peritoneal cavity, evidenced by over 95% bioluminescence live animal imaging (BLI) signal confined to this area (Figures 3K and 3L). In contrast, conventional MCAR-T/FG cells gradually leaked from the peritoneal cavity and infiltrated various other organs, such as the lymph nodes and lungs (Figures 3K and 3L). Notably, in the absence of OVCAR8 tumor cells, $Allo^{15}$ MCAR-NKT/FG cells exhibited significantly reduced expansion and limited *in vivo* persistence, suggesting that their robust and sustained expansion is driven by the presence of tumor antigen (Figures S4A–S4C). In addition to BLI, *in vivo* expansion and persistence of $Allo^{15}$ MCAR-NKT/FG cells were further assessed by i.p. lavage followed by flow-cytometry analysis (Figures S4D and S4E).

On the other hand, when delivered via i.v. administration, $Allo^{15}$ MCAR-NKT/FG cells exhibited a moderate expansion of about 10-fold, reaching its peak around week 3, followed by a gradual decline and a persistence exceeding 100 days (Figures 3I and 3J). In the absence of IL-15 enhancement, $Allo^{15}$ MCAR-NKT/FG cells underwent moderate expansion during the first week but rapidly diminished and eventually disappeared (Figures 3I and 3J). Notably, $Allo^{15}$ MCAR-NKT/FG cells demonstrated superior tumor-homing capabilities, progressively migrating to and localizing within the peritoneal cavity area, where the OC tumor cells are located (Figures 3M and 3N). In contrast, conventional MCAR-T/FG cells exhibited limited tumor-homing ability and mainly dispersed to various organs in experimental mice (Figures 3I, 3J, 3M, and 3N).

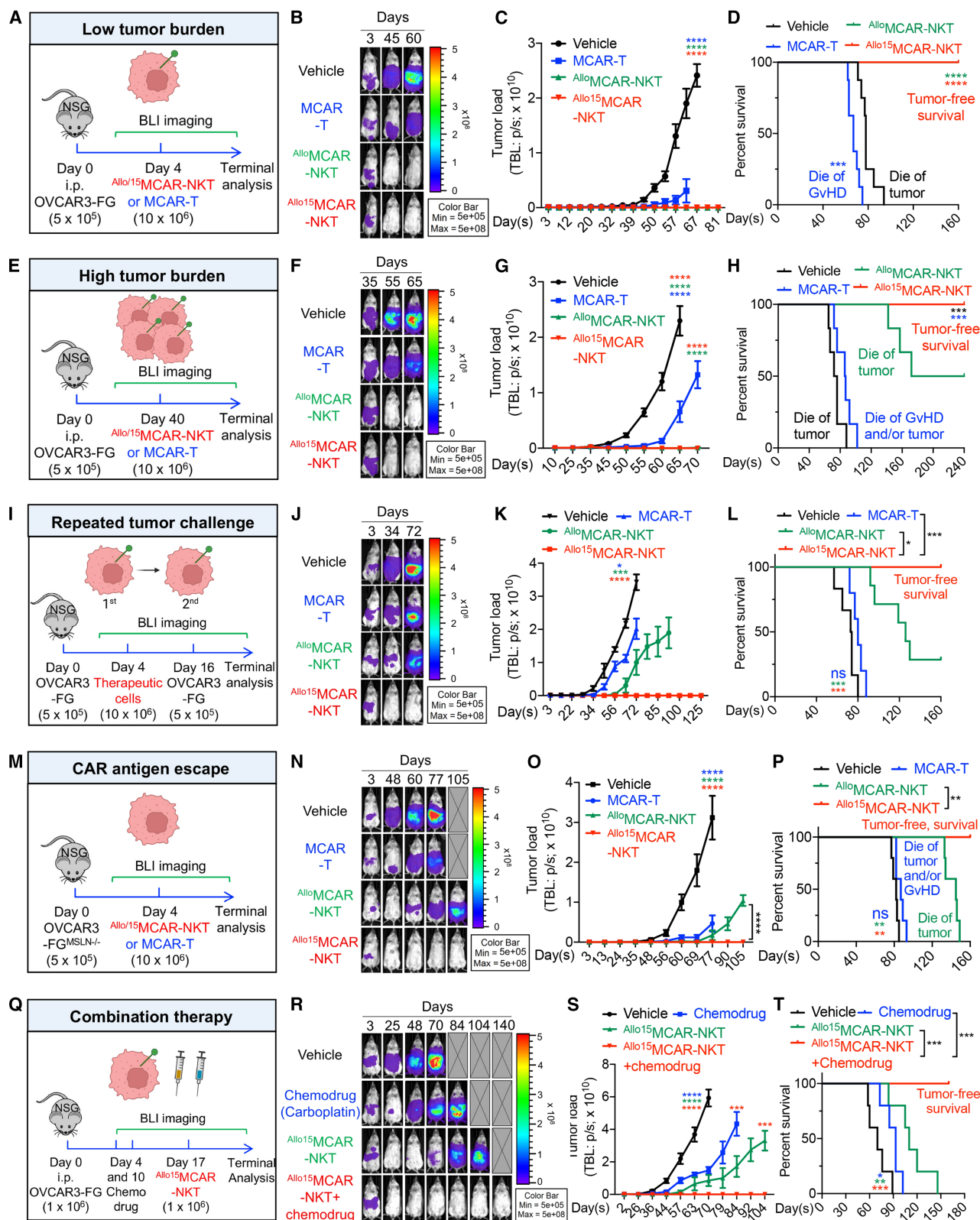
Figure 3. Allogeneic CAR-NKT cells demonstrate superior *in vivo* performance associated with focused OC tumor homing compared to conventional CAR-T cells

(A–G) Studying the *in vivo* antitumor efficacy in an OVCAR8-FG human OC xenograft NSG mouse model. Note that OVCAR8 tumor cells were labeled with the FG. Tumor-bearing experimental mice were treated with $Allo^{15}$ MCAR-NKT or conventional MCAR-T cells, or without therapeutic cells (Vehicle). (A) Experimental design. BLI, bioluminescence live animal imaging. Therapeutic cells were injected into experimental mice via intraperitoneal (i.p.) or intravenous (i.v.) administration route. (B–D) Experimental results generated using the i.p. administration route. (B) BLI images showing tumor loads in experimental mice over time. (C) Quantification of (B) ($n = 6$). (D) Survival curve ($n = 6$). (E–G) Experimental results generated using the i.v. administration route. (E) BLI images showing tumor loads in experimental mice over time. (F) Quantification of (E) ($n = 6$). (G) Survival curve ($n = 6$).

(H–N) Studying the *in vivo* PK/PD in an OVCAR8 human OC xenograft NSG mouse model. Note that therapeutic cells, but not OVCAR8 tumor cells, were labeled with the FG. (H) Experimental design. (I) BLI images showing the presence of therapeutic cells in experimental mice over time. (J) Quantification of (I) ($n = 3–5$). (K and M) Representative BLI images showing the biodistribution of therapeutic cells in the indicated experimental mice, injected via i.p. (K) or i.v. (M) administration route. (L and N) Quantification of the tissue distribution of therapeutic cells, injected via i.p. (L) or i.v. (N) administration route ($n = 3–5$). N.D., not detectable; F.D., found dead.

(O and P) Studying the homing marker pattern of $Allo^{15}$ MCAR-NKT cells related to their focused homing to OC tumor peritoneal sites. (O) FACS detection of S1PR1 and CD69 expressions on the indicated therapeutic cells collected from the peritoneal cavity of OVCAR8-FG tumor-bearing mice on day 50 ($n = 5$). (P) Schematics illustrating the focused homing to OC tumor peritoneal sites of $Allo^{15}$ MCAR-NKT cells driven by their homing marker pattern that is distinct from conventional MCAR-T cells.

Representative of three experiments. Data are presented as the mean $\pm$ SEM. ns, not significant; **** $p < 0.0001$ by Student's *t* test (L, N, and O), one-way ANOVA (C and F), or log rank (Mantel-Cox) test adjusted for multiple comparisons (D and G). See also Figures S4, S5, S8, and S9.



(legend on next page)

To investigate the mechanisms underlying the focused tumor-homing and retention capability of $Allo^{15}$ MCAR-NKT cells, we assessed their homing marker expression. Compared to conventional MCAR-T cells, $Allo^{15}$ MCAR-NKT cells expressed elevated levels of a large set of tissue-inflammatory homing markers, including CCR1, CCR3, CCR5, CXCR3, and CXCR6 (Figure S5A), which may facilitate their homing to the inflammatory tumor sites (Figures 3I, 3M, and 3N).⁹¹ Meanwhile, $Allo^{15}$ MCAR-NKT cells expressed nearly undetectable S1PR1 and markedly higher CD69 (Figures 3O and S5A). This S1PR1^{low}CD69^{high} phenotype has been associated with reduced systemic circulation and enhanced tissue retention.^{92,93} Furthermore, $Allo^{15}$ MCAR-NKT cells displayed reduced levels of lymph-node-homing markers, such as CCR7 and CD62L (Figure S5A), suggesting a reduced capacity for lymph node trafficking.⁹⁴ Importantly, this distinct homing marker profile remains stable in both *in vitro* cell cultures and *in vivo* animal studies, as confirmed by RNA-seq analyses (Figures S5A–S5C).

Collectively, these results demonstrate significant efficacy advantages of $Allo^{15}$ MCAR-NKT cells over conventional MCAR-T cells in the treatment of OC, particularly via i.p. administration. These advantages are associated with the superior tumor homing and retention of $Allo^{15}$ MCAR-NKT cells within the peritoneal cavity, where OC tumor cells are located, mediated by their distinctive homing marker expression patterns (Figures 3P and S5D).

$Allo^{15}$ MCAR-NKT cells exhibit superior potency against OC under high tumor burden, repeated tumor challenge, and tumor antigen escape compared to conventional CAR-T cells

To fully assess and compare the *in vivo* antitumor efficacy of $Allo^{15}$ MCAR-NKT and conventional MCAR-T cells, we employed a series of human OC xenograft models to simulate various disease scenarios, including low and high tumor burdens (Figures 4A–4H), repeated tumor challenges (Figures 4I–4L), tumor CAR antigen escape (Figures 4M–4P), and combination with platinum-based carboplatin treatment (Figures 4Q–4T). The i.p. administration route was used, given its significant efficacy advantages over the i.v. route (Figure 3).

In two OVCAR3-FG human OC xenograft models with low or high tumor loads, a single administration of $Allo^{15}$ MCAR-NKT

cells achieved OC tumor elimination and long-term survival of all mice in the experiment (Figures 4A–4H). In contrast, conventional MCAR-T cells exhibited limited tumor suppression and meanwhile induced GvHD, resulting in only a minimal improvement of mouse survival in both models (Figures 4A–4H, S5E, and S5F).

Remarkably, in an OVCAR3-FG human OC xenograft model with repeated tumor challenges, $Allo^{15}$ MCAR-NKT cells conferred durable protection against a second tumor challenge following initial tumor elimination whereas conventional MCAR-T cells did not, indicating a long-term persistence and robust capacity of $Allo^{15}$ MCAR-NKT cells to prevent tumor relapse (Figures 4I–4L).

In an OVCAR3-FG^{MSLN-/-} human OC xenograft model lacking CAR antigen (i.e., MSLN) expression, $Allo^{15}$ MCAR-NKT cells still achieved complete elimination of OC tumor cells and long-term survival of all experimental mice, different from the conventional MCAR-T cells (Figures 4M–4P), indicating the presence of a potent CAR-independent antitumor capacity of $Allo^{15}$ MCAR-NKT cells that may counteract tumor immune evasion.⁹⁵

In all human OC xenograft models studied, the non-IL-15-enhanced $Allo^{15}$ MCAR-NKT cells demonstrated less impressive antitumor efficacy compared to $Allo^{15}$ MCAR-NKT cells (Figures 4A–4P), confirming IL-15 as a potent augmenting factor for allogeneic MCAR-NKT cells (Figure 3).

Platinum-based agents, such as carboplatin, are the standard treatments for OC.⁸ Therefore, we evaluated the potential synergistic effects of combining $Allo^{15}$ MCAR-NKT cells with carboplatin treatment (Figure 4Q). In an OVCAR3-FG human OC xenograft combination therapy model, treatment with either $Allo^{15}$ MCAR-NKT cells or carboplatin alone resulted in partial tumor suppression and limited survival of the experimental mice. In contrast, the combination therapy achieved complete OC elimination and long-term survival in all experimental mice, suggesting a promising synergy between $Allo^{15}$ MCAR-NKT cells with carboplatin for OC treatment (Figures 4R–4T).

Taken together, across various *in vivo* human OC xenograft mouse models simulating different tumor loads, relapse, and immune evasion scenarios, $Allo^{15}$ MCAR-NKT cells demonstrated superior antitumor efficacy compared to conventional MCAR-T cells. Interestingly, combining $Allo^{15}$ MCAR-NKT cells with carboplatin treatment exhibited synergistic anti-OC efficacy,

Figure 4. Allogeneic CAR-NKT cells showcase superior *in vivo* anti-OC efficacy against high tumor burden, repeated tumor challenge, and tumor antigen escape compared to conventional CAR-T cells

Five human OC xenograft NSG mouse models were utilized. Tumor-bearing experimental mice were treated with $Allo^{15}$ MCAR-NKT or conventional MCAR-T cells, or without therapeutic cells (Vehicle).

(A–D) Studying the *in vivo* antitumor efficacy in a low-tumor-burden OVCAR3-FG human OC xenograft model. (A) Experimental design. (B) BLI images showing tumor loads in experimental mice over time. (C) Quantification of (B) ($n = 8$). (D) Survival curve ($n = 8$). GvHD, graft-versus-host disease.

(E–H) Studying the *in vivo* antitumor efficacy in a high-tumor-burden OVCAR3-FG human OC xenograft model. (E) Experimental design. (F) BLI images. (G) Quantification of (F) ($n = 6$). (H) Survival curve ($n = 6$).

(I–L) Studying the *in vivo* antitumor efficacy in a repeated-tumor-challenge OVCAR3-FG human OC xenograft model. (I) Experimental design. (J) BLI images. (K) Quantification of (J) ($n = 5–7$). (L) Survival curve ($n = 5–7$).

(M–P) Studying the *in vivo* antitumor efficacy in a CAR antigen escape OVCAR3-FG^{MSLN-/-} human OC xenograft model. (M) Experimental design. (N) BLI images. (O) Quantification of (N) ($n = 5$). (P) Survival curve ($n = 5$).

(Q–T) Studying the combination therapy of $Allo^{15}$ MCAR-NKT cells and chemodrug carboplatin in an OVCAR3-FG human OC xenograft model. (Q) Experimental design. (R) BLI images. (S) Quantification of (R) ($n = 5$). (T) Survival curve ($n = 5$).

Representative of three experiments. Data are presented as the mean $\pm$ SEM. ns, not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by one-way ANOVA (C, G, K, O, and S), or log rank (Mantel-Cox) test adjusted for multiple comparisons (D, H, L, P, and T).

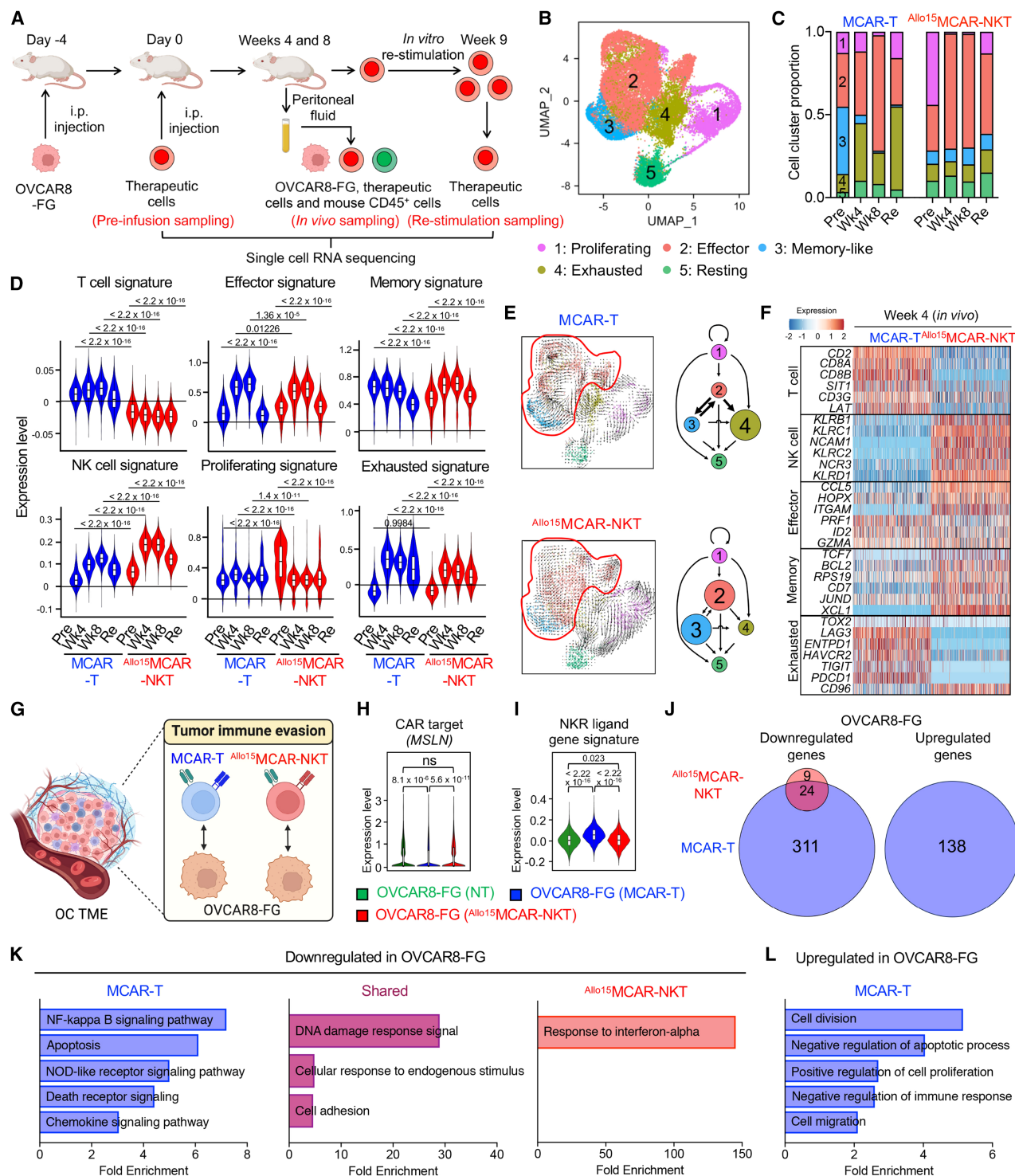


Figure 5. Allogeneic CAR-NKT cells exhibit an *in vivo* gene profile distinct from conventional CAR-T cells and counteract OC immune evasion during anti-OC response

(A–F) Studying the *in vivo* gene profile of Allo15MCAR-NKT cells in an OVCAR8-FG human OC xenograft MSG mouse model using scRNA-seq analyses. (A) Experimental design. Samples were collected at four time points: cryo-recovered prior to infusion (Pre), *in vivo* week 4 post infusion (Wk4), *in vivo* week 8 post infusion (Wk8), and 7 days after *in vitro* re-stimulation of week-8 samples (Re). Therapeutic cells were FACS sorted from collected samples and subjected to scRNA-seq analyses. (B) Combined UMAP plot showing the formation of five major cell clusters. Total cells combined from all samples are included. Each dot represents a single cell and is colored according to its cell-cluster assignment. (C) Bar graphs showing the cell-cluster proportions of the indicated samples. (D)

(legend continued on next page)

underscoring the potential of $Allo^{15}$ MCAR-NKT cells in addressing chemorefractory and recurrent OC cases.

$Allo^{15}$ MCAR-NKT cells display an *in vivo* gene profile distinct from conventional CAR-T cells during anti-OC response

To gain insights into the genomic and molecular regulations controlling the superior *in vivo* anti-OC performance of $Allo^{15}$ MCAR-NKT over conventional MCAR-T cells, we utilized an OVCAR8-FG human OC xenograft mouse model in combination with scRNA-seq analyses (Figure 5A). The two types of therapeutic cells were collected at four time points (week 0, week 4, week 8, and week 9) followed by scRNA-seq analysis (Figure 5A). Week-0 samples were collected prior to infusion, representing the starting status of the therapeutic cells. Week-4 and week-8 samples were collected from the peritoneal tumor sites of the experimental mice, representing the *in vivo* tumor-responding status of the therapeutic cells. Week-9 samples were generated by stimulating week-8 samples *in vitro* with MSLN-expressing artificial antigen-presenting cells (aAPCs) for 1 week, representing the reactivation potential of the therapeutic cells post tumor exposure (Figure 5A).

Uniform manifold approximation and projection (UMAP) analysis of combined samples revealed the formation of five major cell clusters (Figure 5B). The signature gene profiling and gene set enrichment analysis (GSEA) identified cluster 1 as proliferating cells, cluster 2 as effector cells, cluster 3 as memory-like cells, cluster 4 as exhausted cells, and cluster 5 as resting cells (Figures 5B, S6A, and S6B).^{96–103}

An immediate finding is that both types of therapeutic cells displayed dramatic gene-profile changes when comparing their week-0 pre-infusion samples with their week-4/week-8 *in vivo* samples, suggesting an *in vivo* genomic/molecular signature of a cell product that cannot be fully captured by analyzing its pre-infusion product (Figures 5C and S6C). This phenomenon has been previously reported by multiple preclinical and clinical studies.^{104–106} Interestingly, week-9 re-stimulated $Allo^{15}$ MCAR-NKT cells exhibited a potent tumor cell killing capacity comparable to their week-0 pre-infusion counterparts, in contrast to the significantly reduced tumor cell killing capability observed in the week-9 re-stimulated conventional MCAR-T cells (Figures 3G, S6D, and S6E). This disparity aligns with a better preservation of effector and memory cell clusters (clusters 2 and 3) along with a lower accumulation of exhausted cell cluster (cluster 4) when comparing week-9 re-stimulated $Allo^{15}$ MCAR-NKT to conventional MCAR-T cells (Figure 5C). These results

suggest that allogeneic MCAR-NKT cells, despite their prolonged *in vivo* exposure to OC tumors, retain significant potential for reactivation that may result in sustained therapeutic benefits.^{107,108}

Subsequently, we performed detailed analyses of the week-4 and week-8 *in vivo* samples. Compared to conventional MCAR-T cells, $Allo^{15}$ MCAR-NKT cells comprised more effector and memory cells and fewer exhausted cells (Figures 5C and 5D). In support of this notion, RNA velocity analysis revealed the accumulation of $Allo^{15}$ MCAR-NKT cells at the effector/memory stage, while MCAR-T cells showed retention at the exhausted stage (Figure 5E). Gene signature analysis also showed that compared to conventional MCAR-T cells, $Allo^{15}$ MCAR-NKT cells displayed enhanced effector and memory but diminished exhaustion gene profiles (Figures 5D and 5F), aligning with their superior *in vivo* antitumor efficacy and long-term persistence (Figures 3 and 4).

Nevertheless, a fundamental distinction exists between the $Allo^{15}$ MCAR-NKT and conventional MCAR-T cells, with $Allo^{15}$ MCAR-NKT cells exhibiting less T cell feature but significantly elevated NK cell feature, particularly in their *in vivo* week-4/-8 samples (Figures 5D and 5F). The NK cell features of $Allo^{15}$ MCAR-NKT cells were reflected by their heightened expression of NKR and robust NKR-mediated tumor cell killing (Figures S2J, S3C, and S3D). It is notable that MCAR-T cells demonstrated the acquisition of certain NK cell features *in vivo* (Figures 5D and 5F), corresponding with a prior study reporting an NK-like transition and dysfunction in conventional CAR-T cells exposed to repetitive tumor stimulation.¹⁰⁴ Conversely, in the context of the allogeneic CAR-NKT cells, the *in vivo* augmentation of NK features did not correlate with signs of exhaustion (Figures 5D and 5F).

$Allo^{15}$ MCAR-NKT cells counteract OC tumor immune evasion, outperforming conventional CAR-T cells

OC is recognized for its resistance to immunotherapy, employing multiple immune evasion strategies, including the upregulation of immune checkpoints, downregulation of tumor antigens that are recognized by therapeutic cells, and the production of factors that polarize TAMs and MDSCs toward an immunosuppressive state.^{22,109,110} Our previous efficacy study using an OVCAR3-FG^{MSLN-/-} human OC xenograft mouse model demonstrated the efficacy advantages of $Allo^{15}$ MCAR-NKT cells over conventional MCAR-T cells under a CAR antigen escape scenario (Figures 4M–4P). Here, we conducted scRNA-seq

Violin plots showing the expression distribution of signature genes in the indicated therapeutic cell samples. (E) RNA-velocity projected on UMAP plots. Arrows represent the estimates of local average velocity, showing the path (indicated by arrow orientation) and pace (indicated by arrow length) of cell transition. Diagrams summarizing the UMAP cell clustering and RNA-velocity analysis are also presented. The cell-cluster proportions are represented by the sizes of circles, while the transitions between cell clusters are symbolized using connecting arrows. (F) Heatmap showing the expression of representative signature genes. Each column indicates an individual cell. Each row indicates an individual gene.

(G–L) Studying the *in vivo* gene profile of OC tumor cells in an OVCAR8-FG human OC xenograft NSG mouse model using scRNA-seq analyses. OVCAR8-FG cells were collected from the peritoneal cavity of experimental mice on week 4. (G) Diagram showing the scRNA-seq analyses of tumor immune evasion. (H) Violin plots showing the *MSLN* expression levels in the indicated samples. (I) Violin plots showing the NKR ligand expression levels in the indicated samples. (J) Venn diagram showing the numbers of genes downregulated or upregulated in OVCAR8-FG cells under $Allo^{15}$ MCAR-NKT or MCAR-T cell treatment. (K and L) Pathway analyses of genes downregulated (K) or upregulated (L) in OVCAR8-FG cells under $Allo^{15}$ MCAR-NKT or MCAR-T cell treatment.

Representative of one experiment. Cells isolated from 5–10 mice of each experimental group were combined for analyses. *p* values of violin plots were determined by Kruskal-Wallis test for the overall comparison and Dunn's test for post hoc pairwise comparisons between groups. In the violin plots, box-and-whisker plots exhibit the minimum, lower quartile, median, upper quartile, and maximum expression levels of each type of cells. See also Figure S6.

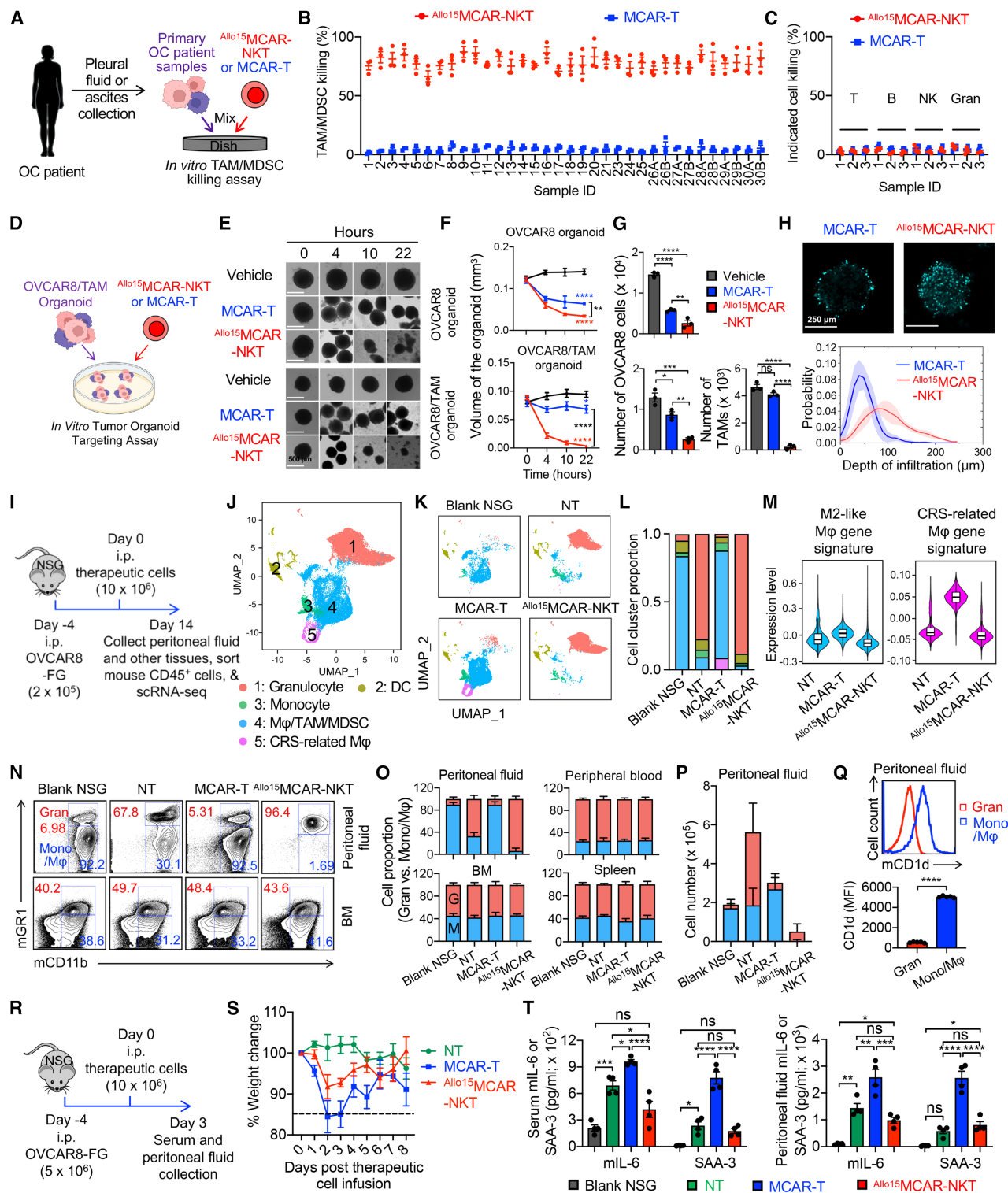


Figure 6. Allogeneic CAR-NKT cells dramatically alter OC TME and minimize CRS toxicity, outperforming conventional CAR-T cells
(A–C) Studying Allo15MCAR-NKT cells' targeting of OC TME using primary OC patient samples. (A) Experimental design. (B) TAM/MDSC killing data at 24 h (n = 3). (C) Killing data of the indicated OC TME cell components at 24 h (n = 3).
(D–H) Studying Allo15MCAR-NKT cells' targeting of OC TME using an OVCAR8/TAM organoid culture. Vehicle indicates no addition of therapeutic cells. (D) Experimental design. (E) Light microscopy images showing the organoid size and morphology over time. Scale bar, 500 μm. (F) Quantification of (E) (n = 3). (G)

(legend continued on next page)

and flow-cytometry analyses of the OVCAR8-FG tumor cells collected from the same *in vivo* experiments (Figures 5A and 5G) and confirmed the reduced CAR antigen gene expression in OC tumors treated with conventional MCAR-T cells, while such a reduction was not observed in tumors treated with Allo^{15} MCAR-NKT cells (Figures 5H and S6F–S6H), which may be attributed to their CAR/NKRs multi-targeting mechanisms (Figures 2K, S3C, and S3D). Notably, OC tumor cells treated with Allo^{15} MCAR-NKT cells retained expression of both CAR antigen and NKR ligands, ensuring sustained susceptibility to the “full-force” CAR/NKR multi-targeting by Allo^{15} MCAR-NKT cells (Figures 5H and 5I). Interestingly, OC tumor cells treated with conventional MCAR-T cells, despite losing CAR antigen, maintained or even upregulated broad NKR ligands (Figures 5H and 5I), consistent with the universal expression of NKR ligands (over 30) on primary and even recurrent OC tumor cells, underscoring the importance of leveraging NK function for effective OC tumor targeting (Figures 1G–1J).

Further *in vivo* gene profiling of OC tumor cells revealed a dramatic change under MCAR-T cell treatment (335 genes downregulated and 138 genes upregulated) and a minimal change under Allo^{15} MCAR-NKT cell treatment (33 genes downregulated and no genes upregulated) (Figure 5J). For the downregulated genes, 24 genes are shared by OC tumor cells under both treatments, involving pathways regulating OC tumor cell DNA damage, response to endogenous stimulus, and cell adhesion; 311 genes are unique to conventional MCAR-T cell treatment, involving pathways regulating OC tumor cell apoptosis, death receptor signaling, chemokine signaling, nuclear factor κ B signaling, and NOD-like receptor signaling; only nine genes are unique to Allo^{15} MCAR-NKT cell treatment, involving pathways primarily regulating IFN- α response (Figures 5J–5L).^{111–115} For the upregulated genes, all the 138 genes are unique to conventional MCAR-T cell treatment, involving pathways regulating OC tumor cell division, proliferation, and migration, as well as negative regulation of apoptosis and immune responses (Figures 5J–5L).^{116–119}

Together, these findings uncover a previously underappreciated CAR-T cell-triggered OC tumor immune evasion that may undermine conventional CAR-T cell therapy for OC.¹⁰⁹ Meanwhile, the remarkable ability of Allo^{15} MCAR-NKT cells to coun-

teract OC tumor evasion positions them as a promising therapeutic candidate, particularly for refractory and recurrent OC.

Allo^{15} MCAR-NKT cells effectively alter OC TME, surpassing conventional CAR-T cells

OC is notoriously known for its immunosuppressive TME, featured by a high abundance of TAMs and MDSCs present in the peritoneal cavity (Figures 1L–1Q).²¹ Given the high expression of CD1d on TAMs and MDSCs (Figures 1L–1Q), these cells are susceptible to NKT TCR-CD1d-mediated killing by Allo^{15} MCAR-NKT cells. We then investigated the interplay between Allo^{15} MCAR-NKT cells and the OC TME using primary OC patient samples (Figure 6A), OC organoids (Figure 6D), and an *in vivo* OC xenograft mouse model (Figure 6I).

In the first study, we co-cultured Allo^{15} MCAR-NKT cells with a total of 34 primary OC patient samples (Figure 6A). In contrast to conventional MCAR-T cells, Allo^{15} MCAR-NKT cells effectively and selectively depleted TAMs and MDSCs, aligning with the high CD1d expression on these cells (Figures 1P, 1Q, and 6B). Importantly, Allo^{15} MCAR-NKT cells spared other immune-cell populations expressing no or low-level CD1d, including granulocytes, T cells, B cells, and NK cells (Figures 1P, 1Q, and 6C). Both Allo^{15} MCAR-NKT and Allo^{15} MCAR-NKT cells exhibited comparable capacities to deplete TAMs and MDSCs, with cytotoxicity significantly reduced by CD1d blockade, indicating a TCR/CD1d-dependent mechanism (Figures S7A and S7B). In contrast, conventional CAR-T cells lack this ability, although IL-15-enhanced CAR-T cells have shown cytotoxicity against tumor-associated myeloid cells, likely mediated by IL-15 transpresentation.¹²⁰

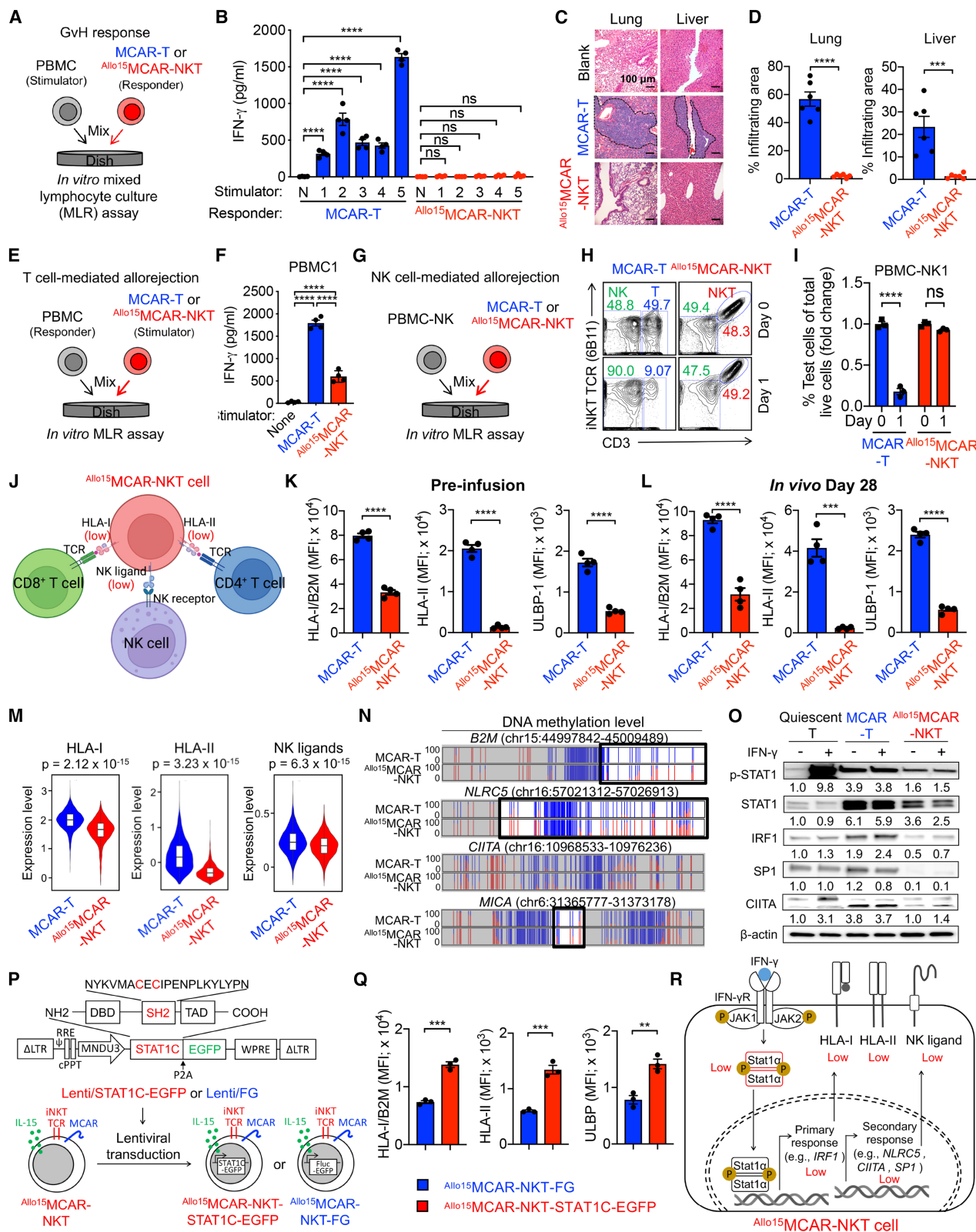
In the second study, we established two types of human OC organoids by 3D culturing the OVCAR8 tumor cells (denoted as OVCAR8 organoids) or a mixture of OVCAR8 and human monocyte-derived M2-polarized macrophages mimicking TAMs (denoted as OVCAR8/TAM organoids) (Figure 6D). Both MCAR-T and Allo^{15} MCAR-NKT cells effectively targeted the OVCAR8 organoids, with Allo^{15} MCAR-NKT cells exhibiting slightly higher killing efficacy (Figures 6E–6G). However, MCAR-T cells showed significantly reduced cytotoxicity against the OVCAR8/TAM organoids, likely due to TAM-induced immunosuppression, while Allo^{15} MCAR-NKT cells maintained high cytotoxicity against the OVCAR8/TAM organoids by targeting both tumor cells and

FACS quantifications of OVCAR8 and TAM cell numbers at 24 h ($n = 3$). (H) Confocal microscopy images (upper) and quantifications (lower) showing the distribution and infiltration depth of the indicated therapeutic cells in the OVCAR8/TAM organoids. Scale bar, 250 μ m.

(I–Q) Studying Allo^{15} MCAR-NKT cells' targeting of OC TME using an OVCAR8 human OC xenograft NSG mouse model. Conventional MCAR-T cells were included as a control. Blank NSG indicates the samples collected from tumor-free NSG mice, and NT indicates the samples collected from tumor-bearing NSG mice receiving no therapeutic cell treatment. (I) Experimental design. (J) Combined UMAP plot showing the formation of five major cell clusters. DC, dendritic cell; CRS, cytokine release syndrome. M ϕ , macrophage. (K) Individual UMAP plots showing cell clusters of the indicated peritoneal fluid samples. (L) Bar graphs showing the cell-cluster proportions of the indicated samples. (M) Violin plots showing the expression levels of M2-like and CRS-related M ϕ genes in the indicated samples. (N) FACS plots showing the identification of mouse granulocytes (Gran) and monocytes/macrophages (Mono/M ϕ) from the indicated samples. (O) Quantification of (N) ($n = 5$ –6). (P) Bar graphs showing the cell counts of Gran and Mono/M ϕ in the indicated peritoneal fluid samples ($n = 5$ –6). (Q) FACS analyses of CD1d expression on mouse Gran and Mono/M ϕ in the peritoneal fluid samples ($n = 5$). Representative data collected from the NT experimental mice are presented.

(R–T) Studying the CRS toxicity induced by Allo^{15} MCAR-NKT cells using an OVCAR8-FG human OC xenograft NSG mouse model. Conventional MCAR-T cells were included as a control. (R) Experimental design. (S) Body-weight measurements of experimental mice over time ($n = 4$). (T) ELISA analyses of mouse IL-6 and SAA3 in the serum and peritoneal fluid samples collected from the indicated experimental mice on day 3 ($n = 4$). SAA-3, serum amyloid A-3.

Representative of one (I–M) and three (A–H and N–T) experiments. Data are presented as the mean $\pm$ SEM. ns, not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by Student's *t* test (Q) or one-way ANOVA (F, G, P, and T). *p* values of violin plots were determined by Wilcoxon rank-sum test (M). See also Figure S7.



(legend on next page)

TAMs (Figures 6E–6G). Immunofluorescence microscopy revealed that MCAR-T cells predominantly accumulated at the outer rim of the OVCAR8/TAM organoids, reflecting the limited solid tumor infiltration of conventional CAR-T cells due to immunosuppressive TME barriers (Figure 6H).^{121–123} In sharp contrast, ^{Allo15}MCAR-NKT cells exhibited extensive infiltration into the OVCAR8/TAM organoids, highlighting their potential to overcome this key barrier in the combat with solid tumors (Figure 6H).

In the third study, ^{Allo15}MCAR-NKT and MCAR-T cells were administered into NSG mice bearing human OC xenografts, followed by analysis of tumor sites (peritoneal fluids) and other tissues (Figure 6I). Notably, although this human OC xenograft mouse model lacks human myeloid cells, it does contain mouse myeloid cells to constitute an OC TME; these mouse myeloid cells express mouse CD1d, an evolutionarily conserved molecule that can be recognized by the human NKT TCR, allowing the study of TME targeting by ^{Allo15}MCAR-NKT cells in this model.¹²⁴

scRNA-seq analysis of peritoneal fluid samples revealed the formation of five major mouse cell clusters, including granulocytes, dendritic cells (DCs), monocytes, macrophages/TAMs/MDSCs, and CRS-related macrophages (Figures 6J and S7C). The engraftment of human OC tumor cells in NSG mice induced a peritoneal TME comprising tumor-associated granulocytes,^{125,126} TAMs, MDSCs, and a small population of DCs (Figure 6K). Conventional MCAR-T and ^{Allo15}MCAR-NKT cell treatments led to markedly different changes in TME cells: MCAR-T cell treatment induced a substantially increased numbers of TAMs/MDSCs with exacerbated M2-like immunosuppressive features as well as macrophages with heightened CRS features, while ^{Allo15}MCAR-NKT cell treatment significantly reduced the numbers of these TME cells associated with decreased M2-like and CRS features, likely attributed to their high expression of CD1d (Figures 6K–6Q and S7D–S7G).^{127–134} Interestingly, despite their high expression of CD1d,⁴⁸ DCs remained intact under ^{Allo15}MCAR-NKT cell treatment, probably due to a DC intrinsic mechanism protecting them from cytotoxic

T cell-mediated killing involving granzyme inhibitors (e.g., *Serpinb9*) (Figure S7H).¹³⁵ Meanwhile, granulocytes also remained intact under ^{Allo15}MCAR-NKT cell treatment, correlating with their low or negative expression of CD1d (Figure 6Q).

Notably, ^{Allo15}MCAR-NKT cell treatment did not result in the depletion of monocytes/macrophages or granulocytes from other tissues, including peripheral blood, spleen, and bone marrow (Figures 6K–6Q). This selective depletion of TME-associated immunosuppressive and CRS-related myeloid cells, while preserving normal tissue-resident and circulating monocytes/macrophages/granulocytes, may be attributed to the preferential localization of ^{Allo15}MCAR-NKT cells to tumor sites within the peritoneal cavity (Figure 3), along with the limited CD1d expression on quiescent monocytes/macrophages/granulocytes.¹³⁶

Taken together, these results suggest that conventional MCAR-T cells are vulnerable to immunosuppression within the OC TME, a condition that could be further exacerbated following MCAR-T treatment, potentially fostering a pro-tumorigenic niche and promoting OC immune evasion.^{21,32,33,137,138} On the other hand, ^{Allo15}MCAR-NKT cells have the unique capacity to significantly alter the OC TME by eliminating immunosuppressive TME cells, overcoming TME-induced OC resistance and immune evasion.

^{Allo15}MCAR-NKT cells minimize CRS toxicity compared to conventional CAR-T cells during anti-OC response

CRS toxicity is a significant side effect associated with CAR-T cell therapy in cancers, particularly OC, as peritoneal CRS-related macrophages have been reported to exacerbate these effects.^{32,33,139} Our scRNA-seq study identified a distinct CRS-related macrophage population induced by MCAR-T cell treatment in the peritoneal fluid of OC-engrafted mice, whereas this population was largely absent in mice treated with ^{Allo15}MCAR-NKT cells (Figures 6J–6M). Indeed, ^{Allo15}MCAR-NKT cell treatment resulted in reduced body-weight loss of the experimental mice and lower levels of CRS-related biomarkers (i.e., mouse IL-6 and SAA-3) in the mouse serum and peritoneal fluid

Figure 7. Allogeneic CAR-NKT cells showcase low GvHD risk and high resistance to alloreactivity compared to conventional CAR-T cells

(A and B) Studying graft-versus-host (GvH) response of ^{Allo15}MCAR-NKT cells using a classical *in vitro* mixed lymphocyte reaction (MLR) assay. (A) Experimental design. (B) ELISA Quantification of IFN- γ production on day 4 ($n = 4$). N, no addition of PBMC stimulator.

(C and D) Studying the GvHD risk of ^{Allo15}MCAR-NKT cells using an OVCAR3-FG human OC xenograft NSG mouse model. Experimental design is shown in Figure 4A. Images of H&E-stained tissue sections are presented. Tissues were collected from experimental mice on day 70. Scale bar, 100 μ m. (D) Quantification of (C) ($n = 6$).

(E and F) Studying the T cell-mediated alloreactivity against ^{Allo15}MCAR-NKT cells using an *in vitro* MLR assay. (E) Experimental design. (F) ELISA analyses of IFN- γ production on day 4 ($n = 4$).

(G–I) Studying the NK cell-mediated alloreactivity against ^{Allo15}MCAR-NKT cells using an *in vitro* MLR assay. (G) Experimental design. (H) FACS quantification of the indicated live cells on day 0 and day 1. (I) Quantification of (H) ($n = 3$).

(J–R) Studying the molecular mechanisms underlying the alloreactivity resistance of ^{Allo15}MCAR-NKT cells. (J) Illustration depicting the “hypoinmunogenicity” working model, characterized by the low expression of surface HLA-I/II and NK ligands on ^{Allo15}MCAR-NKT cells. (K and L) FACS measurements of surface HLA-I/II and ULBP-1 on ^{Allo15}MCAR-NKT and MCAR-T cells prior to infusion (K; $n = 4$) and post-*in vivo* anti-OC response in a human OC xenograft NSG mouse model as described in Figure 4A (L; $n = 4$). (M) scRNA-seq analysis of ^{Allo15}MCAR-NKT and MCAR-T cells collected from human OC xenograft NSG mice on day 28 as described in Figure 5A. (N) Methylation analysis of ^{Allo15}MCAR-NKT and MCAR-T cell product. Vertical lines denote individual CpG sites, wherein red signifies methylation and blue signifies unmethylation. Differentially methylated regions are framed with black boxes. (O) Western blot analysis of key molecules involved in the IFN- γ signaling pathway in ^{Allo15}MCAR-NKT cells. Conventional MCAR-T and healthy donor PBMC-derived quiescent conventional T (quiescent T) cells were included as controls. (P and Q) Rescue experiment by overexpressing a constitutively active STAT1 (STAT1C) in ^{Allo15}MCAR-NKT cells. (P) Experimental design. (Q) FACS measurements of surface HLA-I/II and ULBP-1 on the indicated cells ($n = 4$). (R) Schematics illustrating a desensitized IFN- γ signaling network within an ^{Allo15}MCAR-NKT cell, which contributes to its distinctive “hypoinmunogenicity” phenotype.

Representative of one (M and N) or three (A–L and O–R) experiments. Data are presented as the mean $\pm$ SEM. ns, not significant; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by Student's *t* test (D, I, K, L, and Q) or one-way ANOVA (B and F). See also Figures S8 and S9.

(Figures 6R–6T).^{32,33,139} The underlying mechanisms may be 2-fold: compared to conventional MCAR-T cells, ^{Allo15}MCAR-NKT cells produced lower levels of inflammatory cytokines (e.g., granulocyte-macrophage colony-stimulating factor [GM-CSF], IL-2, and IL-3) associated with CRS triggering (Figure S7I) and selectively depleted established CRS-related macrophages through NKT TCR-mediated CD1d recognition (Figures 6J and 6K).^{32,33,139}

^{Allo}MCAR-NKT cells do not induce GvHD and resist allorejection during anti-OC response

In allogeneic cell therapy, key safety and efficacy concerns include GvHD risk and allorejection. GvHD arises when allogeneic cells attack host tissues, causing inflammation and damage.^{140–144} Conversely, allorejection mediated by host T and NK cells can reduce therapeutic efficacy.^{145,146} We thus assessed the GvHD risk and allorejection potential of ^{Allo15}MCAR-NKT and conventional MCAR-T cells during the anti-OC response.

Allogeneic CAR-NKT cells, leveraging NKT TCR recognition of the nonpolymorphic CD1d molecule, are not expected to induce GvHD.^{147,148} This characteristic was assessed using a classical *in vitro* mixed lymphocyte reaction (MLR) assay (Figure 7A) and an *in vivo* OC xenograft model (Figures 4A, S5E, and S5F). In the *in vitro* MLR assay, ^{Allo15}MCAR-NKT cells were stimulated with irradiated PBMCs from a substantial collection of mismatched healthy donors (more than 15 donors) (Figures 7A and 7B). Minimal IFN- γ productions were detected, in stark contrast to conventional MCAR-T cells that exhibited vigorous IFN- γ production (Figure 7B). In the *in vivo* human OC xenograft NSG mouse model, conventional MCAR-T cells induced severe GvHD, leading to mortality of experimental animals and massive immune-cell infiltration in vital organs such as the liver and lung (Figures 7C and 7D). In sharp contrast, ^{Allo15}MCAR-NKT cell treatment resulted in GvHD-free, long-term animal survival with no detectable immune-cell infiltration in vital organs (Figures 7C, 7D, S5E, and S5F).

We subsequently evaluated the allorejection of ^{Allo15}MCAR-NKT cells, primarily mediated by host CD4 T, CD8 T, and NK cells. Host CD4 and CD8 T cells are acknowledged for rejecting allogeneic cells by recognizing mismatched HLA-II and HLA-I molecules, respectively.^{53,149} Additionally, host NK cell-mediated allorejection is controlled by a double-trigger mechanism: (1) “missing self,” denoting the absence of matching HLA-I molecules on allogeneic cells, which induces the release of inhibitory signals mediated by killer cell immunoglobulin-like receptors (KIRs); and (2) “stress signals,” which involves the upregulation of stress molecules on allogeneic cells to activate NKRs.^{150–152}

Two established *in vitro* MLR assays were performed to assess the allorejection susceptibility of ^{Allo15}MCAR-NKT cells (Figures 7E and 7G). The first MLR assay was designed to study the T cell-mediated allorejection, wherein irradiated ^{Allo15}MCAR-NKT cells were co-cultured with donor-mismatched PBMCs and the IFN- γ production by donor-mismatched PBMCs was measured (Figure 7E). In comparison to MCAR-T cells, ^{Allo15}MCAR-NKT cells induced significantly lower level of IFN- γ (Figure 7F). The second MLR assay was designed to study the NK cell-mediated allorejection, wherein ^{Allo15}MCAR-NKT cells

were co-cultured with donor-mismatched PBMC-NK cells and the viable ^{Allo15}MCAR-NKT cells were quantified (Figure 7G). In comparison to conventional MCAR-T cells, ^{Allo15}MCAR-NKT cells exhibited significantly enhanced survival (Figures 7H and 7I).

To delve into the mechanisms rendering ^{Allo15}MCAR-NKT cell resistance to allorejection, we profiled their cell-surface molecules associated with allorejection.⁵³ Compared to conventional MCAR-T cells, ^{Allo15}MCAR-NKT cells expressed significantly lower levels of surface HLA-I, HLA-II, and NK ligands in both *in vitro* cell cultures and *in vivo* anti-OC studies (Figures 7J–7L). Bulk RNA-seq and scRNA-seq analyses unveiled a sustained downregulation of genes encoding the HLA-I/II molecules and NK ligands (Figures 7M and S2E), signifying an intrinsic and stable “hypoimmunogenic” phenotype of ^{Allo15}MCAR-NKT cells regulated at the gene-transcription level. Subsequently, we conducted further studies exploring epigenetic regulations and signaling networks governing the transcription of relevant genes.

Chromatin remodeling processes, such as DNA methylation, have been implicated in the regulation of HLA and NK ligand gene transcription.^{153,154} Genome-wide methylation sequencing (Methyl-seq) analysis of ^{Allo15}MCAR-NKT cells uncovered the hypermethylation in the promoter regions of many HLA and NK ligand genes (Figure 7N), implying that epigenetic regulation may play a role in shaping the “hypoimmunogenic” phenotype of the ^{Allo15}MCAR-NKT cells.

In addition to epigenetic regulation, signaling networks play a crucial role in governing promoter activities and subsequently influencing gene transcription. We focused on the IFN- γ -JAK-STAT1 signaling pathway, one of the best-characterized pathways that regulate the expression of HLA and NK ligand genes.^{155,156} Interestingly, compared to conventional PBMC-T and MCAR-T cells, ^{Allo15}MCAR-NKT cells demonstrated desensitized IFN- γ -JAK-STAT1 signaling, characterized by low STAT1 phosphorylation both at basal levels and following IFN- γ stimulation (Figure 7O). Correspondingly, transcription factors encoded by the IFN- γ primary responsive genes (e.g., *IRF1*) and the secondary responsive genes (e.g., *CIITA* and *SP-1*) were also maintained at low levels (Figure 7O). Overexpression of a constitutively active STAT1 molecule¹⁵⁷ partially reversed the “hypoimmunogenic” phenotype of ^{Allo15}MCAR-NKT cells, as evidenced by the upregulation of surface HLA-I/II and NK ligands (Figures 7P and 7Q).

Together, these findings reveal an intriguing “hypoimmunogenic” phenotype intrinsic to allogeneic MCAR-NKT cells, which remains stable throughout their *in vivo* anti-OC responses, highlighting their off-the-shelf potential for OC treatment (Figure 7R).

^{Allo15}MCAR-NKT cells exhibit a superior safety profile compared to IL-15-enhanced conventional ¹⁵MCAR-T cells

IL-15 has been incorporated into both autologous CAR-NKT and CAR-T cell therapies in clinical trials, where it has demonstrated enhanced antitumor efficacy across both platforms.^{59,158} However, IL-15 has also been associated with increased toxicity in patients, particularly in the context of GPC3-targeting CAR-T cell therapy for hepatocellular carcinoma (HCC), where it contributed to severe CRS toxicity.^{158,159} To directly compare

efficacy and safety, we evaluated Allo15 MCAR-NKT cells alongside 15 MCAR-T cells (Figures S8A–S8D).

In an *in vitro* tumor cell killing assay, both Allo15 MCAR-NKT and 15 MCAR-T cells exhibited robust cytotoxic activity (Figures S8E and S8F). Notably, Allo15 MCAR-NKT cells outperformed 15 MCAR-T cells, particularly against MSLN-negative OC tumor cells, likely due to their enhanced cytotoxicity and multiple tumor-targeting mechanisms (Figures S8E and S8F).

We next assessed the *in vivo* efficacy and safety of Allo15 MCAR-NKT and 15 MCAR-T cells using an OVCAR8-FG human OC xenograft mouse model (Figure S8G). Both therapeutic cell types effectively suppressed and eliminated tumor growth, confirming that IL-15 enhances the antitumor activity of CAR-T cells (Figures 3A–3D and S8H–S8J). However, mice treated with 15 MCAR-T cells developed severe toxicity, including marked GvHD-like symptoms, significant body-weight loss, and massive T cell expansion in the peripheral blood within 30 days, ultimately resulting in mortality (Figures S8J–S8N). These findings align with clinical observations of IL-15-driven toxicity in GPC3-CAR-T-treated HCC patients.¹⁵⁸ In contrast, treatment with Allo15 MCAR-NKT cells led to complete tumor eradication and long-term survival without observable toxicity, underscoring their superior safety and therapeutic potential over IL-15-enhanced CAR-T cells in OC treatment (Figures S8J–S8N).

Allo15 MCAR-NKT cells exhibit unique features distinct from PBMC-derived CAR-NKT cells

Lastly, we conducted a side-by-side comparison of Allo15 MCAR-NKT cells and PBMC-derived IL-15-enhanced MCAR-NKT (PBMC15 MCAR-NKT) cells (Figures S9A–S9D). Our study revealed several key differences between these two MCAR-NKT cell types. Allo15 MCAR-NKT cells expressed significantly higher levels of NKR (e.g., NKG2D, DNAM-1, and NKp44), as validated by flow-cytometry and scRNA-seq analyses (Figure S9E). Moreover, Allo15 MCAR-NKT cells demonstrated superior cytotoxicity against MSLN[−] OC tumor cell lines and primary OC patient-derived tumor cells, highlighting their multiple tumor-targeting mechanisms (Figures S9F–S9I). Additionally, Allo15 MCAR-NKT cells differed from PBMC15 MCAR-NKT cells in the expression of homing markers (e.g., S1PR1 and CD69) (Figure S9J). Furthermore, the “hypoimmunogenic” phenotype of Allo15 MCAR-NKT cells is distinct, as PBMC15 MCAR-NKT cells exhibited higher levels of surface HLA-I, HLA-II, and NK ligands, leading to strong alloreactivity mediated by host T and NK cells (Figures S9K–S9O). Collectively, these distinctive features position allogeneic HSPC-engineered MCAR-NKT cells as a unique cell type, presenting a promising off-the-shelf therapeutic candidate for treating OC with high efficacy and safety.

DISCUSSION

Currently, clinical investigations are actively exploring conventional CAR-T cell-based therapy in the treatment of OC, targeting antigens such as MSLN (NCT01583686 and NCT02159716), MUC16 (NCT02498912), and CLDN6 (NCT04503278).^{10,12,17,18,31,160} These clinical studies have demonstrated the feasibility of conventional CAR-T cell products, showing engraftment and tumor

trafficking but limited antitumor efficacy.^{10,17,18} The limited efficacy of conventional CAR-T cell therapy in OC may be attributed to several factors, including the inherent extreme heterogeneity of OC tumor cells and their variable antigen expression,²⁰ the presence of therapy-resistant and immuno-evasive CSCs,^{21–23} and the existence of a hostile TME characterized by an exceedingly high numbers of immunosuppressive cells, such as TAMs and MDSCs, in the peritoneal cavity.^{24–26} Furthermore, conventional CAR-T cell treatment can induce adverse effects, such as CRS toxicity, which may be exacerbated by interactions between CAR-T cells and the abundant macrophage population in the peritoneal cavity during the treatment of OC.^{32,33} Here, we present the evaluation of an alternative cell platform, HSPC-engineered allogeneic CAR-NKT cells, which display distinctive attributes that enable them to overcome OC resistance and evasion encountered by conventional CAR-T cells, leading to superior anti-OC efficacy and prominent safety.

The first challenge encountered by conventional CAR-T cell therapy for OC is the extensive heterogeneity of OC tumor cells, rendering them resistant to a single CAR targeting strategy.²⁰ To address this limitation, CAR-T cells have been engineered with dual-specific or tandem CARs to simultaneously target two antigens; however, the effectiveness of this two-antigen targeting strategy against solid tumors like those in OC remains to be demonstrated.^{161,162} Our analysis of primary OC tumor samples revealed a diverse repertoire of NKR ligands that are universally expressed on OC tumor cells, including those from platinum-resistant patients (Figures 1G–1K). In contrast to CAR-T cells, which target OC tumor cells solely by recognizing the single CAR antigen, allogeneic CAR-NKT cells expressed a broad array of NKRs, allowing them to target OC tumor cells through recognizing either the CAR antigen or/and the diverse NKR ligands (Figures 2E–2K, S2E, and S2J). The CAR/NKRs multi-targeting mechanism enables allogeneic MCAR-NKT cells to overcome OC resistance associated with CAR antigen heterogeneity, as demonstrated by *in vitro* MOA studies and *in vivo* human OC xenograft models that mimic CAR antigen loss in OC tumor cells (Figures 2E–2K, 4M–4P, S2E, S2J, S3C, and S3D).

Another major challenge faced by CAR-T cell therapy for solid tumors is the limited ability of therapeutic cells to effectively home to and infiltrate tumor sites.^{121,163} In the case of OC, which primarily manifests as a peritoneal disease, successful cell therapy requires therapeutic cells to effectively traffic to and be retained at peritoneal tumor sites.^{160,164} Over the past decade, alongside the classical i.v. administration, the i.p. administration of therapeutic agents via a catheter or port has emerged as a more targeted approach for the treatment of OC.^{7,86,87} We therefore evaluated both administration routes and found that the i.p. route outperformed the i.v. route in anti-OC efficacy for both MCAR-T and allogeneic MCAR-NKT cells, with allogeneic MCAR-NKT cells showing superior efficacy compared to MCAR-T cells (Figures 3A–3G). Following i.p. administration, conventional MCAR-T cells gradually leaked from the peritoneal cavity and infiltrated other organs, whereas allogeneic MCAR-NKT cells demonstrated highly focused retention within the peritoneal tumor sites, likely associated with their distinct homing marker expression pattern (e.g., S1PR1^{low}CD69^{high}) that reduces blood recirculation and promotes tumor retention

(Figures 3H–3P and S5A–S5D). The specific localization of allogeneic MCAR-NKT cells at peritoneal sites, particularly when MSLN is chosen as the CAR antigen, offers another advantage of avoiding the “on-target, off-tumor” lung toxicity reported in a recent clinical trial of conventional MCAR-T cell therapy for solid tumors.¹⁶⁵

A major safety concern of conventional CAR-T cell therapy is CRS, which is particularly pronounced in OC due to the abundance of peritoneal macrophages that can significantly amplify CRS toxicity (Figures 1L–1Q).^{32,33} Current strategies to mitigate CAR-T cell-induced CRS include the use of glucocorticoids that suppress inflammatory reactions, tocilizumab that blocks the IL-6 receptor, and anakinra that blocks the IL-1 receptor.¹⁶⁶ However, these interventions may dampen the CAR-T cell immune response, and patients may experience resistance and rebound effects following treatment.¹⁶⁶ Allogeneic MCAR-NKT cells present a promising alternative, as these cells have the potential to minimize CRS toxicity without the need for additional immunosuppressive agents (Figures 6R–6T). The low CRS risk of allogeneic MCAR-NKT cells arises from their NK-like characteristics (e.g., lower production of CRS inducers such as GM-CSF, IL-2, and IL-3) (Figure S7I) as well as their capacity to deplete CRS-related macrophages through CD1d recognition (Figures 6K–6Q).^{32,33,139} These capacities ensure a more favorable safety profile while maintaining high anti-OC efficacy for allogeneic MCAR-NKT cells.

Importantly, beyond their unique potential to overcome OC resistance and evasion faced by conventional MCAR-T cells, HSPC-engineered allogeneic MCAR-NKT cells also offer the opportunity for off-the-shelf application. These cells do not risk inducing GvHD due to their invariant TCR and resist host T and NK cell-mediated alloreactivity due to their intrinsic and stable “hypoinmunogenic” signature regulated by epigenetic and signaling network mechanisms (Figure 7).^{48,167} Collectively, allogeneic MCAR-NKT cells provide superior efficacy, safety, and off-the-shelf potential compared to conventional CAR-T cells for OC treatment.

Limitations of the study

Despite its promise, the reported off-the-shelf CAR-NKT cell therapy for OC still encounters certain limitations that require further refinement. The potential safety concerns related to “on-target, off-tumor” toxicity of MSLN CAR target could be addressed by switching to other OC CAR targets (e.g., CLDN6 and MUC16), which should not encounter manufacturing difficulties thanks to the versatility and robustness of our clinically guided allogeneic CAR-NKT cell-culture method (Figures 1F–1H, and 2A).^{17,18} Additionally, besides IL-15, the incorporation of genes encoding other immune-enhancing molecules (e.g., IL-7, IL-12, IL-18, and IL-21), along with immunosuppression-resistant factors (e.g., dominant-negative transforming growth factor β receptor), can be considered to enhance the antitumor capacities of allogeneic CAR-NKT cells.^{88,168–170} In addition, suicide switches (e.g., inducible caspase 9) can be incorporated into the allogeneic CAR-NKT cell product to further enhance its safety profile.¹⁵⁸ Overall, clinical investigations are essential to fully evaluate the potential of allogeneic CAR-NKT cells as promising off-the-shelf candidates for treating OC.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for new reagents generated in this study may be directed to and will be fulfilled by the lead contact, Lili Yang (liliyang@ucla.edu).

Materials availability

All materials and reagents will be made available upon reasonable request and the execution of a material transfer agreement.

Data and code availability

All data associated with this study are present in the paper or [supplemental information](#). The genomics data generated during this study will be available from the Gene Expression Omnibus database under accession numbers GEO: GSE272764 (scRNA-seq for the primary OC patient sample profiling study), GSE254262 (single-cell TCR sequencing), GSE252379 and GSE164425 (bulk RNA-seq), GSE270820 (scRNA-seq for the *in vivo* therapeutic cell profiling study), GSE255431 (scRNA-seq for the *in vivo* tumor cell profiling study), GSE268749 (scRNA-seq for the *in vivo* TME profiling study), and GSE251909 (Methyl-seq). This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

ACKNOWLEDGMENTS

We thank the UCLA animal facility for providing animal support; the UCLA Translational Pathology Core Laboratory for providing histology support; the UCLA Technology Center for Genomics & Bioinformatics facility for providing RNA-seq services; the UCLA CFAR Virology Core for providing human cells; and the UCLA BSCRC Flow Cytometry Core Facility for cell-sorting support. This work was supported by Partnering Opportunity for Discovery Stage Research Projects awards and Translational Research Projects awards from the California Institute for Regenerative Medicine (DISC2-11157, DISC2-13015, TRAN1-12250, and TRAN1-16050 to L.Y.), a Department of Defense CDMRP PRCRP Impact award (CA200456 to L.Y.), a UCLA BSCRC Innovation award (to L.Y.), and an Ablon Scholars award (to L.Y.). Y.-R.L. is a postdoctoral fellow supported by a UCLA MIMG M. John Pickett Post-Doctoral Fellow award, a CIRM-BSCRC Postdoctoral Fellowship, a UCLA Sydney Finegold Postdoctoral award, a UCLA Chancellor's Award for Postdoctoral Research, and a Goodman-Luskin Microbiome Center Collaborative Research Fellowship award. C.O. is supported by the UCLA BSCRC Rose Hills Foundation Graduate Scholarship Training Program. S.M., T.S., and G.D. are partially supported by research funding from the Department of Veterans Affairs (I01BX006019 and I01BX006411 to S.M.).

AUTHOR CONTRIBUTIONS

Y.-R.L., S.M., and L.Y. designed the experiments, analyzed the data, wrote the manuscript, and had unrestricted access to all data. L.Y. conceived and oversaw the study, with assistance from Y.-R.L. and with suggestions from A.W., A.J.L., X.J.Z., J.J.Z., M.P., P.W., and S.M. Y.-R.L. performed all experiments, including the statistical analyses, with assistance from Z.L., Y. Zhu, M.L., Y.C., D.L., C.J.O., T.S., G.D., W.G., S.L., Y. Zhou, Y.Y., A.K., M.W., K.Z., Z.S.D., Y.F., J.Y., Y.J.K., J.H., X.S., Y.-C.W., R.H., T.B., E.C., S.L., and A.N. N.A.M. helped with pathology analyses of primary OC patient samples. S.S.O. and S.S. screened potential study participants, performed informed consent, and collected primary samples. E.Z. helped with scanning electron microscope imaging. W.G., Y.-C.W., C.P., and A.J.L. helped with analysis of RNA-seq data. S.L. and X.J.Z. helped with the study of DNA methylation. J.J.Z. helped with the statistical analysis of data. All authors read and approved the final article and take responsibility for its content.

DECLARATION OF INTERESTS

Y.-R.L., Z.L., D.L., Y. Zhou, J.Y., Y.J.K., P.W., and L.Y. are inventors on patents relating to this study filed by UCLA. Z.L. is currently an employee of Allogene.

Y. Zhou is currently an employee of Amberstone Biosciences. Y.Y. is currently an employee of Retro Biosciences. Z.S.D. is currently an employee of Guggenheim Securities. P.W. is a co-founder, stockholder, and advisory board member of Appia Bio. L.Y. is a scientific advisor to AlzChem and Amberstone Biosciences and a co-founder, 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.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 - Mice
 - Cell lines
 - Human cord blood CD34⁺ hematopoietic stem and progenitor cells (HSPCs) and peripheral blood mononuclear cells (PBMCs)
 - Primary ovarian cancer patient samples
 - Media and reagents
- METHOD DETAILS
 - Lentiviral vectors
 - Antibodies and flow cytometry
 - Enzyme-linked immunosorbent cytokine assays (ELISA)
 - Generation of allogeneic mesothelin-targeting CAR-engineered NKT (Allo¹⁵MCAR-NKT) cells and their IL-15-enhanced derivatives (Allo¹⁵MCAR-NKT cells)
 - Generation of PBMC-derived conventional αβ T, NKT, and NK cells
 - Generation of MSLN-targeting CAR-engineered conventional αβ T (MCAR-T) cells
 - Generation of PBMC-derived IL-15-enhanced MSLN-targeting CAR-engineered NKT (PBMC¹⁵MCAR-NKT) cells
 - *In vitro* assays using OC patient samples
 - *In vitro* tumor cell killing assay
 - *In vitro* serial tumor cell killing assay
 - *In vitro* tumor organoid targeting assay
 - *In vitro* scanning electron microscopy (SEM)
 - *In vitro* mixed lymphocyte reaction (MLR) assay: Studying graft-versus-host (GvH) response
 - *In vitro* MLR assay: Studying T cell-mediated alloreactivity
 - *In vitro* MLR assay: Studying NK cell-mediated alloreactivity
 - *In vivo* bioluminescence live animal imaging (BLI)
 - *In vivo* antitumor efficacy study of Allo¹⁵MCAR-NKT cells: OVCAR8-FG human OC xenograft NSG mouse model with intraperitoneal administration route
 - *In vivo* antitumor efficacy study of Allo¹⁵MCAR-NKT cells: OVCAR8-FG human OC xenograft NSG mouse model with intravenous administration route
 - *In vivo* antitumor efficacy study of Allo¹⁵MCAR-NKT cells: OVCAR3-FG low tumor burden human OC xenograft NSG mouse model
 - *In vivo* antitumor efficacy study of Allo¹⁵MCAR-NKT cells: OVCAR3-FG high tumor burden human OC xenograft NSG mouse model
 - *In vivo* antitumor efficacy study of Allo¹⁵MCAR-NKT cells: OVCAR3-FG repeated tumor challenge human OC xenograft NSG mouse model
 - *In vivo* antitumor efficacy study of Allo¹⁵MCAR-NKT cells combined with carboplatin: OVCAR3-FG human OC xenograft NSG mouse model
 - *In vivo* antitumor efficacy study of Allo¹⁵MCAR-NKT cells: OVCAR3-FG^{MSLN-/-} human OC xenograft NSG mouse model
 - *In vivo* pharmacokinetics/pharmacodynamics (PK/PD) study of Allo¹⁵MCAR-NKT/FG cells: Human OC xenograft NSG mouse model
 - *In vivo* TME targeting study
 - *In vivo* cytokine release syndrome (CRS) study
 - Single cell TCR sequencing (scTCR-seq)

- Bulk RNA sequencing (bulk RNA-seq)
- Single cell RNA sequencing (scRNA-seq)
- Methylation sequencing (Methyl-seq)
- Histological analysis
- Western blot (WB)
- Generation and analysis of Allo¹⁵MCAR-NKT cells overexpressing a constitutively active STAT1

● QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.medj.2025.100752>.

Received: June 16, 2025

Revised: July 10, 2025

Accepted: July 16, 2025

Published: August 12, 2025

REFERENCES

1. Piechocki, M., Koziol, W., Sroka, D., Matrejka, A., Miziolek, P., Saiuk, N., Sledzik, M., Jaworska, A., Bereza, K., Pluta, E., and Banas, T. (2022). Trends in Incidence and Mortality of Gynecological and Breast Cancers in Poland (1980-2018). *Clin. Epidemiol.* 14, 95–114. <https://doi.org/10.2147/CLEP.S330081>.
2. Siegel, R.L., Miller, K.D., Wagle, N.S., and Jemal, A. (2023). Cancer statistics, 2023. *CA Cancer J. Clin.* 73, 17–48. <https://doi.org/10.3322/caac.21763>.
3. Lheureux, S., Braunstein, M., and Oza, A.M. (2019). Epithelial ovarian cancer: Evolution of management in the era of precision medicine. *CA Cancer J. Clin.* 69, 280–304. <https://doi.org/10.3322/caac.21559>.
4. Matulonis, U.A., Sood, A.K., Fallowfield, L., Howitt, B.E., Sehouli, J., and Karlan, B.Y. (2016). Ovarian cancer. *Nat. Rev. Dis. Primers* 2, 16061. <https://doi.org/10.1038/nrdp.2016.61>.
5. Agarwal, R., and Kaye, S.B. (2003). Ovarian cancer: strategies for overcoming resistance to chemotherapy. *Nat. Rev. Cancer* 3, 502–516. <https://doi.org/10.1038/nrc1123>.
6. Mei, S., Chen, X., Wang, K., and Chen, Y. (2023). Tumor microenvironment in ovarian cancer peritoneal metastasis. *Cancer Cell Int.* 23, 11. <https://doi.org/10.1186/s12935-023-02854-5>.
7. Marth, C., Reimer, D., and Zeimet, A.G. (2017). Front-line therapy of advanced epithelial ovarian cancer: standard treatment. *Ann. Oncol.* 28, viii36–viii39. <https://doi.org/10.1093/annonc/mdx450>.
8. Pignata, S., C Cecere, S., Du Bois, A., Harter, P., and Heitz, F. (2017). Treatment of recurrent ovarian cancer. *Ann. Oncol.* 28, viii51–viii56. <https://doi.org/10.1093/annonc/mdx441>.
9. Nersesian, S., Glazebrook, H., Toulany, J., Grantham, S.R., and Boudreau, J.E. (2019). Naturally Killing the Silent Killer: NK Cell-Based Immunotherapy for Ovarian Cancer. *Front. Immunol.* 10, 1782. <https://doi.org/10.3389/fimmu.2019.01782>.
10. Tanyi, J.L., Haas, A.R., Beatty, G.L., Stashwick, C.J., O'Hara, M.H., Morgan, M.A., Porter, D.L., Melenhorst, J.J., Plesa, G., Lacey, S.F., and June, C.H. (2016). Anti-mesothelin chimeric antigen receptor T cells in patients with epithelial ovarian cancer. *J. Clin. Oncol.* 34, 5511. https://doi.org/10.1200/JCO.2016.34.15_suppl.5511.
11. Wu, J.W.Y., Dand, S., Doig, L., Papenfuss, A.T., Scott, C.L., Ho, G., and Ooi, J.D. (2021). T-Cell Receptor Therapy in the Treatment of Ovarian Cancer: A Mini Review. *Front. Immunol.* 12, 672502. <https://doi.org/10.3389/fimmu.2021.672502>.
12. Zhu, X., Cai, H., Zhao, L., Ning, L., and Lang, J. (2017). CAR-T cell therapy in ovarian cancer: from the bench to the bedside. *Oncotarget* 8, 64607–64621. <https://doi.org/10.18632/oncotarget.19929>.

13. Lang, S.M., and Dorigo, O. (2022). Cell-based immunotherapies in gynecologic cancers. *Curr. Opin. Obstet. Gynecol.* **34**, 10–14. <https://doi.org/10.1097/GCO.0000000000000760>.
14. Maurer, K., Grabski, I.N., Houot, R., Gohil, S.H., Miura, S., Redd, R., Lyu, H., Lu, W., Arihara, Y., Budka, J., et al. (2024). Baseline immune state and T-cell clonal kinetics are associated with durable response to CAR-T therapy in large B-cell lymphoma. *Blood* **144**, 2490–2502. <https://doi.org/10.1182/blood.2024024381>.
15. Majzner, R.G., and Mackall, C.L. (2019). Clinical lessons learned from the first leg of the CAR T cell journey. *Nat. Med.* **25**, 1341–1355. <https://doi.org/10.1038/s41591-019-0564-6>.
16. Li, Y.-R., Zhu, Y., Halladay, T., and Yang, L. (2025). In vivo CAR engineering for immunotherapy. *Nat. Rev. Immunol.* <https://doi.org/10.1038/s41577-025-01174-1>.
17. Mun, S.S., Peraro, L., Meyerberg, J., Korontsvit, T., Malviya, M., Gardner, T., Kyi, C., O’Cearbhaill, R.E., Liu, C., Dao, T., and Scheinberg, D.A. (2023). Dual targeting ovarian cancer by Muc16 CAR-T cells secreting a bispecific T cell engager antibody for an intracellular tumor antigen WT1. *Cancer Immunol. Immunother.* **72**, 3773–3786. <https://doi.org/10.1007/s00262-023-03529-w>.
18. Mackensen, A., Haanen, J.B.A.G., Koenecke, C., Alsdorf, W., Wagner-Drouet, E., Borchmann, P., Heudobler, D., Ferstl, B., Klobuch, S., Bokenmeyer, C., et al. (2023). CLDN6-specific CAR-T cells plus amplifying RNA vaccine in relapsed or refractory solid tumors: the phase 1 BNT211-01 trial. *Nat. Med.* **29**, 2844–2853. <https://doi.org/10.1038/s41591-023-02612-0>.
19. Chen, J., Hu, J., Gu, L., Ji, F., Zhang, F., Zhang, M., Li, J., Chen, Z., Jiang, L., Zhang, Y., et al. (2023). Anti-mesothelin CAR-T immunotherapy in patients with ovarian cancer. *Cancer Immunol. Immunother.* **72**, 409–425. <https://doi.org/10.1007/s00262-022-03238-w>.
20. Veneziani, A.C., Gonzalez-Ochoa, E., Alqaisi, H., Madariaga, A., Bhat, G., Rouzbahman, M., Sneha, S., and Oza, A.M. (2023). Heterogeneity and treatment landscape of ovarian carcinoma. *Nat. Rev. Clin. Oncol.* **20**, 820–842. <https://doi.org/10.1038/s41571-023-00819-1>.
21. Li, Y.-R., Ochoa, C.J., Zhu, Y., Kramer, A., Wilson, M., Fang, Y., Chen, Y., Singh, T., Di Bernardo, G., Zhu, E., et al. (2023). Profiling ovarian cancer tumor and microenvironment during disease progression for cell-based immunotherapy design. *iScience* **26**, 107952. <https://doi.org/10.1016/j.isci.2023.107952>.
22. Li, Y.-R., Fang, Y., Lyu, Z., Zhu, Y., and Yang, L. (2023). Exploring the dynamic interplay between cancer stem cells and the tumor microenvironment: implications for novel therapeutic strategies. *J. Transl. Med.* **21**, 686. <https://doi.org/10.1186/s12967-023-04575-9>.
23. Ma, H., Tian, T., and Cui, Z. (2023). Targeting ovarian cancer stem cells: a new way out. *Stem Cell Res. Ther.* **14**, 28. <https://doi.org/10.1186/s13287-023-03244-4>.
24. Lin, Y., Xu, J., and Lan, H. (2019). Tumor-associated macrophages in tumor metastasis: biological roles and clinical therapeutic applications. *J. Hematol. Oncol.* **12**, 76. <https://doi.org/10.1186/s13045-019-0760-3>.
25. Li, Y.-R., Wilson, M., and Yang, L. (2022). Target tumor microenvironment by innate T cells. *Front. Immunol.* **13**, 999549. <https://doi.org/10.3389/fimmu.2022.999549>.
26. Kamat, K., Krishnan, V., and Dorigo, O. (2022). Macrophage-derived CCL23 upregulates expression of T-cell exhaustion markers in ovarian cancer. *Br. J. Cancer* **127**, 1026–1033. <https://doi.org/10.1038/s41416-022-01887-3>.
27. Giuntoli, R.L., 2nd, Webb, T.J., Zoso, A., Rogers, O., Diaz-Montes, T.P., Bristow, R.E., and Oelke, M. (2009). Ovarian cancer-associated ascites demonstrates altered immune environment: implications for antitumor immunity. *Anticancer Res.* **29**, 2875–2884.
28. Worzfeld, T., Pogge von Strandmann, E., Huber, M., Adhikary, T., Wagner, U., Reinartz, S., and Müller, R. (2017). The Unique Molecular and Cellular Microenvironment of Ovarian Cancer. *Front. Oncol.* **7**, 24. <https://doi.org/10.3389/fonc.2017.00024>.
29. Ghoneum, A., Afify, H., Salih, Z., Kelly, M., and Said, N. (2018). Role of tumor microenvironment in ovarian cancer pathobiology. *Oncotarget* **9**, 22832–22849. <https://doi.org/10.18632/oncotarget.25126>.
30. Raghavan, S., Mehta, P., Xie, Y., Lei, Y.L., and Mehta, G. (2019). Ovarian cancer stem cells and macrophages reciprocally interact through the WNT pathway to promote pro-tumoral and malignant phenotypes in 3D engineered microenvironments. *J. Immunother. Cancer* **7**, 190. <https://doi.org/10.1186/s40425-019-0666-1>.
31. Zhang, X.-W., Wu, Y.-S., Xu, T.-M., and Cui, M.-H. (2023). CAR-T Cells in the Treatment of Ovarian Cancer: A Promising Cell Therapy. *Biomolecules* **13**, 465. <https://doi.org/10.3390/biom13030465>.
32. Giavridis, T., Van Der Stegen, S.J.C., Eyquem, J., Hamieh, M., Piersigilli, A., and Sadelain, M. (2018). CAR T cell-induced cytokine release syndrome is mediated by macrophages and abated by IL-1 blockade letter. *Nat. Med.* **24**, 731–738. <https://doi.org/10.1038/s41591-018-0041-7>.
33. Norelli, M., Camisa, B., Barbiera, G., Falcone, L., Purevdorj, A., Genua, M., Sanvito, F., Ponzoni, M., Doglioni, C., Cristofori, P., et al. (2018). Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells. *Nat. Med.* **24**, 739–748. <https://doi.org/10.1038/s41591-018-0036-4>.
34. Baci, D., Bosi, A., Gallazzi, M., Rizzi, M., Noonan, D.M., Poggi, A., Bruno, A., and Mortara, L. (2020). The Ovarian Cancer Tumor Immune Microenvironment (TIME) as Target for Therapy: A Focus on Innate Immunity Cells as Therapeutic Effectors. *Int. J. Mol. Sci.* **21**, 3125. <https://doi.org/10.3390/ijms21093125>.
35. Yang, Y., Yang, Y., Yang, J., Zhao, X., and Wei, X. (2020). Tumor Microenvironment in Ovarian Cancer: Function and Therapeutic Strategy. *Front. Cell Dev. Biol.* **8**, 758. <https://doi.org/10.3389/fcell.2020.00758>.
36. Zheng, X., Wang, X., Cheng, X., Liu, Z., Yin, Y., Li, X., Huang, Z., Wang, Z., Guo, W., Ginhoux, F., et al. (2023). Single-cell analyses implicate ascites in remodeling the ecosystems of primary and metastatic tumors in ovarian cancer. *Nat. Cancer* **4**, 1138–1156. <https://doi.org/10.1038/s43018-023-00599-8>.
37. Yang, B., Li, X., Zhang, W., Fan, J., Zhou, Y., Li, W., Yin, J., Yang, X., Guo, E., Li, X., et al. (2022). Spatial heterogeneity of infiltrating T cells in high-grade serous ovarian cancer revealed by multi-omics analysis. *Cell Rep. Med.* **3**, 100856. <https://doi.org/10.1016/j.xcrm.2022.100856>.
38. Deng, Y., Tan, Y., Zhou, D., Bai, Y., Cao, T., Zhong, C., Huang, W., Ou, Y., Guo, L., Liu, Q., et al. (2022). Single-Cell RNA-Sequencing Atlas Reveals the Tumor Microenvironment of Metastatic High-Grade Serous Ovarian Carcinoma. *Front. Immunol.* **13**, 923194. <https://doi.org/10.3389/fimmu.2022.923194>.
39. Bae, E.-A., Seo, H., Kim, I.-K., Jeon, I., and Kang, C.-Y. (2019). Roles of NKT cells in cancer immunotherapy. *Arch. Pharm. Res. (Seoul)* **42**, 543–548. <https://doi.org/10.1007/s12272-019-01139-8>.
40. Bendelac, A., Savage, P.B., and Teyton, L. (2007). The biology of NKT cells. *Annu. Rev. Immunol.* **25**, 297–336. <https://doi.org/10.1146/annurev.immunol.25.022106.141711>.
41. Zhou, X., Wang, Y., Dou, Z., Delfanti, G., Tsaouridis, O., Pellegrini, C.M., Zingarelli, M., Atassi, G., Woodcock, M.G., Casorati, G., et al. (2024). CAR-redirectioned natural killer T cells demonstrate superior antitumor activity to CAR-T cells through multimodal CD1d-dependent mechanisms. *Nat. Cancer* **5**, 1607–1621. <https://doi.org/10.1038/s43018-024-00830-0>.
42. Rotolo, A., Caputo, V.S., Holubova, M., Baxan, N., Dubois, O., Chaudhry, M.S., Xiao, X., Goudevenou, K., Pitcher, D.S., Petev, K., et al. (2018). Enhanced Anti-lymphoma Activity of CAR19-INKT Cells Underpinned by Dual CD19 and CD1d Targeting. *Cancer Cell* **34**, 596–610.e11. <https://doi.org/10.1016/j.ccell.2018.08.017>.
43. Courtney, A.N., Tian, G., and Metelitsa, L.S. (2023). Natural killer T cells and other innate-like T lymphocytes as emerging platforms for allogeneic

- cancer cell therapy. *Blood* 141, 869–876. <https://doi.org/10.1182/blood.2022016201>.
44. Hadiloo, K., Tahmasebi, S., and Esmaeilzadeh, A. (2023). CAR-NKT cell therapy: a new promising paradigm of cancer immunotherapy. *Cancer Cell Int.* 23, 86. <https://doi.org/10.1186/s12935-023-02923-9>.
45. Heczey, A., Liu, D., Tian, G., Courtney, A.N., Wei, J., Marinova, E., Gao, X., Guo, L., Yvon, E., Hicks, J., et al. (2014). Invariant NKT cells with chimeric antigen receptor provide a novel platform for safe and effective cancer immunotherapy. *Blood* 124, 2824–2833. <https://doi.org/10.1182/blood-2013-11-541235>.
46. Dorigo, O., Moser, J., Contag, P., Patel, S., and Weng, W.-K. (2024). Safety and tolerability of CRX100, an NKT cell therapy combined with tumor-specific oncolytic vaccinia virus, among patients with recurrent, platinum-resistant ovarian cancer. *Gynecol. Oncol.* 190, S297. <https://doi.org/10.1016/j.ygyno.2024.07.444>.
47. Vivier, E., Ugolini, S., Blaise, D., Chabannon, C., and Brossay, L. (2012). Targeting natural killer cells and natural killer T cells in cancer. *Nat. Rev. Immunol.* 12, 239–252. <https://doi.org/10.1038/nri3174>.
48. Li, Y.-R., Zhou, Y., Yu, J., Kim, Y.J., Li, M., Lee, D., Zhou, K., Chen, Y., Zhu, Y., Wang, Y.-C., et al. (2025). Generation of allogeneic CAR-NKT cells from hematopoietic stem and progenitor cells using a clinically guided culture method. *Nat. Biotechnol.* 43, 329–344. <https://doi.org/10.1038/s41587-024-02226-y>.
49. Xu, J., Fang, Y., Chen, K., Li, S., Tang, S., Ren, Y., Cen, Y., Fei, W., Zhang, B., Shen, Y., and Lu, W. (2022). Single-Cell RNA Sequencing Reveals the Tissue Architecture in Human High-Grade Serous Ovarian Cancer. *Clin. Cancer Res.* 28, 3590–3602. <https://doi.org/10.1158/1078-0432.CCR-22-0296>.
50. Zhang, K., Erkan, E.P., Jamalzadeh, S., Dai, J., Andersson, N., Kaipio, K., Lamminen, T., Mansuri, N., Huhtinen, K., Carpén, O., et al. (2022). Longitudinal single-cell RNA-seq analysis reveals stress-promoted chemoresistance in metastatic ovarian cancer. *Sci. Adv.* 8, eabm1831. <https://doi.org/10.1126/sciadv.abm1831>.
51. (2011). Integrated genomic analyses of ovarian carcinoma. *Nature* 474, 609–615. <https://doi.org/10.1038/nature10166>.
52. Hilliard, T.S., Kowalski, B., Iwamoto, K., Agadi, E.A., Liu, Y., Yang, J., Asem, M., Klymenko, Y., Johnson, J., Shi, Z., et al. (2021). Host Mesothelin Expression Increases Ovarian Cancer Metastasis in the Peritoneal Microenvironment. *Int. J. Mol. Sci.* 22, 12443. <https://doi.org/10.3390/ijms222212443>.
53. Li, Y.-R., Zhou, Y., Kim, Y.J., Zhu, Y., Ma, F., Yu, J., Wang, Y.-C., Chen, X., Li, Z., Zeng, S., et al. (2021). Development of allogeneic HSC-engineered iNKT cells for off-the-shelf cancer immunotherapy. *Cell Rep. Med.* 2, 100449. <https://doi.org/10.1016/j.xcrm.2021.100449>.
54. Mackensen, A., Koenecke, C., Haanen, J., Alsdorf, W., Desuki, A., Wagner-Drouet, E., Heudobler, D., Borchmann, P., Wiegert, E., Schulz, C., et al. (2021). 958 BNT211: a phase I/II trial to evaluate safety and efficacy of CLDN6 CAR-T cells and vaccine-mediated in vivo expansion in patients with CLDN6-positive advanced solid tumors. *J. Immunother. Cancer* 9, A1008. <https://doi.org/10.1136/jitc-2021-SITC2021.958>.
55. Zhu, Y., Smith, D.J., Zhou, Y., Li, Y.R., Yu, J., Lee, D., Wang, Y.C., Di Biase, S., Wang, X., Hardoy, C., et al. (2019). Development of Hematopoietic Stem Cell-Engineered Invariant Natural Killer T Cell Therapy for Cancer. *Cell Stem Cell* 25, 542–557.e9. <https://doi.org/10.1016/j.stem.2019.08.004>.
56. Qu, Y., Siegler, E., Cheng, C., Liu, J., Cinay, G., Bagrodia, N., and Wang, P. (2019). Engineering CAR-expressing natural killer cells with cytokine signaling and synthetic switch for an off-the-shelf cell-based cancer immunotherapy. *MRS Commun.* 9, 433–440. <https://doi.org/10.1557/mrc.2019.31>.
57. Xu, X., Huang, W., Heczey, A., Liu, D., Guo, L., Wood, M., Jin, J., Courtney, A.N., Liu, B., Di Pierro, E.J., et al. (2019). NKT cells coexpressing a GD2-specific chimeric antigen receptor and IL15 show enhanced in vivo persistence and antitumor activity against neuroblastoma. *Clin. Cancer Res.* 25, 7126–7138. <https://doi.org/10.1158/1078-0432.CCR-19-0421>.
58. Heczey, A., Courtney, A.N., Montalbano, A., Robinson, S., Liu, K., Li, M., Ghatwai, N., Dakhova, O., Liu, B., Raveh-Sadka, T., et al. (2020). Anti-GD2 CAR-NKT cells in patients with relapsed or refractory neuroblastoma: an interim analysis. *Nat. Med.* 26, 1686–1690. <https://doi.org/10.1038/s41591-020-1074-2>.
59. Heczey, A., Xu, X., Courtney, A.N., Tian, G., Barragan, G.A., Guo, L., Amador, C.M., Ghatwai, N., Rathi, P., Wood, M.S., et al. (2023). Anti-GD2 CAR-NKT cells in relapsed or refractory neuroblastoma: updated phase 1 trial interim results. *Nat. Med.* 29, 1379–1388. <https://doi.org/10.1038/s41591-023-02363-y>.
60. Montoya, C.J., Pollard, D., Martinson, J., Kumari, K., Wasserfall, C., Mulder, C.B., Rugeles, M.T., Atkinson, M.A., Landay, A.L., and Wilson, S.B. (2007). Characterization of human invariant natural killer T subsets in health and disease using a novel invariant natural killer T cell-clonotypic monoclonal antibody, 6B11. *Immunology* 122, 1–14. <https://doi.org/10.1111/j.1365-2567.2007.02647.x>.
61. Coman, T., Rossignol, J., D'Aveni, M., Fabiani, B., Dussiot, M., Rignault, R., Babbord, J., Bouillé, M., Herbelin, A., Coté, F., et al. (2018). Human CD4- invariant NKT lymphocytes regulate graft versus host disease. *Oncoimmunology* 7, e1470735. <https://doi.org/10.1080/2162402X.2018.1470735>.
62. Bendelac, A., Savage, P.B., and Teyton, L. (2007). The Biology of NKT Cells. *Annu. Rev. Immunol.* 25, 297–336. <https://doi.org/10.1146/annurev.immunol.25.022106.141711>.
63. Kitayama, S., Zhang, R., Liu, T.Y., Ueda, N., Iriguchi, S., Yasui, Y., Kawai, Y., Tatsumi, M., Hirai, N., Mizoro, Y., et al. (2016). Cellular Adjuvant Properties, Direct Cytotoxicity of Re-differentiated Vα24 Invariant NKT-like Cells from Human Induced Pluripotent Stem Cells. *Stem Cell Rep.* 6, 213–227. <https://doi.org/10.1016/j.stemcr.2016.01.005>.
64. Godfrey, D.I., and Berzins, S.P. (2007). Control points in NKT-cell development. *Nat. Rev. Immunol.* 7, 505–518. <https://doi.org/10.1038/nri2116>.
65. Fujii, S.I., Liu, K., Smith, C., Bonito, A.J., and Steinman, R.M. (2004). The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J. Exp. Med.* 199, 1607–1618. <https://doi.org/10.1084/jem.20040317>.
66. Fujii, S.I., Shimizu, K., Hemmi, H., and Steinman, R.M. (2007). Innate Vα14+ natural killer T cells mature dendritic cells, leading to strong adaptive immunity. *Immunol. Rev.* 220, 183–198. <https://doi.org/10.1111/j.1600-065X.2007.00561.x>.
67. Giannoni, F., Hardee, C.L., Wherley, J., Gschweng, E., Senadheera, S., Kaufman, M.L., Chan, R., Bahner, I., Gersuk, V., Wang, X., et al. (2013). Allelic exclusion and peripheral reconstitution by TCR transgenic T cells arising from transduced human hematopoietic stem/progenitor cells. *Mol. Ther.* 21, 1044–1054. <https://doi.org/10.1038/mt.2013.8>.
68. Labanieh, L., Majzner, R.G., and Mackall, C.L. (2018). Programming CAR-T cells to kill cancer. *Nat. Biomed. Eng.* 2, 377–391. <https://doi.org/10.1038/s41551-018-0235-9>.
69. Alonzo, E.S., and Sant'Angelo, D.B. (2011). Development of PLZF-expressing invariant T cells. *Curr. Opin. Immunol.* 23, 220–227. <https://doi.org/10.1016/j.coi.2010.12.016>.
70. Zhang, Y., Zhang, Y., Gu, W., and Sun, B. (2014). Th1/Th2 cell differentiation and molecular signals. *Adv. Exp. Med. Biol.* 841, 15–44. https://doi.org/10.1007/978-94-017-9487-9_2.
71. Gagliani, N., and Huber, S. (2017). Basic aspects of T helper cell differentiation. *Methods Mol. Biol.* 1514, 19–30. https://doi.org/10.1007/978-1-4939-6548-9_2.
72. Kamata, T., Suzuki, A., Mise, N., Ihara, F., Takami, M., Makita, Y., Hori-naka, A., Harada, K., Kunii, N., Yoshida, S., et al. (2016). Blockade of programmed death-1/programmed death ligand pathway enhances the

- antitumor immunity of human invariant natural killer T cells. *Cancer Immunol. Immunother.* 65, 1477–1489. <https://doi.org/10.1007/s00262-016-1901-y>.
73. Durgan, K., Ali, M., Warner, P., and Latchman, Y.E. (2011). Targeting NKT cells and PD-L1 pathway results in augmented anti-tumor responses in a melanoma model. *Cancer Immunol. Immunother.* 60, 547–558. <https://doi.org/10.1007/s00262-010-0963-5>.
74. Furukawa, Y., Hamano, Y., Shirane, S., Kinoshita, S., Azusawa, Y., Ando, J., Nakauchi, H., and Ando, M. (2022). Advances in Allogeneic Cancer Cell Therapy and Future Perspectives on “Off-the-Shelf” T Cell Therapy Using iPSC Technology and Gene Editing. *Cells* 11, 269. <https://doi.org/10.3390/cells11020269>.
75. Ozdemir, Z.N., and Civriz Bozdağ, S. (2018). Graft failure after allogeneic hematopoietic stem cell transplantation. *Transfus. Apher. Sci.* 57, 163–167. <https://doi.org/10.1016/j.transci.2018.04.014>.
76. Lam, P.Y., Nissen, M.D., and Mattarollo, S.R. (2017). Invariant natural killer T cells in immune regulation of blood cancers: Harnessing their potential in immunotherapies. *Front. Immunol.* 8, 1355. <https://doi.org/10.3389/fimmu.2017.01355>.
77. Krijgsman, D., Hokland, M., and Kuppen, P.J.K. (2018). The Role of Natural Killer T Cells in Cancer-A Phenotypical and Functional Approach. *Front. Immunol.* 9, 367. <https://doi.org/10.3389/fimmu.2018.00367>.
78. Kronenberg, M. (2005). Toward an understanding of NKT cell biology: progress and paradoxes. *Annu. Rev. Immunol.* 23, 877–900. <https://doi.org/10.1146/annurev.immunol.23.021704.115742>.
79. Li, Y.-R., Zhou, Y., Wilson, M., Kramer, A., Hon, R., Zhu, Y., Fang, Y., and Yang, L. (2022). Tumor-Localized Administration of α -GalCer to Recruit Invariant Natural Killer T Cells and Enhance Their Antitumor Activity against Solid Tumors. *Int. J. Mol. Sci.* 23, 7547. <https://doi.org/10.3390/ijms23147547>.
80. Lee, P.T., Benlagha, K., Teyton, L., and Bendelac, A. (2002). Distinct functional lineages of human V α 24 natural killer T cells. *J. Exp. Med.* 195, 637–641. <https://doi.org/10.1084/jem.20011908>.
81. Wolf, B.J., Choi, J.E., and Exley, M.A. (2018). Novel Approaches to Exploiting Invariant NKT Cells in Cancer Immunotherapy. *Front. Immunol.* 9, 384. <https://doi.org/10.3389/fimmu.2018.00384>.
82. Rafiq, S., Hackett, C.S., and Brentjens, R.J. (2020). Engineering strategies to overcome the current roadblocks in CAR T cell therapy. *Nat. Rev. Clin. Oncol.* 17, 147–167. <https://doi.org/10.1038/s41571-019-0297-y>.
83. Sterner, R.C., and Sterner, R.M. (2021). CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J.* 11, 69. <https://doi.org/10.1038/s41408-021-00459-7>.
84. Dhatchinamoorthy, K., Colbert, J.D., and Rock, K.L. (2021). Cancer Immune Evasion Through Loss of MHC Class I Antigen Presentation. *Front. Immunol.* 12, 636568. <https://doi.org/10.3389/fimmu.2021.636568>.
85. Liu, D., Song, L., Wei, J., Courtney, A.N., Gao, X., Marinova, E., Guo, L., Heczey, A., Asgharzadeh, S., Kim, E., et al. (2012). IL-15 protects NKT cells from inhibition by tumor-associated macrophages and enhances antitumorigenic activity. *J. Clin. Invest.* 122, 2221–2233. <https://doi.org/10.1172/JCI59535>.
86. Gourley, C., Walker, J.L., and Mackay, H.J. (2016). Update on Intraperitoneal Chemotherapy for the Treatment of Epithelial Ovarian Cancer. *Am. Soc. Clin. Oncol. Educ. Book.* 35, 143–151. https://doi.org/10.1200/EDBK_158927.
87. Wolinsky, J.B., Colson, Y.L., and Grinstaff, M.W. (2012). Local drug delivery strategies for cancer treatment: gels, nanoparticles, polymeric films, rods, and wafers. *J. Control. Release* 159, 14–26. <https://doi.org/10.1016/j.jconrel.2011.11.031>.
88. Labanieh, L., and Mackall, C.L. (2023). CAR immune cells: design principles, resistance and the next generation. *Nature* 614, 635–648. <https://doi.org/10.1038/s41586-023-05707-3>.
89. Li, Y.-R., Zeng, S., Dunn, Z.S., Zhou, Y., Li, Z., Yu, J., Wang, Y.-C., Ku, J., Cook, N., Kramer, A., and Yang, L. (2022). Off-the-shelf third-party HSC-engineered iNKT cells for ameliorating GvHD while preserving GvL effect in the treatment of blood cancers. *iScience* 25, 104859. <https://doi.org/10.1016/j.isci.2022.104859>.
90. Sommer, C., Boldajipour, B., Kuo, T.C., Bentley, T., Sutton, J., Chen, A., Geng, T., Dong, H., Galetto, R., Valton, J., et al. (2019). Preclinical Evaluation of Allogeneic CAR T Cells Targeting BCMA for the Treatment of Multiple Myeloma. *Mol. Ther.* 27, 1126–1138. <https://doi.org/10.1016/j.ymthe.2019.04.001>.
91. Thomas, S.Y., Hou, R., Boyson, J.E., Means, T.K., Hess, C., Olson, D.P., Strominger, J.L., Brenner, M.B., Gumperz, J.E., Wilson, S.B., and Luster, A.D. (2003). CD1d-Restricted NKT Cells Express a Chemokine Receptor Profile Indicative of Th1-Type Inflammatory Homing Cells. *J. Immunol.* 171, 2571–2580. <https://doi.org/10.4049/jimmunol.171.5.2571>.
92. Baeyens, A., Fang, V., Chen, C., and Schwab, S.R. (2015). Exit Strategies: S1P Signaling and T Cell Migration. *Trends Immunol.* 36, 778–787. <https://doi.org/10.1016/j.it.2015.10.005>.
93. Schwab, S.R., and Cyster, J.G. (2007). Finding a way out: lymphocyte egress from lymphoid organs. *Nat. Immunol.* 8, 1295–1301. <https://doi.org/10.1038/ni1545>.
94. Woodland, D.L., and Kohlmeier, J.E. (2009). Migration, maintenance and recall of memory T cells in peripheral tissues. *Nat. Rev. Immunol.* 9, 153–161. <https://doi.org/10.1038/nri2496>.
95. Li, Y.-R., Halladay, T., and Yang, L. (2024). Immune evasion in cell-based immunotherapy: unraveling challenges and novel strategies. *J. Biomed. Sci.* 31, 5. <https://doi.org/10.1186/s12929-024-00998-8>.
96. Miller, B.C., Sen, D.R., Al Abosy, R., Bi, K., Virkud, Y.V., LaFleur, M.W., Yates, K.B., Lako, A., Felt, K., Naik, G.S., et al. (2019). Subsets of exhausted CD8(+) T cells differentially mediate tumor control and respond to checkpoint blockade. *Nat. Immunol.* 20, 326–336. <https://doi.org/10.1038/s41590-019-0312-6>.
97. Zheng, L., Qin, S., Si, W., Wang, A., Xing, B., Gao, R., Ren, X., Wang, L., Wu, X., Zhang, J., et al. (2021). Pan-cancer single-cell landscape of tumor-infiltrating T cells. *Science* 374, abe6474. <https://doi.org/10.1126/science.abe6474>.
98. Li, H., van der Leun, A.M., Yofe, I., Lubling, Y., Gelbard-Solodkin, D., van Akkooi, A.C.J., van den Braber, M., Rozeman, E.A., Haanen, J.B.A.G., Blank, C.U., et al. (2019). Dysfunctional CD8 T Cells Form a Proliferative, Dynamically Regulated Compartment within Human Melanoma. *Cell* 176, 775–789.e18. <https://doi.org/10.1016/j.cell.2018.11.043>.
99. Milner, J.J., Toma, C., He, Z., Kurd, N.S., Nguyen, Q.P., McDonald, B., Quezada, L., Widjaja, C.E., Witherden, D.A., Crowl, J.T., et al. (2020). Heterogenous Populations of Tissue-Resident CD8(+) T Cells Are Generated in Response to Infection and Malignancy. *Immunity* 52, 808–824.e7. <https://doi.org/10.1016/j.immuni.2020.04.007>.
100. Beltra, J.-C., Manne, S., Abdel-Hakeem, M.S., Kurachi, M., Giles, J.R., Chen, Z., Casella, V., Ngio, S.F., Khan, O., Huang, Y.J., et al. (2020). Developmental Relationships of Four Exhausted CD8(+) T Cell Subsets Reveals Underlying Transcriptional and Epigenetic Landscape Control Mechanisms. *Immunity* 52, 825–841.e8. <https://doi.org/10.1016/j.immuni.2020.04.014>.
101. Zheng, C., Zheng, L., Yoo, J.-K., Guo, H., Zhang, Y., Guo, X., Kang, B., Hu, R., Huang, J.Y., Zhang, Q., et al. (2017). Landscape of Infiltrating T Cells in Liver Cancer Revealed by Single-Cell Sequencing. *Cell* 169, 1342–1356.e16. <https://doi.org/10.1016/j.cell.2017.05.035>.
102. Wang, X., Peticone, C., Kotsopoulou, E., Göttgens, B., and Calero-Nieto, F. J. (2021). Single-cell transcriptome analysis of CAR T-cell products reveals subpopulations, stimulation, and exhaustion signatures. *Oncotarget* 10, 1866287. <https://doi.org/10.1080/2162402X.2020.1866287>.
103. Bai, Z., Woodhouse, S., Zhao, Z., Arya, R., Govek, K., Kim, D., Lundh, S., Baysoy, A., Sun, H., Deng, Y., et al. (2022). Single-cell antigen-specific landscape of CAR T infusion product identifies determinants of

- CD19-positive relapse in patients with ALL. *Sci. Adv.* 8, eabj2820. <https://doi.org/10.1126/sciadv.abj2820>.
104. Good, C.R., Aznar, M.A., Kuramitsu, S., Samareh, P., Agarwal, S., Donahue, G., Ishiyama, K., Wellhausen, N., Rennels, A.K., Ma, Y., et al. (2021). An NK-like CAR T cell transition in CAR T cell dysfunction. *Cell* 184, 6081–6100.e26. <https://doi.org/10.1016/j.cell.2021.11.016>.
 105. Cazaux, M., Grandjean, C.L., Lemaître, F., Garcia, Z., Beck, R.J., Milo, I., Postat, J., Beltman, J.B., Cheadle, E.J., and Bousso, P. (2019). Single-cell imaging of CAR T cell activity in vivo reveals extensive functional and anatomical heterogeneity. *J. Exp. Med.* 216, 1038–1049. <https://doi.org/10.1084/jem.20182375>.
 106. Haradhvala, N.J., Leick, M.B., Maurer, K., Gohil, S.H., Larson, R.C., Yao, N., Gallagher, K.M.E., Katsis, K., Frigault, M.J., Southard, J., et al. (2022). Distinct cellular dynamics associated with response to CAR-T therapy for refractory B cell lymphoma. *Nat. Med.* 28, 1848–1859. <https://doi.org/10.1038/s41591-022-01959-0>.
 107. Schuster, S.J., Svoboda, J., Chong, E.A., Nasta, S.D., Mato, A.R., Anak, Ö., Brogdon, J.L., Pruteanu-Malinici, I., Bhoj, V., Landsburg, D., et al. (2017). Chimeric Antigen Receptor T Cells in Refractory B-Cell Lymphomas. *N. Engl. J. Med.* 377, 2545–2554. <https://doi.org/10.1056/NEJMoa1708566>.
 108. Guo, X., Zhang, Y., Zheng, L., Zheng, C., Song, J., Zhang, Q., Kang, B., Liu, Z., Jin, L., Xing, R., et al. (2018). Global characterization of T cells in non-small-cell lung cancer by single-cell sequencing. *Nat. Med.* 24, 978–985. <https://doi.org/10.1038/s41591-018-0045-3>.
 109. Gupta, R., Kumar, R., Penn, C.A., and Wajapeyee, N. (2025). Immune evasion in ovarian cancer: implications for immunotherapy and emerging treatments. *Trends Immunol.* 46, 166–181. <https://doi.org/10.1016/j.it.2024.12.006>.
 110. Allavena, P., Digifico, E., and Belgiovine, C. (2021). Macrophages and cancer stem cells: a malevolent alliance. *Mol. Med.* 27, 121. <https://doi.org/10.1186/s10020-021-00383-3>.
 111. Xia, Y., Shen, S., and Verma, I.M. (2014). NF- κ B, an active player in human cancers. *Cancer Immunol. Res.* 2, 823–830. <https://doi.org/10.1158/2326-6066.CIR-14-0112>.
 112. Xu, P., Zhang, G., Hou, S., and Sha, L.-G. (2018). MAPK8 mediates resistance to temozolomide and apoptosis of glioblastoma cells through MAPK signaling pathway. *Biomed. Pharmacother.* 106, 1419–1427. <https://doi.org/10.1016/j.biopha.2018.06.084>.
 113. Joshi, V., Lakhani, S.R., and McCart Reed, A.E. (2022). NDRG1 in Cancer: A Suppressor, Promoter, or Both? *Cancers (Basel)* 14, 5739. <https://doi.org/10.3390/cancers14235739>.
 114. Hutchenreuther, J., Nguyen, J., Quesnel, K., Vincent, K.M., Petitjean, L., Bourgeois, S., Boyd, M., Bou-Gharios, G., Postovit, L.-M., and Leask, A. (2024). Cancer-associated Fibroblast-specific Expression of the Matricellular Protein CCN1 Coordinates Neovascularization and Stroma Deposition in Melanoma Metastasis. *Cancer Res. Commun.* 4, 556–570. <https://doi.org/10.1158/2767-9764.CRC-23-0571>.
 115. Kawai, T., and Akira, S. (2006). Innate immune recognition of viral infection. *Nat. Immunol.* 7, 131–137. <https://doi.org/10.1038/ni1303>.
 116. Montalto, F.I., and De Amicis, F. (2020). Cyclin D1 in Cancer: A Molecular Connection for Cell Cycle Control, Adhesion and Invasion in Tumor and Stroma. *Cells* 9, 2648. <https://doi.org/10.3390/cells9122648>.
 117. Chen, J.-C., Chang, Y.-W., Hong, C.-C., Yu, Y.-H., and Su, J.-L. (2012). The role of the VEGF-C/VEGFRs axis in tumor progression and therapy. *Int. J. Mol. Sci.* 14, 88–107. <https://doi.org/10.3390/ijms14010088>.
 118. Jiang, C., Ding, Z., Joy, M., Chakraborty, S., Kim, S.H., Bottcher, R., Condeelis, J., Singh, S., and Roy, P. (2017). A balanced level of profilin-1 promotes stemness and tumor-initiating potential of breast cancer cells. *Cell Cycle* 16, 2366–2373. <https://doi.org/10.1080/15384101.2017.1346759>.
 119. Schröder, B. (2016). The multifaceted roles of the invariant chain CD74—More than just a chaperone. *Biochim. Biophys. Acta* 1863, 1269–1281. <https://doi.org/10.1016/j.bbamer.2016.03.026>.
 120. Zannikou, M., Duffy, J.T., Levine, R.N., Seblani, M., Liu, Q., Presser, A., Arrieta, V.A., Chen, C.J., Sonabend, A.M., Horbinski, C.M., et al. (2023). IL15 modification enables CAR T cells to act as a dual targeting agent against tumor cells and myeloid-derived suppressor cells in GBM. *J. Immunother. Cancer* 11, e006239. <https://doi.org/10.1136/jitc-2022-006239>.
 121. Tahmasebi, S., Elahi, R., and Esmaeilzadeh, A. (2019). Solid Tumors Challenges and New Insights of CAR T Cell Engineering. *Stem Cell Rev. Rep.* 15, 619–636. <https://doi.org/10.1007/s12015-019-09901-7>.
 122. Yan, T., Zhu, L., and Chen, J. (2023). Current advances and challenges in CAR T-Cell therapy for solid tumors: tumor-associated antigens and the tumor microenvironment. *Exp. Hematol. Oncol.* 12, 14. <https://doi.org/10.1186/s40164-023-00373-7>.
 123. Martinez, M., and Moon, E.K. (2019). CAR T Cells for Solid Tumors: New Strategies for Finding, Infiltrating, and Surviving in the Tumor Microenvironment. *Front. Immunol.* 10, 128. <https://doi.org/10.3389/fimmu.2019.00128>.
 124. Matsuda, J.L., Malleval, T., Scott-Browne, J., and Gapin, L. (2008). CD1d-restricted iNKT cells, the “Swiss-Army knife” of the immune system. *Curr. Opin. Immunol.* 20, 358–368. <https://doi.org/10.1016/j.coi.2008.03.018>.
 125. Mayer, C., Darb-Esfahani, S., Meyer, A.-S., Hübner, K., Rom, J., Sohn, C., Braicu, I., Sehouli, J., Hänsch, G.M., and Gaida, M.M. (2016). Neutrophil Granulocytes in Ovarian Cancer - Induction of Epithelial-To-Mesenchymal-Transition and Tumor Cell Migration. *J. Cancer* 7, 546–554. <https://doi.org/10.7150/jca.14169>.
 126. Xiong, S., Dong, L., and Cheng, L. (2021). Neutrophils in cancer carcinogenesis and metastasis. *J. Hematol. Oncol.* 14, 173. <https://doi.org/10.1186/s13045-021-01187-y>.
 127. Arlauckas, S.P., Garren, S.B., Garriss, C.S., Kohler, R.H., Oh, J., Pittet, M. J., and Weissleder, R. (2018). Arg1 expression defines immunosuppressive subsets of tumor-associated macrophages. *Theranostics* 8, 5842–5854. <https://doi.org/10.7150/thno.26888>.
 128. Revel, M., Sautès-Fridman, C., Fridman, W.-H., and Roumenina, L.T. (2022). C1q+ macrophages: passengers or drivers of cancer progression. *Trends Cancer* 8, 517–526. <https://doi.org/10.1016/j.trecan.2022.02.006>.
 129. Yadav, M.K., Inoue, Y., Nakane-Otani, A., Tsunakawa, Y., Jeon, H., Samir, O., Teramoto, A., Kulathunga, K., Kusakabe, M., Nakamura, M., et al. (2020). Transcription factor MafB is a marker of tumor-associated macrophages in both mouse and humans. *Biochem. Biophys. Res. Commun.* 521, 590–595. <https://doi.org/10.1016/j.bbrc.2019.10.125>.
 130. Gong, Q., Lv, X., Liao, C., Liang, A., Luo, C., Wu, J., Zhou, Y., Huang, Y., and Tong, Z. (2024). Single-cell RNA sequencing combined with proteomics of infected macrophages reveals prothymosin- α as a target for treatment of apical periodontitis. *J. Adv. Res.* 66, 349–361. <https://doi.org/10.1016/j.jare.2024.01.018>.
 131. Jin, F.Y., Nathan, C., Radzioch, D., and Ding, A. (1997). Secretory leukocyte protease inhibitor: a macrophage product induced by and antagonistic to bacterial lipopolysaccharide. *Cell* 88, 417–426. [https://doi.org/10.1016/s0092-8674\(00\)81880-2](https://doi.org/10.1016/s0092-8674(00)81880-2).
 132. Metzloff, A.E., Padilla, M.S., Gong, N., Billingsley, M.M., Han, X., Merolle, M., Mai, D., Figueroa-Espada, C.G., Thatte, A.S., Haley, R.M., et al. (2024). Antigen Presenting Cell Mimetic Lipid Nanoparticles for Rapid mRNA CAR T Cell Cancer Immunotherapy. *Adv. Mater.* 36, e2313226. <https://doi.org/10.1002/adma.202313226>.
 133. Jones, A.E., and Divakaruni, A.S. (2020). Macrophage activation as an archetype of mitochondrial repurposing. *Mol. Aspect. Med.* 71, 100838. <https://doi.org/10.1016/j.mam.2019.100838>.
 134. Neelapu, S.S., Tummala, S., Kebriaei, P., Wierda, W., Gutierrez, C., Locke, F.L., Komanduri, K.V., Lin, Y., Jain, N., Daver, N., et al. (2018). Chimeric antigen receptor T-cell therapy—assessment and management of toxicities. *Nat. Rev. Clin. Oncol.* 15, 47–62. <https://doi.org/10.1038/nrclinonc.2017.148>.

135. Mangan, M.S., Vega-Ramos, J., Joeckel, L.T., Mitchell, A.J., Rizzitelli, A., Roediger, B., Kaiserman, D., Weninger, W.W., Villadangos, J.A., and Bird, P.I. (2017). Serpinb9 is a marker of antigen cross-presenting dendritic cells. *Mol. Immunol.* 82, 50–56. <https://doi.org/10.1016/j.molimm.2016.12.011>.
136. Li, Y.-R., Brown, J., Yu, Y., Lee, D., Zhou, K., Dunn, Z.S., Hon, R., Wilson, M., Kramer, A., Zhu, Y., et al. (2022). Targeting Immunosuppressive Tumor-Associated Macrophages Using Innate T Cells for Enhanced Antitumor Reactivity. *Cancers* 14, 2749. <https://doi.org/10.3390/cancers14112749>.
137. Fan, Q.-M., Jing, Y.-Y., Yu, G.-F., Kou, X.-R., Ye, F., Gao, L., Li, R., Zhao, Q.-D., Yang, Y., Lu, Z.-H., and Wei, L.X. (2014). Tumor-associated macrophages promote cancer stem cell-like properties via transforming growth factor-beta1-induced epithelial-mesenchymal transition in hepatocellular carcinoma. *Cancer Lett.* 352, 160–168. <https://doi.org/10.1016/j.canlet.2014.05.008>.
138. Hoves, S., Ooi, C.-H., Wolter, C., Sade, H., Bissinger, S., Schmittnaegel, M., Ast, O., Giusti, A.M., Wartha, K., Runza, V., et al. (2018). Rapid activation of tumor-associated macrophages boosts preexisting tumor immunity. *J. Exp. Med.* 215, 859–876. <https://doi.org/10.1084/jem.20171440>.
139. Sterner, R.M., Sakemura, R., Cox, M.J., Yang, N., Khadka, R.H., Forsman, C.L., Hansen, M.J., Jin, F., Ayasoufi, K., Hefazi, M., et al. (2019). GM-CSF inhibition reduces cytokine release syndrome and neuroinflammation but enhances CAR-T cell function in xenografts. *Blood* 133, 697–709. <https://doi.org/10.1182/blood-2018-10-881722>.
140. Hill, G.R., Betts, B.C., Tkachev, V., Kean, L.S., and Blazar, B.R. (2021). Current Concepts and Advances in Graft-Versus-Host Disease Immunology. *Annu. Rev. Immunol.* 39, 19–49. <https://doi.org/10.1146/annurev-immunol-102119-073227>.
141. Chang, Y.-J., Zhao, X.-Y., and Huang, X.-J. (2018). Strategies for Enhancing and Preserving Anti-leukemia Effects Without Aggravating Graft-Versus-Host Disease. *Front. Immunol.* 9, 3041. <https://doi.org/10.3389/fimmu.2018.03041>.
142. Tugues, S., Amorim, A., Spath, S., Martin-Blondel, G., Schreiner, B., De Feo, D., Lutz, M., Guscetti, F., Apostolova, P., Haftmann, C., et al. (2018). Graft-versus-host disease, but not graft-versus-leukemia immunity, is mediated by GM-CSF-licensed myeloid cells. *Sci. Transl. Med.* 10, eaat8410. <https://doi.org/10.1126/scitranslmed.aat8410>.
143. Shlomchik, W.D. (2007). Graft-versus-host disease. *Nat. Rev. Immunol.* 7, 340–352. <https://doi.org/10.1038/nri2000>.
144. Chen, Y., Zhu, Y., Kramer, A., Fang, Y., Wilson, M., Li, Y.-R., and Yang, L. (2023). Genetic engineering strategies to enhance antitumor reactivity and reduce alloreactivity for allogeneic cell-based cancer therapy. *Front. Med.* 10, 1135468. <https://doi.org/10.3389/fmed.2023.1135468>.
145. Lanza, R., Russell, D.W., and Nagy, A. (2019). Engineering universal cells that evade immune detection. *Nat. Rev. Immunol.* 19, 723–733. <https://doi.org/10.1038/s41577-019-0200-1>.
146. Wagner, D.L., Fritsche, E., Pulsipher, M.A., Ahmed, N., Hamieh, M., Hegde, M., Ruella, M., Savoldo, B., Shah, N.N., Turtle, C.J., et al. (2021). Immunogenicity of CAR T cells in cancer therapy. *Nat. Rev. Clin. Oncol.* 18, 379–393. <https://doi.org/10.1038/s41571-021-00476-2>.
147. Fang, Y., Zhu, Y., Kramer, A., Chen, Y., Li, Y.-R., and Yang, L. (2023). Graft-versus-Host Disease Modulation by Innate T Cells. *Int. J. Mol. Sci.* 24, 4084. <https://doi.org/10.3390/ijms24044084>.
148. Pillai, A.B., George, T.I., Dutt, S., Teo, P., and Strober, S. (2007). Host NKT Cells Can Prevent Graft-versus-Host Disease and Permit Graft Antitumor Activity after Bone Marrow Transplantation. *J. Immunol.* 178, 6242–6251. <https://doi.org/10.4049/jimmunol.178.10.6242>.
149. Ren, J., Liu, X., Fang, C., Jiang, S., June, C.H., and Zhao, Y. (2017). Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition. *Clin. Cancer Res.* 23, 2255–2266. <https://doi.org/10.1158/1078-0432.CCR-16-1300>.
150. Paul, S., and Lal, G. (2017). The molecular mechanism of natural killer cells function and its importance in cancer immunotherapy. *Front. Immunol.* 8, 1124. <https://doi.org/10.3389/fimmu.2017.01124>.
151. Torikai, H., Reik, A., Soldner, F., Warren, E.H., Yuen, C., Zhou, Y., Crossland, D.L., Huls, H., Littman, N., Zhang, Z., et al. (2013). Toward eliminating HLA class I expression to generate universal cells from allogeneic donors. *Blood* 122, 1341–1349. <https://doi.org/10.1182/blood-2013-03-478255>.
152. Braud, V.M., Allan, D.S., O'Callaghan, C.A., Söderström, K., D'Andrea, A., Ogg, G.S., Lazetic, S., Young, N.T., Bell, J.I., Phillips, J.H., et al. (1998). HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 391, 795–799.
153. Suárez-Alvarez, B., Rodríguez, R.M., Calvanese, V., Blanco-Gelaz, M.A., Suhr, S.T., Ortega, F., Otero, J., Cibelli, J.B., Moore, H., Fraga, M.F., and López-Larrea, C. (2010). Epigenetic mechanisms regulate MHC and antigen processing molecules in human embryonic and induced pluripotent stem cells. *PLoS One* 5, e10192. <https://doi.org/10.1371/journal.pone.0010192>.
154. Majumder, P., and Boss, J.M. (2011). DNA methylation dysregulates and silences the HLA-DQ locus by altering chromatin architecture. *Genes Immun.* 12, 291–299. <https://doi.org/10.1038/gene.2010.77>.
155. Liu, X., Ye, L., Bai, Y., Mojidi, H., Simister, N.E., and Zhu, X. (2008). Activation of the JAK/STAT-1 signaling pathway by IFN-gamma can down-regulate functional expression of the MHC class I-related neonatal Fc receptor for IgG. *J. Immunol.* 181, 449–463. <https://doi.org/10.4049/jimmunol.181.1.449>.
156. Hu, X., Li, J., Fu, M., Zhao, X., and Wang, W. (2021). The JAK/STAT signaling pathway: from bench to clinic. *Signal Transduct. Targeted Ther.* 6, 402. <https://doi.org/10.1038/s41392-021-00791-1>.
157. Liddle, F.J., Alvarez, J.V., Poli, V., and Frank, D.A. (2006). Tyrosine phosphorylation is required for functional activation of disulfide-containing constitutively active STAT mutants. *Biochemistry* 45, 5599–5605. <https://doi.org/10.1021/bi0525674>.
158. Steffin, D., Ghatwai, N., Montalbano, A., Rathi, P., Courtney, A.N., Arnett, A.B., Fleurence, J., Sweidan, R., Wang, T., Zhang, H., et al. (2025). Interleukin-15-armoured GPC3 CAR T cells for patients with solid cancers. *Nature* 637, 940–946. <https://doi.org/10.1038/s41586-024-08261-8>.
159. Tian, G., Courtney, A.N., Yu, H., Bhar, S., Xu, X., Barragán, G.A., Martínez Amador, C., Ghatwai, N., Wood, M.S., Schady, D., et al. (2025). Hyperleukocytosis in a neuroblastoma patient after treatment with natural killer T cells expressing a GD2-specific chimeric antigen receptor and IL-15. *J. Immunother. Cancer* 13, e010156. <https://doi.org/10.1136/jitc-2024-010156>.
160. Domínguez-Prieto, V., Qian, S., Villarejo-Campos, P., Meliga, C., González-Soares, S., Guijo Castellano, I., Jiménez-Galanes, S., García-Arranz, M., Guadalajara, H., and García-Olmo, D. (2023). Understanding CAR T cell therapy and its role in ovarian cancer and peritoneal carcinomatosis from ovarian cancer. *Front. Oncol.* 13, 1104547. <https://doi.org/10.3389/fonc.2023.1104547>.
161. Tong, C., Zhang, Y., Liu, Y., Ji, X., Zhang, W., Guo, Y., Han, X., Ti, D., Dai, H., Wang, C., et al. (2020). Optimized tandem CD19/CD20 CAR-engineered T cells in refractory/relapsed B-cell lymphoma. *Blood* 136, 1632–1644. <https://doi.org/10.1182/blood.2020005278>.
162. Shah, N.N., Zhu, F., Taylor, C., Schneider, D., Krueger, W., Worden, A., Yim, S., Fenske, T.S., Hamadani, M., Johnson, B., et al. (2018). A Phase 1 Study with Point-of-Care Manufacturing of Dual Targeted, Tandem Anti-CD19, Anti-CD20 Chimeric Antigen Receptor Modified T (CAR-T) Cells for Relapsed, Refractory, Non-Hodgkin Lymphoma. *Blood* 132, 4193. <https://doi.org/10.1182/blood-2018-99-110194>.
163. Dagher, O.K., and Posey, A.D. (2023). Forks in the road for CAR T and CAR NK cell cancer therapies. *Nat. Immunol.* 24, 1994–2007. <https://doi.org/10.1038/s41590-023-01659-y>.
164. van Stein, R.M., Aalbers, A.G.J., Sonke, G.S., and van Driel, W.J. (2021). Hyperthermic Intraperitoneal Chemotherapy for Ovarian and Colorectal

- Cancer: A Review. *JAMA Oncol.* 7, 1231–1238. <https://doi.org/10.1001/jamaoncol.2021.0580>.
165. Haas, A.R., Golden, R.J., Litzky, L.A., Engels, B., Zhao, L., Xu, F., Taraszka, J.A., Ramones, M., Granda, B., Chang, W.-J., et al. (2023). Two cases of severe pulmonary toxicity from highly active mesothelin-directed CAR T cells. *Mol. Ther.* 31, 2309–2325. <https://doi.org/10.1016/j.ymthe.2023.06.006>.
166. Leclercq, G., Steinhoff, N., Haegel, H., De Marco, D., Bacac, M., and Klein, C. (2022). Novel strategies for the mitigation of cytokine release syndrome induced by T cell engaging therapies with a focus on the use of kinase inhibitors. *OncolImmunology* 11, 2083479. <https://doi.org/10.1080/2162402X.2022.2083479>.
167. Li, Y.-R., Fang, Y., Niu, S., Zhu, Y., Chen, Y., Lyu, Z., Zhu, E., Tian, Y., Huang, J., Rezek, V., et al. (2025). Allogeneic CD33-directed CAR-NKT cells for the treatment of bone marrow-resident myeloid malignancies. *Nat. Commun.* 16, 1248. <https://doi.org/10.1038/s41467-025-56270-6>.
168. Landoni, E., Woodcock, M.G., Barragan, G., Casirati, G., Cinella, V., Stucchi, S., Flick, L.M., Withers, T.A., Hudson, H., Casorati, G., et al. (2024). IL-12 reprograms CAR-expressing natural killer T cells to long-lived Th1-polarized cells with potent antitumor activity. *Nat. Commun.* 15, 89. <https://doi.org/10.1038/s41467-023-44310-y>.
169. Chmielewski, M., and Abken, H. (2017). CAR T Cells Releasing IL-18 Convert to T-Bet(high) FoxO1(low) Effectors that Exhibit Augmented Activity against Advanced Solid Tumors. *Cell Rep.* 21, 3205–3219. <https://doi.org/10.1016/j.celrep.2017.11.063>.
170. Svoboda, J., Landsburg, D.J., Gerson, J., Nasta, S.D., Barta, S.K., Chong, E.A., Cook, M., Frey, N.V., Shea, J., Cervini, A., et al. (2025). Enhanced CAR T-Cell Therapy for Lymphoma after Previous Failure. *N. Engl. J. Med.* 392, 1824–1835. <https://doi.org/10.1056/NEJMoa2408771>.
171. Lan, P., Tonomura, N., Shimizu, A., Wang, S., and Yang, Y.G. (2006). Reconstitution of a functional human immune system in immunodeficient mice through combined human fetal thymus/liver and CD34+ cell transplantation. *Blood* 108, 487–492. <https://doi.org/10.1182/blood-2005-11-4388>.
172. Smith, D.J., Liu, S., Ji, S., Li, B., McLaughlin, J., Cheng, D., Witte, O.N., and Yang, L. (2015). Genetic engineering of hematopoietic stem cells to generate invariant natural killer T cells. *Proc. Natl. Acad. Sci. USA* 112, 1523–1528. <https://doi.org/10.1073/pnas.1424877112>.
173. Haas, A.R., Tanyi, J.L., O'Hara, M.H., Gladney, W.L., Lacey, S.F., Torigian, D.A., Soulen, M.C., Tian, L., McGarvey, M., Nelson, A.M., et al. (2019). Phase I Study of Lentiviral-Transduced Chimeric Antigen Receptor-Modified T Cells Recognizing Mesothelin in Advanced Solid Cancers. *Mol. Ther.* 27, 1919–1929. <https://doi.org/10.1016/j.ymthe.2019.07.015>.
174. Wu, C., Molavi, O., Zhang, H., Gupta, N., Alshareef, A., Bone, K.M., Gopal, K., Wu, F., Lewis, J.T., Douglas, D.N., et al. (2015). STAT1 is phosphorylated and downregulated by the oncogenic tyrosine kinase NPM-ALK in ALK-positive anaplastic large-cell lymphoma. *Blood* 126, 336–345. <https://doi.org/10.1182/blood-2014-10-603738>.
175. Butler, A., Hoffman, P., Smibert, P., Papalexi, E., and Satija, R. (2018). Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* 36, 411–420. <https://doi.org/10.1038/nbt.4096>.
176. Tran, H.T.N., Ang, K.S., Chevrier, M., Zhang, X., Lee, N.Y.S., Goh, M., and Chen, J. (2020). A benchmark of batch-effect correction methods for single-cell RNA sequencing data. *Genome Biol.* 21, 12. <https://doi.org/10.1186/s13059-019-1850-9>.
177. Jia, C., Hu, Y., Kelly, D., Kim, J., Li, M., and Zhang, N.R. (2017). Accounting for technical noise in differential expression analysis of single-cell RNA sequencing data. *Nucleic Acids Res.* 45, 10978–10988. <https://doi.org/10.1093/nar/gkx754>.
178. Yu, G., Wang, L.-G., Han, Y., and He, Q.-Y. (2012). clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* 16, 284–287. <https://doi.org/10.1089/omi.2011.0118>.
179. Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., Feng, T., Zhou, L., Tang, W., Zhan, L., et al. (2021). clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *Innovation* 2, 100141. <https://doi.org/10.1016/j.xinn.2021.100141>.
180. La Manno, G., Soldatov, R., Zeisel, A., Braun, E., Hochgerner, H., Petukhov, V., Lidschreiber, K., Kastrioti, M.E., Lönnerberg, P., Furlan, A., et al. (2018). RNA velocity of single cells. *Nature* 560, 494–498. <https://doi.org/10.1038/s41586-018-0414-6>.
181. Jin, S., Guerrero-Juarez, C.F., Zhang, L., Chang, I., Ramos, R., Kuan, C.-H., Myung, P., Plikus, M.V., and Nie, Q. (2021). Inference and analysis of cell-cell communication using CellChat. *Nat. Commun.* 12, 1088. <https://doi.org/10.1038/s41467-021-21246-9>.
182. Krueger, F., and Andrews, S.R. (2011). Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics* 27, 1571–1572. <https://doi.org/10.1093/bioinformatics/btr167>.
183. Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R.; 1000 Genome Project Data Processing Subgroup (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078–2079. <https://doi.org/10.1093/bioinformatics/btp352>.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human IFN- γ (ELISA, capture; Clone NIB42)	BD Biosciences	CAT#551221, RRID: AB_394099
Anti-human IFN- γ (ELISA, detection; Clone 4S.B3)	BD Biosciences	CAT#554550, RRID: AB_395472
Anti-human TNF- α (ELISA, capture; Clone MAb1)	BD Biosciences	CAT#551220, RRID: AB_394098
Anti-human TNF- α (ELISA, detection; Clone MAb11)	BD Biosciences	CAT#554511, RRID: AB_395442
Anti-human IL-2 (ELISA, capture; Clone MQ1-17H12)	BD Biosciences	CAT#554563; RRID: AB_398570
Anti-human IL-2 (ELISA, detection; Clone B33-2)	BD Biosciences	CAT#555040; RRID: AB_395666
Anti-human IL-4 (ELISA, capture; Clone 8D4-8)	BD Biosciences	CAT#554515; RRID: AB_398567
Anti-human IL-4 (ELISA, detection; Clone MP4-25D2)	BD Biosciences	CAT#554483; RRID: AB_395422
Anti-human IL-15 (ELISA, capture; Clone G243-935)	BD Biosciences	CAT#554712; RRID: AB_2561319
Anti-human IL-15 (ELISA, detection; Clone G243-886)	BD Biosciences	CAT#554713; RRID: AB_2561320
Anti-mouse IL-6 (ELISA, capture; Clone MP5-20F3)	BD Biosciences	CAT#554400; RRID: AB_315336
Anti-mouse IL-6 (ELISA, detection; Clone MP5-32C11)	BD Biosciences	CAT#554402; RRID: AB_2127458
Anti-human CD45 (Clone HI30)	Biolegend	CAT#304026, RFID: AB_893337
Anti-human TCR $\alpha\beta$ (Clone IP26)	Biolegend	CAT#306716, RRID: AB_1953257
Anti-human CD3 (Clone HIT3a)	Biolegend	CAT#300330, RRID: AB_10552893
Anti-human CD4 (Clone OKT4)	Biolegend	CAT#317414, RRID: AB_571959
Anti-human CD8 (Clone SK1)	Biolegend	CAT#344714, RRID: AB_2044006
Anti-human CD45RO (Clone UCHL1)	Biolegend	CAT#304216, RRID: AB_493659
Anti-human CD161 (Clone HP-3G10)	Biolegend	CAT#339928, RRID: AB_2563967
Anti-human CD69 (Clone FN50)	Biolegend	CAT#310914, RRID: AB_314849
Anti-human CD56 (Clone HCD56)	Biolegend	CAT#318304, RRID: AB_604100
Anti-human CD62L (Clone DREG-56)	Biolegend	CAT#304822, RRID: AB_830801
Anti-human CD14 (Clone HCD14)	Biolegend	CAT#325608, RRID: AB_830681
Anti-human CD11b (Clone ICRF44)	Biolegend	CAT#301330, RRID: AB_2561703
Anti-human CD11c (Clone Bu15)	Biolegend	CAT#337234, RRID: AB_2566656
Anti-human CD1d (Clone 51.1)	Biolegend	CAT#350308, RRID: AB_10642829
Anti-human CD206 (Clone 15-2)	Biolegend	CAT#321110, RRID: AB_571885
Anti-human CD163 (Clone GHI/61)	Biolegend	CAT#333621, RRID: AB_2563611
Anti-human CD19 (Clone SJ25C1)	Biolegend	CAT#363006, RRID: AB_2564127
Anti-human CD20 (Clone 2H7)	Biolegend	CAT#302306, RRID: AB_2924581
Anti-human NKG2D (Clone 1D11)	Biolegend	CAT#320812, RRID: AB_2234394
Anti-human DNAM-1 (Clone 11A8)	Biolegend	CAT#338312, RRID: AB_2561952
Anti-human IFN- γ (Clone B27)	Biolegend	CAT#506518, RRID: AB_2123321

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-human Granzyme B (Clone QA16A02)	Biologend	CAT#372204, RRID: AB_2687028
Anti-human Perforin (Clone dG9)	Biologend	CAT#308126, RRID: AB_2572049
Anti-human TNF- α (Clone MAb11)	Biologend	CAT#502912, RRID: AB_315264
Anti-human IL-2 (Clone MQ1-17H12)	Biologend	CAT#500342, RRID: AB_2562854
Anti-human β 2-microglobulin (B2M) (Clone 2M2)	Biologend	CAT#316312, RRID: AB_10641281
Anti-human HLA-DR (Clone L243)	Biologend	CAT#307618, RRID: AB_493586
Anti-human MICA/B (Clone 6D4)	Biologend	CAT#320908, RRID: AB_493195
Anti-human HLA-A,B,C (Clone W6/32)	Biologend	CAT#311422, RRID: AB_1501265
Anti-human TCR V δ 2 (Clone B6)	Biologend	CAT#331418, RRID: AB_2687323
Anti-mouse CD45 (Clone QA17A26)	Biologend	CAT#157608, RRID: AB_2832554
Anti-mouse CD11b (Clone M1/70)	Biologend	CAT#101206, RRID: AB_312789
Anti-mouse Gr-1 (Clone RB6-8C5)	Biologend	CAT#108412, RRID: AB_313377
Anti-mouse F4/80 (Clone BM8)	Biologend	CAT#123126, RRID: AB_893495
Anti-human CD107a (Clone H4A3)	Biologend	CAT#328620, RRID: AB_1279055
Anti-human CD34 (Clone 581)	BD Biosciences	CAT#555822, RRID: AB_396151
Anti-human TCR V α 24-J β 18 (Clone 6B11)	BD Biosciences	CAT#552825, RRID: AB_394478
Anti-human mesothelin (MSLN) (Clone 420411)	R&D Systems	CAT#FAB32652P, RRID: AB_1151946
Anti-human fibroblast activation protein alpha (FAP) (Clone 427819)	R&D Systems	CAT# MAB3715-SP
Anti-human ULBP-1 (Clone 170818)	R&D Systems	CAT#FAB1380P, RRID: AB_2687471
Anti-human ULBP-2,5,6 (Clone 165903)	R&D Systems	CAT#FAB1298A, RRID: AB_2257142
Anti-human V β 11	Beckman-Coulter	CAT#A66905
Human Fc Receptor Blocking Solution (TruStain FcX)	Biologend	CAT#422302
Mouse Fc Block (anti-mouse CD16/32)	BD Biosciences	CAT#553142, RRID: AB_394657
Ultra-LEAF purified anti-human CD1d antibody (Clone 51.1)	Biologend	CAT#350322
Ultra-LEAF purified Mouse IgG2b, k isotype ctrl (Clone MG2b-57)	Biologend	CAT#401220
Anti-human NKG2D antibody (Clone 1D11)	Biologend	CAT#320806, RRID: AB_492960
Mouse IgG1, κ isotype control antibody (Clone MOPC-21)	Biologend	CAT#981804
Anti-human DNAM-1 antibody (Clone DX11)	BD Biosciences	CAT#559787, RRID: AB_397327
CIITA Polyclonal Antibody	Invitrogen	CAT#PA5-21031, RRID: AB_11154145
Anti-human p-STAT1(Y701) (Clone 58D6)	Cell Signaling Technology	CAT#9167, RRID: AB_561284
Anti-human STAT1 (Clone D1K9Y)	Cell Signaling Technology	CAT#14994, RRID: AB_2737027
Anti-human IRF-1 (Clone D5E4)	Cell Signaling Technology	CAT#8478, RRID: AB_10949108
Anti-human SP1 (Clone D4C3)	Cell Signaling Technology	CAT#9389, RRID: AB_11220235
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling Technology	CAT#7074, RRID: AB_2099233
Anti- β -Actin (Clone D6A8)	Cell Signaling Technology	CAT#8457, RRID: AB_10950489
Anti-GAPDH (Clone D16H11)	Cell Signaling Technology	CAT#5174, RRID: AB_10622025
Bacterial and virus strains		
Lenti/iNKT-MCAR	This paper	N/A
Lenti/iNKT-MCAR-IL15	This paper	N/A
Lenti/MCAR	This paper	N/A
Lenti/MCAR-IL15	This paper	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Lenti/FG	This paper	N/A
Lenti/CD1d	This paper	N/A
Lenti/MSLN	This paper	N/A
Lenti/STAT1C-EGFP	This paper	N/A
Biological samples		
Human peripheral blood mononuclear cells (PBMCs)	UCLA	N/A
Primary ovarian cancer (OC) patient samples	UCLA	N/A
Cord Blood CD34 ⁺ Stem/Progenitor Cells	HemaCare (Charles River)	CAT#CB34C-3
Chemicals, peptides, and recombinant proteins		
Streptavidin-HRP conjugate	Invitrogen	CAT#SA10001
IFN- γ (ELISA, standard)	eBioscience	CAT#29-8319-65
TNF α (ELISA, standard)	eBioscience	CAT#29-8329-65
IL-2 (ELISA, standard)	eBioscience	CAT#29-8029-65
IL-4 (ELISA, standard)	eBioscience	CAT#39-8049-65
IL-17A (ELISA, standard)	eBioscience	CAT#29-8179-65
Tetramethylbenzidine (TMB)	KPL	CAT#5120-0053
Ganciclovir (GCV)	Sigma	CAT#G2536
α -Galactosylceramide (KRN7000)	Avanti Polar Lipids	SKU#867000P-1mg
Recombinant human IL-2	Peptotech	CAT#200-02
Recombinant human IL-3	Peptotech	CAT#200-03
Recombinant human IL-4	Peptotech	CAT#200-04
Recombinant human IL-7	Peptotech	CAT#200-07
Recombinant human IL-13	Peptotech	CAT#200-13
Recombinant human IL-15	Peptotech	CAT#200-15
Recombinant human IFN- γ	Peptotech	CAT#300-02
Recombinant human Flt3-Ligand	Peptotech	CAT#300-19
Recombinant human SCF	Peptotech	CAT#300-07
Recombinant human TPO	Peptotech	CAT#300-18
Recombinant human GM-CSF	Peptotech	CAT#300-03
Recombinant human M-CSF	Peptotech	CAT#300-25
L-ascorbic acid 2-phosphate	Sigma	CAT#A8960-5G
B27 TM Supplement (50X), serum free	ThermoFisher	CAT#17504044
Cas9-NLS purified protein	UC Berkeley	N/A
X-VIVO 15 Serum-free Hematopoietic Cell Medium	Lonza	CAT#04-418Q
RPMI1640 cell culture medium	Corning Cellgro	CAT#10-040-CV
DMEM cell culture medium	Corning Cellgro	CAT#10-013-CV
Fetal Bovine Serum (FBS)	Sigma	CAT#F2442
MACS BSA stock solution	Miltenyi	CAT#130-091-376
30% BSA	Gemini	CAT#700-110-100
Penicillin-Streptomycin-Glutamine (P/S/G)	Gibco	CAT#10378016
Penicillin: streptomycin (pen:strep) solution (P/S)	Gemini Bio-products	CAT#400-109-100
MEM non-essential amino acids (NEAA)	Gibco	CAT#11140050
HEPES Buffer Solution	Gibco	CAT#15630080
Sodium Pyruvate	Gibco	CAT#11360070
Beta-Mercaptoethanol	Sigma	SKU#M6250

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Normocin	Invivogen	CAT#ant-nr-2
Fixable Viability Dye eFluor506	eBioscience	CAT#65-0866-14
Cell Fixation/Permeabilization Kit	BD Biosciences	CAT#554714
RetroNectin recombination human fibronectin fragment, 2.5mg	Takara	CAT#T100B
10% neutral-buffered formalin	Richard-Allan Scientific	CAT#5705
D-Luciferin Potassium Salt Bioluminescent Substrate	Revivi	CAT#122799-100MG
Fluriso (Isoflurane)	Vet One	CAT#502017
Phosphate Buffered Saline (PBS) pH 7.4 (1X)	Gibco	CAT#10010-023
Formaldehyde	Sigma-Aldrich	CAT#F8775
Golgistop Protein Transport Inhibitor	BD Biosciences	CAT#554724
Poloxamer Synperonic F108	Millipore Sigma	CAT#07579-250G-F
Prostaglandin E2 (PGE2)	Cayman Chemical	CAT#14010
TrypLE™ Express Enzyme	ThermoFisher Scientific	CAT#12605010
Phorbol-12-myristate-13-acetate (PMA)	Sigma	CAT#524400
Ionomycin, Calcium salt, <i>Streptomyces conglobatus</i>	Sigma	CAT#407952

Critical commercial assays

Human NK Cell Isolation Kit	Miltenyi Biotec	CAT#130-092-657
Human CD34 MicroBeads Kit	Miltenyi Biotec	CAT#130-046-703
Human CD14 MicroBeads Kit	Miltenyi Biotec	CAT#130-050-201
Human Anti-NKT MicroBeads	Miltenyi Biotec	CAT#130-094-842
Human Anti-HLA-DR MicroBeads	Miltenyi Biotec	CAT#130-046-101
Human tumor cell isolation kit	Miltenyi Biotec	CAT#130-108-339
Fixation/Permeabilization Solution Kit	BD Sciences	CAT#55474
Amata™ P3 Primary Cell 4D-Nucleofector™ X Kit S	Lonza	CAT#V4XP-3032
Dynabeads Human T-Activator CD3/CD28	ThermoFisher	CAT#11131D
miRNeasy Mini Kit	Qiagen	CAT#217004
Chromium single cell V(D)J enrichment kit, human T cell	10 x Genomics	CAT#1000005
Cryostor cell cryopreservation media	Sigma	CAT#C2874-100ML
StemSpan™ Lymphoid Differentiation Coating Material (100X)	Stem Cell Technologies	CAT#9925
StemSpan™ SFEM II	Stem Cell Technologies	CAT#9605
ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator	Stem Cell Technologies	CAT#10970
TransIT-Lenti Transfection Reagent	Mirus Bio	CAT#MIR 6600
Amicon Ultra-15 Centrifugal Filter Unit	Sigma	CAT#UFC910024
Nunclon Sphera-Treated 96-Well Microplate	ThermoFisher	CAT#174929
CellQART 6-Well Cell Culture Insert	Millicell	CAT#9301002
Human IL-17A ELISA MAX Deluxe Kit	Biolegend	CAT#433915

Deposited data

scRNA-seq for the primary OC patient sample profiling study	This paper	GEO: GSE272764
scRNA-seq for the <i>in vivo</i> therapeutic cell profiling study	This paper	GEO: GSE270820

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
scRNA-seq for the <i>in vivo</i> tumor cell profiling study	This paper	GEO: GSE255431
scRNA-seq for the <i>in vivo</i> TME profiling study	This paper	GEO: GSE268749
scTCR-seq	This paper	GEO: GSE254262
Bulk RNA-seq (dataset 1)	This paper	GEO: GSE252379
Bulk RNA-seq (dataset 2)	This paper	GEO: GSE164425
Methyl-seq	This paper	GEO: GSE251909
scRNA-seq for primary OC patient sample profiling study (public dataset B)	Zhang et al. ⁵⁰	GEO: GSE165897
scRNA-seq for primary OC patient sample profiling study (public dataset C)	Xu et al. ⁴⁹	GEO: GSE184800
scRNA-seq for primary OC patient sample profiling study (public dataset D)	Zheng et al. ³⁶	GSA-Human, PRJCA005422
Experimental models: Cell lines		
Human multiple myeloma (MM) cell line MM.1S	ATCC	CRL-2974
Human chronic myelogenous leukemia cancer cell line K562	ATCC	CCL-243
Human ovarian cancer cell line OVCAR3	ATCC	HTB-161
Human ovarian cancer cell line OVCAR8	NIH	N/A
Human ovarian cancer cell line SKOV3	ATCC	HTB-77
Human multiple myeloma (MM) cell line MM.1S-FG	This paper	N/A
Human multiple myeloma (MM) cell line MM.1S-MSLN-FG	This paper	N/A
Human ovarian cancer cell line OVCAR3-FG	This paper	N/A
Human ovarian cancer cell line OVCAR3-FG ^{MSLN-/-}	This paper	N/A
Human ovarian cancer cell line OVCAR8-FG	This paper	N/A
Human ovarian cancer cell line SKOV3-FG	This paper	N/A
Human artificial APC cell line	This paper	N/A
Experimental models: Organisms/strains		
NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG)	The Jackson Laboratory	Stock #: 005557
Oligonucleotides		
gRNA (<i>MSLN</i>): GCCAACGGCTCGAC CCCTGT	Synthego	N/A
Recombinant DNA		
Vector: parental lentivector pMNDW	Giannoni et al. ⁶⁷ ; Lan et al. ¹⁷¹	N/A
Software and algorithms		
FlowJo Software	FlowJo	https://www.flowjo.com/solutions/flowjo/downloads
AURA imaging software	Spectral Instruments Imaging	https://spectralin vivo.com/software/
ImageJ	ImageJ	https://imagej.net/Downloads
Prism 9	Graphpad	https://www.graphpad.com/scientific-software/prism/
Matlab	The MathWorks, Inc	http://www.mathworks.com/products/matlab.html
R	R	http://www.R-project.org/
RStudio	RStudio	https://posit.co

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

This study utilized the NOD.Cg-Prkdc^{SCID}Il2rg^{tm1Wjl}/SzJ (NOD/SCID/IL-2Rγ^{-/-}, NSG) mice housed by the animal facilities of the University of California, Los Angeles (UCLA). The mice used in the experiments aged between 6–10 weeks, unless otherwise specified. Although female mice are physiologically more relevant for modeling ovarian cancer, no significant differences were observed between female and male mice in the peritoneal ovarian cancer models used in this study, including tumor burden, immune cell infiltration, and therapeutic response. All protocols of animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of UCLA. All mice were housed under specific pathogen-free conditions, and all experimental procedures were conducted in accordance with the regulations established by the Division of Laboratory Animal Medicine (DLAM) at UCLA.

Cell lines

Human multiple myeloma cancer cell line MM.1S, human ovarian cancer cell lines SKOV3 and OVCAR3, and human embryonic kidney (HEK) cell line 293T were obtained from the American Type Culture Collection (ATCC). Human ovarian cancer cell line OVCAR8 was generously provided by the Division of Cancer Treatment and Diagnosis (DCTD) Tumor Repository at the National Institutes of Health (NIH). To generate stable tumor cell lines overexpressing mesothelin (MSLN) and/or the firefly luciferase (Fluc) and enhanced green fluorescent protein (EGFP) dual-reporters (FG), the parental tumor cell lines were transduced with lentiviral vectors encoding the intended gene(s). 72h post lentivector transduction, tumor cells were subjected to flow cytometry sorting to isolate gene-engineered cells for making stable cell lines. Five stable tumor cell lines were generated for this study, including MM.1S-FG, MM.1S-MSLN-FG, SKOV3-FG, OVCAR3-FG, and OVCAR8-FG. The OVCAR3-FG^{MSLN^{-/-}} cell line was generated by knocking out the MSLN gene from the parental OVCAR3-FG cell line using CRISPR/Cas9. The single guide RNA (sgRNA) targeting the *MSLN* gene (GCCAACGGCTCGACCCCTGT) was designed and purchased from Synthego, and was introduced into OVCAR3-FG cells via electroporation using an Amaxa 4D Nucleofection X Unit (Lonza), according to the manufacturer's instructions. All tumor cell lines used in this study were tested mycoplasma-free and frequently STR verified during experiments.

Human cord blood CD34⁺ hematopoietic stem and progenitor cells (HSPCs) and peripheral blood mononuclear cells (PBMCs)

Purified CB CD34⁺ HSPCs were purchased from HemaCare (now a Charles River company). Healthy donor PBMCs were obtained from the UCLA/CFAR Virology Core Laboratory, without identification information under federal and state regulations. CD34⁺ HSPCs and PBMCs were cryopreserved in Cryostor CS10 (Sigma St. Louis, MO, USA) using CoolCell (BioCision, Larkspur, CA, USA), and were frozen in liquid nitrogen for storage and to supply all experiments.

Primary ovarian cancer patient samples

Primary OC patient ascites or pleural fluid samples were collected from patients who provided informed consent under protocols approved by the UCLA Office of the Human Research Protection Program (IRB#10-0727 and IRB#20-1659). Clinical information was obtained from the medical records. The study population consisted of platinum resistant and platinum sensitive high grade serous ovarian cancer (HGSOC) patients who were chemo-naïve, post-neoadjuvant chemotherapy or recurrent. A total of 35 primary OC samples were studied, and the sample information are described in [Table S1](#).

The determination of platinum sensitivity of primary OC samples was described previously.²¹ Chemo-naïve samples were collected from patients with new diagnosis of HGSOC and no prior exposure to chemotherapy. All patients received platinum-based chemotherapy. Disease recurrence in chemo-naïve patient samples were prospectively assessed to determine platinum sensitivity. Specimens from those patients with disease relapse < 6 months after the last platinum-based chemotherapy infusion were considered platinum resistant. Specimens collected from patients who did not experience relapse of disease or from patients who experienced disease relapse ≥ 6 months after the last platinum-based chemotherapy were categorized as platinum sensitive. On the other hand, recurrent samples were collected from patients who have experienced relapse of disease following primary treatment. Past treatment history from recurrent patient samples was reviewed retrospectively to determine platinum sensitivity. If at the time of the collection of a recurrent tumor, the disease had relapsed ≥ 6 months after the last platinum-based chemotherapy infusion, the specimen was categorized as platinum sensitive. If at the time of the collection of a recurrent tumor, the patient had evidence of recurrent disease < 6 months after the last platinum-based chemotherapy at any point during course of therapy or had evidence of disease progression on platinum drugs, the specimen was categorized platinum resistant.

The OC sample processing was described previously.²¹ Briefly, freshly-collected OC samples were centrifuged, and then resuspended in RBC Lysis Buffer (BioLegend) to lyse red blood cells. The RBC-lysed samples were analyzed immediately or cryopreserved for long-term storage and future analyses. To prepare single cells, each OC sample was resuspended in 10 mL of DMEM medium supplemented with 2 mg/ml Collagenase Type I (ThermoFisher Scientific), 2 mg/ml Dispase II (ThermoFisher Scientific), and 1 mg/ml DNase I (Sigma-Aldrich), and incubated at 37°C for 60 minutes with mechanical agitation. The resulting OC sample single cells were utilized for relevant assays.

Media and reagents

The X-VIVO 15 Serum-Free Hematopoietic Cell Medium was purchased from Lonza. The StemSpan™ T Cell Generation Kit, comprising the StemSpan™ SFEM II Medium, the StemSpan™ Lymphoid Progenitor Expansion Supplement, the StemSpan™ Lymphoid Progenitor Maturation Supplement, the StemSpan™ Lymphoid Progenitor Differentiation Coating Material, and the ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator, was purchased from StemCell Technologies. The CTS™ OpTmizer™ T-Cell Expansion SFM (no phenol red, bottle format), the RPMI 1640 cell culture medium, and the DMEM cell culture medium were purchased from ThermoFisher Scientific. The CryoStor® Cell Cryopreservation Media CS10 was purchased from MilliporeSigma.

α -Galactosylceramide (α -GalCer or α GC, KR7000) was purchased from Avanti Polar Lipids. Recombinant human IL-2, IL-3, IL-7, IL-15, IL-21, IFN- γ , Flt3 ligand (Flt3L), stem cell factor (SCF), and thrombopoietin (TPO) were purchased from Peprotech. Fetal Bovine Serum (FBS) and Beta-Mercaptoethanol (β -ME) were purchased from Sigma. Penicillin-Streptomycin-Glutamine (P/S/G), MEM non-essential amino acids (NEAA), HEPES Buffer Solution, and Sodium Pyruvate were purchased from Gibco. Normocin was purchased from Invivogen.

The homemade C10 medium for culturing immune cells was made of RPMI 1640 cell culture medium, supplemented with FBS (10% vol/vol), P/S/G (1% vol/vol), MEM NEAA (1% vol/vol), HEPES (10 mM), Sodium Pyruvate (1 mM), β -ME (50 μ M), and Normocin (100 μ g/ml). The homemade D10 medium for culturing the HEK 293T cells was made of DMEM supplemented with FBS (10% vol/vol), P/S/G (1% vol/vol), and Normocin (100 μ g/ml). The homemade R10 medium for culturing human tumor cell lines was made of RPMI supplemented with FBS (10% vol/vol), P/S/G (1% vol/vol), and Normocin (100 μ g/ml).

METHOD DETAILS

Lentiviral vectors

Lentiviral vectors used in this study were all constructed from a parental lentivector pMNDW.^{67,172} The 2A sequences derived from foot-and-mouth disease virus (F2A), porcine teschovirus-1 (P2A), and thogotovirus (T2A) were used to link the inserted genes to achieve co-expression.

The Lenti/MCAR vector was constructed by inserting into the pMNDW parental vector a synthetic gene encoding mesothelin-targeting chimeric antigen receptor (MCAR). The MCAR design contains a mesothelin-targeting single-chain variable fragment (scFv),¹⁷³ a CD8 hinge, a CD28 transmembrane domain, a CD28 and a 4-1BB co-stimulatory domains, and a CD3 ζ domain. The Lenti/MCAR-IL15 vector was constructed by inserting into the pMNDW parental vector a synthetic bicistronic gene encoding MCAR-F2A-IL15. The Lenti/iNKT-MCAR vector was constructed by inserting into the pMNDW parental vector a synthetic tricistronic gene encoding human iNKT TCR α -F2A-TCR β -P2A-MCAR; the Lenti/iNKT-MCAR-IL15 vector was constructed by inserting into the pMNDW parental vector a synthetic tetracistronic gene encoding human iNKT TCR α -F2A-TCR β -P2A-MCAR-T2A-IL15; the Lenti/MSLN vector was constructed by inserting into the pMNDW parental vector a synthetic gene encoding human MSLN; the Lenti/FG vector was constructed by inserting into the pMNDW parental vector a synthetic bicistronic gene encoding Fluc-P2A-EGFP; and the Lenti/STAT1C-EGFP vector was constructed by inserting into the pMNDW parental vector a synthetic bicistronic gene encoding human STAT1C-P2A-EGFP (STAT1C indicates a human constitutively active STAT1¹⁷⁴). The synthetic gene fragments were obtained from GenScript or IDT. Lentiviruses were produced using HEK 293T cells, following a standard transfection protocol using the Trans-IT-Lenti Transfection Reagent (Mirus Bio) and a centrifugation concentration protocol using the Amicon™ Ultra Centrifugal Filter Units (MilliporeSigma), according to the manufacturers' instructions.

Antibodies and flow cytometry

Fluorochrome-conjugated antibodies specific for human CD45 (Clone HI30), TCR $\alpha\beta$ (Clone IP26), CD3 (Clone HIT3a), CD4 (Clone OKT4), CD8 (Clone SK1), CD20 (Clone 2H7), CD45RO (Clone UCHL1), CD161 (Clone HP-3G10), CD56 (Clone HCD56), CD14 (Clone HCD14), CD69 (Clone FN50), CD62L (Clone DREG-56), CD107a (Clone H4A3), CD1d (Clone 51.1), CD11b (Clone ICRF44), CD11c (Clone Bu15), CD206 (Clone 15-2), CD163 (Clone GHI/61), NKG2D (Clone 1D11), DNAM-1 (Clone 11A8), CD19 (Clone HIB19), β 2-microglobulin (B2M) (Clone 2M2), HLA-DR (Clone L243), HLA-A,B,C (Clone W6/32), TCR V δ 2 (Clone B6), MICA/B (Clone 6D4), IFN- γ (Clone B27), TNF- α (clone MAb11), IL-2 (Clone MQ1-17H12), Granzyme B (Clone QA16A02), Perforin (Clone dG9), and Streptavidin were purchased from BioLegend. Fluorochrome-conjugated antibodies specific for human mesothelin (MSLN; Clone 420411), fibroblast activation protein alpha (FAP; Clone 427819), ULBP-1 (Clone 170818), and ULBP-2,5,6 (Clone 165903) were purchased from R&D Systems. Fluorochrome-conjugated antibodies specific for mouse CD45 (Clone QA17A26), F4/80 (Clone BM8), CD11b (Clone M1/70), Gr-1 (Clone RB6-8C5), IgG2b, κ Isotype control (Clone MG2b-57) were purchased from Biolegend. Fluorochrome-conjugated antibodies specific for human TCR V α 24-J β 18 (Clone 6B11) were purchased from BD Biosciences. Fixable Viability Dye eFluor506 was purchased from Affymetrix eBioscience. Human Fc Receptor Blocking Solution (TrueStain FcX) was purchased from BioLegend, and Mouse Fc Block (anti-mouse CD16/32) was purchased from BD Biosciences. The DAPI Solution and the goat anti-mouse IgG F(ab')₂ secondary antibody were purchased from Invitrogen. A fluorochrome-conjugated antibody specific for human V β 11 was purchased from Beckman-Coulter.

Most flow cytometry stains were performed in PBS for 15 min at 4°C. The samples were stained with Fixable Viability Dye eFluor506 (e506) mixed with Mouse Fc Block (anti-mouse CD16/32) or Human Fc Receptor Blocking Solution (TrueStain FcX) prior to antibody

staining. Antibody staining was performed at a dilution according to the manufacturer's instructions. For intracellular cytokine staining, cells were stimulated with Phorbol-12-myristate-13-acetate (PMA) and Ionomycin for 2 hours, followed by the addition of Golgi-stop Protein Transport Inhibitor, and further incubated for 4 hours. The cells were then stained using a Cell Fixation/Permeabilization Kit (BD Biosciences). For the degranulation marker CD107a staining, cells were initially treated with an anti-human CD107a antibody, followed by a 2-hour incubation, and subsequent collection and staining with other antibodies. Stained cells were analyzed using a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec), following the manufacturer's guidelines. FlowJo software version 9 (BD Biosciences) was used for data analysis.

Enzyme-linked immunosorbent cytokine assays (ELISA)

The ELISAs for detecting human or mouse cytokines were performed following a standard protocol from BD Biosciences.⁵⁵ Supernatants from cell culture assays were collected and assayed to quantify human IFN- γ , TNF- α , IL-2, IL-4, and IL-15. Mouse serum and peritoneal fluid samples were collected and assayed to quantify mouse IL-6. The capture and biotinylated antibody pairs were purchased from BD Biosciences. The streptavidin-HRP conjugate was purchased from Invitrogen. Cytokine standards were purchased from eBioscience. Tetramethylbenzidine (TMB) substrate was purchased from KPL. Human IL-17a was quantified using a Human IL-17A ELISA MAX Deluxe Kit (BioLegend), following the manufacturer's instructions. Mouse SAA-3 was quantified using a Mouse SAA-3 ELISA Kit (MilliporeSigma), per the manufacturer's instructions. The samples were analyzed for absorbance at 450 nm using an Infinite M1000 microplate reader (Tecan).

Generation of allogeneic mesothelin-targeting CAR-engineered NKT (^{Allo}MCAR-NKT) cells and their IL-15-enhanced derivatives (^{Allo}IL-15⁺MCAR-NKT cells)

^{Allo}IL-15⁺MCAR-NKT cells were generated from gene-engineered human cord blood CD34⁺ HSPCs using a 5-stage clinically guided culture method.⁴⁸ The starting HSPCs were engineered to overexpress a transgenic human iNKT TCR, a MSLN-targeting CAR and/or the secreted form of human IL-15.

At Stage 0, frozen-thawed human CD34⁺ HSPCs were revived and cultured in X-VIVO 15 Serum-Free Hematopoietic Stem Cell Medium supplemented with Flt3L (50 ng/ml), SCF (50 ng/ml), TPO (50 ng/ml), and IL-3 (20 ng/ml) in non-tissue culture treated 24-well plate for 24 hours, then transduced with Lenti/iNKT-MCAR-(IL15) viruses for another 24 hours following a previously established protocol.^{48,53,55} Concentrated lentivirus supernatants were mixed with Poloxamer Synperonic F108 (1:100 dilution) and Prostaglandin E2 (PGE2; 1:1000 dilution), and then gently added to the HSPCs cultures.

At Stage 1, gene-engineered HSPCs collected from Stage 0 were cultured in the StemSpanTM SFEM II Medium supplemented with StemSpanTM Lymphoid Progenitor Expansion Supplement (denoted as LPE medium) for two weeks. CELLSTAR[®] 24-well Cell Culture Nontreated Multiwell Plates (VWR) were used. The plates were pre-coated with 500 μ l/well StemSpanTM Lymphoid Differentiation Coating Material for 2 hours at room temperature or alternatively, overnight at 4°C. Gene-engineered HSPCs were suspended at 2×10^4 cells/ml and 500 μ l of cell suspension was added into each pre-coated well. Twice per week, half of the medium from each well was removed and replaced with fresh LPE medium.

At Stage 2, cells collected from the Stage 1 were cultured in the StemSpanTM SFEM II Medium supplemented with StemSpanTM Lymphoid Progenitor Maturation Supplement (denoted as LPM medium) for one week. Non-Treated FalconTM Polystyrene 6-well Microplates (ThermoFisher Scientific) were coated with 1 ml/well of StemSpanTM Lymphoid Differentiation Coating Material. The Stage 1 cells were collected and resuspended at 1×10^5 cells/ml; 2 ml of cell suspension was added into each pre-coated well. Cells were passaged 1-2 times per week to maintain a cell density at $0.5-1 \times 10^6$ cells/ml; fresh LPM medium was added at every passage.

At Stage 3, the cells collected from the Stage 2 were cultured in the LPM medium supplemented with CD3/CD28/CD2 T Cell Activator and 20 ng/ml human recombinant IL-15 for one week. Cells were resuspended at 5×10^5 cells/ml; 2 ml cell suspension was added into Non-Treated FalconTM Polystyrene 6-well Microplates pre-coated with 1 ml/well of StemSpanTM Lymphoid Differentiation Coating Material. Cells were passaged 2-3 times per week to maintain a cell density at $0.5-1 \times 10^6$ cells/ml; fresh LPM medium supplemented with human IL-15 was added at every passage.

At Stage 4, cells collected from the Stage 3, now mature ^{Allo}IL-15⁺MCAR-NKT cells, were expanded using an artificial APC (aAPC) expansion approach. The aAPC was generated by engineering the K562 human chronic myelogenous leukemia cell line (ATCC).⁴⁸ aAPCs were irradiated at 10,000 rads using a Rad Source RS-2000 X-Ray Irradiator (Rad Source Technologies). Mature ^{Allo}IL-15⁺MCAR-NKT cells collected from the Stage 3 culture were mixed with the irradiated aAPCs at 1:1 - 1:2 ratio, resuspended in expansion medium supplemented with 10 ng/ml IL-7 and IL-15 at $0.5-1 \times 10^6$ cells/ml, and seeded into the 150-mm cell culture dishes (ThermoFisher Scientific) at 30 ml per plate. Cells were passaged 2-3 times per week to maintain a cell density at $0.5-1 \times 10^6$ cells/ml; fresh expansion medium was added at every passage. The expansion stage lasted for 2 weeks. At stage 4, cells could be cultured in 150-mm cell culture dishes (ThermoFisher Scientific) or G-Rex[®]6M Well Plates (Wilson Wolf). The expansion can happen in a feeder-free, serum-free CTSTM OpTmizerTM T-Cell Expansion SFM (ThermoFisher Scientific), or a homemade C10 medium. The resulting ^{Allo}IL-15⁺MCAR-NKT cell products were aliquoted and cryopreserved in CryoStor[®] Cell Cryopreservation Media CS10 using a Thermo ScientificTM CryoMedTM Controlled-Rate Freezer 7450 (Thermo scientific) for future use, following the manufacturers' instructions. All allogeneic MCAR-NKT cell products were cryopreserved and used post-thaw to reflect their intended use as an off-the-shelf therapeutic.

Generation of PBMC-derived conventional $\alpha\beta$ T, NKT, and NK cells

Healthy donor PBMCs were used to generate the PBMC-derived conventional $\alpha\beta$ T, NKT, and NK cells (denoted as PBMC- $\alpha\beta$ Tc cells, PBMC-NKT cells, and PBMC-NK cells, respectively).

To generate PBMC- $\alpha\beta$ Tc cells, PBMCs were stimulated with the Dynabeads™ Human T-Activator CD3/CD28 (ThermoFisher Scientific) according to the manufacturer's instructions, followed by culturing in the C10 medium supplemented with human IL-2 (20 ng/mL) for 2–3 weeks.

To generate PBMC-NKT cells, PBMCs were MACS-sorted via Anti-iNKT MicroBeads (Miltenyi Biotec) labeling to enrich NKT cells, following the manufacturer's instructions. The enriched NKT cells were mixed with donor-matched irradiated α GC-loaded PBMCs at a ratio of 1:1 - 1:2, followed by culturing in C10 medium supplemented with 10 ng/mL IL-7 and IL-15 for 2–3 weeks. If needed, the resulting cultured cells could be further purified using Fluorescence-Activated Cell Sorting (FACS) via human NKT TCR antibody (Clone 6B11; BD Biosciences) staining.

To generate PBMC-NK cells, PBMCs were FACS-sorted using a FACSaria III Sorter (BD Biosciences) via human CD56 antibody (Clone HCD56; BioLegend) labeling, or MACS-sorted using a Human NK Cell Isolation Kit (Miltenyi Biotec), following the manufacturers' instructions.

Generation of MSLN-targeting CAR-engineered conventional $\alpha\beta$ T (MCAR-T) cells

Non-treated tissue culture 24-well or 12-well plates (Corning) were coated with Ultra-LEAF™ Purified Anti-Human CD3 Antibody (Clone OKT3; BioLegend) at 1 μ g/mL (500 μ L/well), at room temperature for 2 hours or alternatively, overnight at 4°C. Healthy donor PBMCs were resuspended in the C10 medium supplemented with 1 μ g/mL Ultra-LEAF™ Purified Anti-Human CD28 Antibody (Clone CD28.2, BioLegend) and 30 ng/mL IL-2, followed by seeding in the pre-coated plates at 1 $\times$ 10⁶ cells/mL (1 mL/well). On day 2, cells were transduced with Lenti/MCAR virus for 24 hours. The resulting MCAR-T cells were expanded for about 2 weeks in C10 medium supplemented with human IL-2, and cryopreserved for future use, following established protocols.^{48,53}

Generation of PBMC-derived IL-15-enhanced MSLN-targeting CAR-engineered NKT (PBMC¹⁵MCAR-NKT) cells

Healthy donor PBMCs were sorted with MACS via Anti-iNKT Microbeads (Miltenyi Biotec) labeling to enrich NKT cells, following the manufacturer's instructions. The enriched NKT cells were mixed with donor-matched irradiated α GC/PBMCs at a ratio of 1:1 - 1:2, followed by culturing in C10 medium supplemented with 10 ng/mL IL-7 and IL-15. On day 3, NKT cells were transduced with Lenti/MCAR-IL15 viruses for 24 h. The resulting CAR-NKT cells were expanded for about 2 weeks in C10 medium supplemented with 10 ng/mL IL-7 and IL-15 and cryopreserved for future use.

In vitro assays using OC patient samples

In one assay, the primary OC patient samples were analyzed for tumor cell phenotype and the TME composition using flow cytometry. OC tumor cells were sorted using a Human Tumor Cell Isolation Kit (Miltenyi Biotec) and/or identified as CD45⁺CD31⁺FAP(fibroblast activation protein)⁺ cells,²¹ T cells were identified as CD45⁺CD3⁺ cells, CD4 T cells were identified as CD4⁺ T cells, CD8 T cells were identified as CD8⁺ T cells, B cells were identified as CD45⁺CD19⁺ or CD45⁺CD20⁺ cells, NK cells were identified as CD45⁺CD56⁺CD3⁺ cells, monocytes and macrophages were identified as CD45⁺CD11b⁺CD14⁺ cells, granulocytes were identified as CD45⁺CD11b⁺CD14⁺CD15⁺ cells, tumor-associated macrophages (TAMs) were identified as HLA-DR^{high}CD206^{high} monocytes, and myeloid-derived suppressor cells (MDSCs) were identified as HLA-DR^{low}CD206^{low} monocytes. Surface expression of MSLN, CD1d, and NK ligands on tumor or/and immune cells were also analyzed using flow cytometry. A total of 34 primary OC patient samples were included in this assay.

In another assay, the primary OC patient samples were used to study tumor cell killing by Allo/¹⁵MCAR-NKT cells. Tumor cells were pre-sorted using a Human Tumor Cell Isolation Kit (Miltenyi Biotec), followed by co-culturing with various therapeutic cells (E:T ratio 1:1) in C10 medium in Corning 96-well Round Bottom Cell Culture plates for 24 hours. At the end of culture, cells were collected and live OC tumor cells (identified as CD45⁺CD3⁺6B11⁺ cells) was analyzed using flow cytometry. A total of 20 primary OC patient samples were included in this assay.

In another assay, the primary OC patient samples were used to study the TME targeting by Allo/¹⁵MCAR-NKT cells. Patient samples were directly co-cultured with Allo/¹⁵MCAR-NKT cells (ratio 1:1) in C10 medium in Corning 96-well Round Bottom Cell Culture plates for 24 hours. At the end of culture, cells were collected, and the TME targeting of Allo/¹⁵MCAR-NKT cells was assessed using flow cytometry by quantifying live human TAM/MDSCs (identified as 6B11⁺CD45⁺CD14⁺CD11b⁺ cells), T cells (identified as 6B11⁺CD45⁺CD3⁺ cells), B cells (identified as 6B11⁺CD45⁺CD3⁺CD19⁺ cells or 6B11⁺CD45⁺CD3⁺CD20⁺ cells), granulocytes (identified as 6B11⁺CD45⁺CD14⁺CD11b⁺ cells), and NK cells (identified as 6B11⁺CD45⁺CD3⁺CD56⁺ cells). A total of 34 primary OC patient samples were included in this assay.

In vitro tumor cell killing assay

Tumor cells (1 $\times$ 10⁴ cells per well) were co-cultured with therapeutic cells (e.g., Allo/¹⁵MCAR-NKT, MCAR-T, and T cells) in Corning 96-well clear bottom black plates for 24 hours in C10 medium. The E:T ratios were indicated in the figures or figure legends. At the end of culture, live tumor cells were quantified by adding D-luciferin (150 μ g/mL; Caliper Life Science) to cell cultures and reading out luciferase activities using an Infinite M1000 microplate reader (Tecan).

To study the NKR-mediated tumor cell killing *in vitro*, 10 µg/ml LEAFTM purified anti-human NKG2D (Clone 1D11, BioLegend), anti-human DNAM-1 antibody (Clone 11A8, BioLegend), or LEAFTM purified mouse IgG2bk isotype control antibody (Clone MG2B-57, BioLegend) was added to co-cultures one hour prior to adding ^{Allo}15MCAR-NKT cells.

***In vitro* serial tumor cell killing assay**

1 × 10⁴ non-engineered tumor cells (e.g., OVCAR3 cells; referred to as stimulator cells) were co-cultured with 2 × 10⁵ therapeutic cells in a Corning 96-well clear bottom black plate within C10 medium. Cultures were supplemented with a dose of 1 × 10⁴ stimulator cells every 2 days. Stimulator cells were then substituted with 1 × 10⁴ of FG-engineered tumor cells (e.g., OVCAR3-FG cells; referred to as indicator cells) 24 hours prior to luminescent read-out of tumor killing. On the day of imaging, remaining live indicator cells were quantified through addition of 100 µL of D-Luciferin (150 µg/ml) with subsequent readout using an Infinite M1000 microplate reader (Tecan) to measure luciferase activity from residual indicator cells.

***In vitro* tumor organoid targeting assay**

Human M2-polarized macrophages were generated from healthy donor PBMCs. 1 × 10⁸ PBMCs were resuspended in 10 ml of RPMI medium and seeded in a 10-cm tissue culture plate (Corning) to allow monocyte adherence. After 1 hour of incubation, the plate was washed to remove non-adherent cells, followed by adding back 10 ml of fresh C10 medium supplemented with recombinant human M-CSF (10 ng/ml). The culture medium was replaced every 2-3 days during a 6-day culture to generate monocyte-derived macrophages (MDMs). Subsequently, recombinant human IL-4 (10 ng/ml) and IL-13 (10 ng/ml) were added, and MDMs were cultured for another 48 hours to induce M2 polarization.

The resulting M2-polarized macrophages were then utilized as a surrogate of TAMs for human OC organoid cultures. Two types of OC organoids were generated, including OVCAR8 organoids and OVCAR8/TAM organoids. To generate the OVCAR8 organoids, 2 × 10⁵ OVCAR8 tumor cells were resuspended in 200 µl of C10 medium and seeded in each well of a Nunclon Sphera-Treated 96-Well Microplate (ThermoFisher Scientific). To generate the OVCAR8/TAM organoids, 1 × 10⁵ M2-polarized human macrophages and 1 × 10⁵ OVCAR8 tumor cells were mixed, resuspended in 200 µl of C10 medium, and seeded in each well of a Nunclon Sphera-Treated 96-Well Microplate. The plates were centrifuged at 300 × g for 10 minutes to allow cell pellet formation. The supernatants were then removed, and each cell pellet was resuspended in 5 µl of C10 medium and transferred onto a microporous CellQART 6-Well Cell Culture Insert (Millicell). These inserts were placed in a 6-well cell culture plate (Corning), one insert per well containing 2 ml of C10 medium supplemented with recombinant human M-CSF (2 ng/ml). Organoids were generated over a 7-day culture.

To set up the *in vitro* tumor organoid targeting assay, 3 × 10⁶ FG-labeled therapeutic cells (i.e., ^{Allo}15MCAR-NKT/FG and conventional MCAR-T/FG cells) were resuspended in 100 µl of C10 medium and dropped onto an organoid, followed by a 24-hour co-culture. Throughout the co-culture, the organoids were periodically imaged by widefield imaging using an EVOS M5000 imaging system (Invitrogen) and analyzed using Image J (Image J). Therapeutic cell infiltration into the organoids was imaged using a Leica Confocal SP8-STED/FLIM/FCS microscope and analyzed using customized Matlab algorithms (MathWorks). At the end of the co-culture, organoids were collected. 20 µl of 1× TrypLETM Express Enzyme (ThermoFisher Scientific) was added to each organoid, followed by a 10-minute incubation. The organoids were then disaggregated by gently pipetting and passing through a 50-µm nylon strainer (Fisher Scientific). The resulting single cells were analyzed using flow cytometry to quantify OVCAR8 tumor cells (identified as human CD45⁺ cells) and macrophages (identified as human CD45⁺CD11b⁺ cells).

***In vitro* scanning electron microscopy (SEM)**

SEM was used to visualize the ^{Allo}MCAR-NKT cell targeting of OVCAR3-FG human OC cells. The ^{Allo}MCAR-NKT cells were co-cultured with OVCAR3-FG cells in C10 medium on a Cover Glass Slide (Bioland Scientific LLC) for 8 hours. After the culture, the cells were rinsed with warm HBSS and then fixed with warm 3% glutaraldehyde in the SEM buffer, moved to 4 degrees, and stored overnight. The SEM buffer (pH 7.4) was prepared using 0.1 M Na-phosphate buffer containing 0.1M Sucrose. On the second day, the cells were washed twice with SEM buffer for five minutes each time. Then, the cells were fixed with 2% osmium tetroxide in SEM buffer on ice for one hour and washed again with SEM buffer twice with five minutes each time. After the washing step, the cells were dehydrated successively with 50%, 70%, 95%, and 100% ethanol, each for 15 minutes. The final 100% ethanol was replaced with hexamethyldisilazane, and then the cells were evaporated in the hood. The processed cells were then subjected to Low-Vacuum Scanning Electron Microscopy (LV-SEM) on a FEI Nova Nano 230 SEM (ThermoFisher Scientific), following the manufacturer's instructions.

***In vitro* mixed lymphocyte reaction (MLR) assay: Studying graft-versus-host (GvH) response**

PBMCs of multiple healthy donors were irradiated at 2,500 rads and used as stimulators, to study the GvH response of ^{Allo}15MCAR-NKT cells as responders. MCAR-T cells were included as a responder control. Stimulators (5 × 10⁵ cells/well) and responders (2 × 10⁴ cells/well) were co-cultured in 96-well round bottom plates in C10 medium for 4 days; the cell culture supernatants were then collected to measure IFN-γ production using ELISA.

***In vitro* MLR assay: Studying T cell-mediated allorejection**

PBMCs of multiple healthy donors were used as responders, to study the T cell-mediated allorejection of $Allo^{15}$ MCAR-NKT cells as stimulators (irradiated at 2,500 rads). MCAR-T cells were included as stimulator controls. Stimulators (5×10^5 cells/well) and responders (2×10^4 cells/well) were co-cultured in 96-well round bottom plates in C10 medium for 4 days; the cell culture supernatants were then collected to measure IFN- γ production using ELISA.

***In vitro* MLR assay: Studying NK cell-mediated allorejection**

PBMC-NK cells isolated from PBMCs of multiple healthy donors were used to study the NK cell-mediated allorejection of $Allo^{15}$ MCAR-NKT cells. MCAR-T cells were included as controls. PBMC-NK cells (2×10^4 cells/well) and the corresponding effector cells (2×10^4 cells/well) were co-cultured in 96-well round bottom plates in C10 medium for 24 h; the cell cultures were then collected to quantify live cells using flow cytometry.

***In vivo* bioluminescence live animal imaging (BLI)**

BLI was performed using a Spectral Advanced Molecular Imaging (AMI) HTX imaging system (Spectral instrument Imaging). Live animal images were acquired 5 minutes after intraperitoneal (i.p.) injection of D-Luciferin for total body bioluminescence. To monitor the signal of tumor cells (e.g., OVCAR3-FG cells), 1 mg/mouse of D-Luciferin was injected; To monitor the signal of therapeutic cells (e.g., $Allo^{15}$ MCAR-NKT/FG cells), 3 mg/mouse of D-Luciferin was injected. Imaging data were analyzed using an AURA imaging software (Spectral Instrument Imaging, version 3.2.0). Due to the large number of experimental mice used in this study, representative images from individual mice are shown in the figures.

***In vivo* antitumor efficacy study of $Allo^{15}$ MCAR-NKT cells: OVCAR8-FG human OC xenograft NSG mouse model with intraperitoneal administration route**

Experimental design is shown in Figure 3A. Briefly, on Day 0, NSG mice received i.p. inoculation of OVCAR8-FG human OC cells (2×10^5 cells per mouse). On Day 4, the experimental mice received i.p. injection of vehicle (100 μ l PBS per mouse), $Allo^{15}$ MCAR-NKT cells (10×10^6 CAR $^+$ cells in 100 μ l PBS per mouse), or control MCAR-T cells (10×10^6 CAR $^+$ cells in 100 μ l PBS per mouse). In this experiment, MCAR-T cells were pre-sorted to isolate CAR $^+$ cells, which were then injected into the experimental mice. Over the experiment, mice were monitored for survival and their tumor loads were measured twice per week using BLI.

***In vivo* antitumor efficacy study of $Allo^{15}$ MCAR-NKT cells: OVCAR8-FG human OC xenograft NSG mouse model with intravenous administration route**

Experimental design is shown in Figure 3A. Briefly, on Day 0, NSG mice received i.p. inoculation of OVCAR8-FG human OC cells (2×10^5 cells per mouse). On Day 4, the experimental mice received Intravenous (i.v.) injection of vehicle (100 μ l PBS per mouse), $Allo^{15}$ MCAR-NKT cells (10×10^6 CAR $^+$ cells in 100 μ l PBS per mouse), or control MCAR-T cells (10×10^6 CAR $^+$ cells in 100 μ l PBS per mouse). Over the experiment, mice were monitored for survival and their tumor loads were measured twice per week using BLI.

***In vivo* antitumor efficacy study of $Allo^{15}$ MCAR-NKT cells: OVCAR3-FG low tumor burden human OC xenograft NSG mouse model**

Experimental design is shown in Figure 4A. Briefly, on Day 0, NSG mice received i.p. inoculation of OVCAR3-FG human OC cells (5×10^5 cells per mouse). On Day 4, the experimental mice received i.p. injection of vehicle (100 μ l PBS per mouse), $Allo^{15}$ MCAR-NKT cells (10×10^6 CAR $^+$ cells in 100 μ l PBS per mouse), or control MCAR-T cells (10×10^6 CAR $^+$ cells in 100 μ l PBS per mouse). Over the experiment, mice were monitored for survival and their tumor loads were measured twice per week using BLI.

***In vivo* antitumor efficacy study of $Allo^{15}$ MCAR-NKT cells: OVCAR3-FG high tumor burden human OC xenograft NSG mouse model**

Experimental design is shown in Figure 4E. Briefly, on Day 0, NSG mice received i.p. inoculation of OVCAR3-FG human OC cells (5×10^5 cells per mouse). The tumor cells were allowed to grow for 40 days, establishing a high tumor burden. On Day 40, the experimental mice received i.p. injection of vehicle (100 μ l PBS per mouse), $Allo^{15}$ MCAR-NKT cells (10×10^6 CAR $^+$ cells in 100 μ l PBS per mouse), or control MCAR-T cells (10×10^6 CAR $^+$ cells in 100 μ l PBS per mouse). Over the experiment, mice were monitored for survival and their tumor loads were measured twice per week using BLI.

***In vivo* antitumor efficacy study of $Allo^{15}$ MCAR-NKT cells: OVCAR3-FG repeated tumor challenge human OC xenograft NSG mouse model**

Experimental design is shown in Figure 4I. Briefly, on Day 0, NSG mice received i.p. inoculation of OVCAR3-FG human OC cells (5×10^5 cells per mouse). On Day 4, the experimental mice received i.p. injection of vehicle (100 μ l PBS per mouse), $Allo^{15}$ MCAR-NKT cells (10×10^6 CAR $^+$ cells in 100 μ l PBS per mouse), or control MCAR-T cells (10×10^6 CAR $^+$ cells in 100 μ l PBS per mouse). On Day 16, the experimental mice received another i.p. inoculation of OVCAR3-FG human OC cells (5×10^5 cells per mouse). Over the experiment, mice were monitored for survival and their tumor loads were measured twice per week using BLI.

***In vivo* antitumor efficacy study of $Allo^{15}$ MCAR-NKT cells combined with carboplatin: OVCAR3-FG human OC xenograft NSG mouse model**

Experimental design is shown in Figure 4Q. Briefly, on Day 0, NSG mice received i.p. inoculation of OVCAR8-FG human OC cells (1×10^6 cells per mouse). On Day 4 and Day 10, the experimental mice received i.p. injection of chemodrug carboplatin (10 mg/kg per mouse). On Day 17, the experimental mice received i.p. injection of $Allo^{15}$ MCAR-NKT cells (1×10^6 CAR⁺ cells in 100 μ l PBS per mouse). Over the experiment, mice were monitored for survival and their tumor loads were measured twice per week using BLI.

***In vivo* antitumor efficacy study of $Allo^{15}$ MCAR-NKT cells: OVCAR3-FG^{MSLN} human OC xenograft NSG mouse model**

Experimental design is shown in Figure 4M. Briefly, on Day 0, NSG mice received i.p. inoculation of OVCAR3-FG^{MSLN} human OC cells (5×10^5 cells per mouse). On Day 4, the experimental mice received i.p. injection of vehicle (100 μ l PBS per mouse), $Allo^{15}$ MCAR-NKT cells (10×10^6 CAR⁺ cells in 100 μ l PBS per mouse), or control MCAR-T cells (10×10^6 CAR⁺ cells in 100 μ l PBS per mouse). Over the experiment, mice were monitored for survival and their tumor loads were measured twice per week using BLI.

***In vivo* pharmacokinetics/pharmacodynamics (PK/PD) study of $Allo^{15}$ MCAR-NKT/FG cells: Human OC xenograft NSG mouse model**

Experimental design is shown in Figure 3H. Briefly, on Day -4, NSG mice received i.p. inoculation of OVCAR8 cells (2×10^5 cells/mouse). On Day 0, the experimental mice received i.p. or i.v. injection of $Allo^{15}$ MCAR-NKT/FG cells (10×10^6 CAR⁺ cells in 100 μ l PBS per mouse), or control MCAR-T/FG cells (10×10^6 CAR⁺ cells in 100 μ l PBS per mouse). Over the experiment, mice were monitored for survival and their therapeutic cells were measured twice per week using BLI. Note in this study, therapeutic immune cells, but not the tumor cells, were labeled with FG. MCAR-T/FG cells were included as a control.

***In vivo* TME targeting study**

Experimental design is shown in Figure 6I. Briefly, on Day -4, NSG mice received i.p. inoculation of OVCAR8-FG cells (2×10^5 cells/mouse). On Day 0, the experimental mice received i.p. injection of $Allo^{15}$ MCAR-NKT cells (10×10^6 CAR⁺ cells in 100 μ l PBS per mouse), or control MCAR-T cells (10×10^6 CAR⁺ cells in 100 μ l PBS per mouse). On Day 14, experimental mice were euthanized and multiple tissues (i.e., peritoneal fluid, blood, spleen, and bone marrow) were collected for the detection of immune cell composition using flow cytometry.

***In vivo* cytokine release syndrome (CRS) study**

Experimental design is shown in Figure 6R. Briefly, on Day -4, NSG mice received i.p. inoculation of OVCAR8-FG cells (5×10^6 cells per mouse). On Day 0, the experimental mice received i.p. injection of vehicle (100 μ l PBS per mouse), $Allo^{15}$ MCAR-NKT cells (10×10^6 CAR⁺ cells in 100 μ l PBS per mouse), or control BCAR-T cells (10×10^6 CAR⁺ cells in 100 μ l PBS per mouse). On Day 3, blood and peritoneal fluid samples were collected from the experimental mice, and their serum and peritoneal fluid IL-6 and SAA-3 were measured using ELISA. A Mouse SAA-3 ELISA Kit (Millipore Sigma) was used to measure SAA-3, following the manufacturer's instructions.

Single cell TCR sequencing (scTCR-seq)

$Allo^{15}$ MCAR-NKT (6B11⁺TCR $\alpha\beta$ ⁺), $Allo^{15}$ MCAR-NKT (6B11⁺TCR $\alpha\beta$ ⁺), PBMC-NKT (6B11⁺TCR $\alpha\beta$ ⁺), and PBMC-Tc (6B11⁺TCR $\alpha\beta$ ⁺) cells were sorted using a FACSaria II flow cytometer. Sorted cells were immediately delivered to the UCLA TCGB (Technology Center for Genomics and Bioinformatics) Core to perform single cell TCR sequencing using a 10X Genomics ChromiumTM Controller Single Cell Sequencing System (10X Genomics), following the manufacturer's instructions and the TCGB Core's standard protocol. Briefly, the cells were encapsulated in gel beads and lysed to release RNA, which was then reverse-transcribed and amplified. The resulting cDNA was fragmented and barcoded, and libraries were constructed using the Illumina Tru-Seq RNA Sample Prep Kit (Cat#FC-122-1001). The libraries were sequenced with 150 bp paired-end reads, with 5,000 reads/cell on an Illumina Nova-Seq. The raw sequencing data were processed and mapped to the human TCR reference genome (hg38) using the Cell Ranger VDJ software. The frequencies of the unique α or β chains were plotted to show the TCR repertoire diversity in each cell population.

Bulk RNA sequencing (bulk RNA-seq)

A total of 20 cell samples were analyzed, as described in the following table.

Sample name	Number of replicates	FACS sorting markers	Description
$Allo^{15}$ MCAR-NKT	4	6B11 ⁺ TCR $\alpha\beta$ ⁺	Allogeneic HSPC-derived MCAR-engineered NKT cells
$Allo^{15}$ MCAR-NKT	4	6B11 ⁺ TCR $\alpha\beta$ ⁺	Allogeneic IL15-enhanced HSPC-derived MCAR-engineered NKT cells

(Continued on next page)

Continued

Sample name	Number of replicates	FACS sorting markers	Description
PBMC-NKT (CD4 ⁺)	3	6B11 ⁺ TCRαβ ⁺ CD4 ⁺	PBMC-NKT cells derived from healthy donor PBMCs (CD4 ⁺ cells were analyzed)
PBMC-αβTc (CD4 ⁺)	7	6B11 ⁺ TCRαβ ⁺ CD4 ⁺	PBMC-αβTc cells derived from healthy donor PBMCs (CD4 ⁺ cells were analyzed)
PBMC-NK	2	CD56 ⁺ CD3 ⁺	PBMC-NK cells derived from healthy donor PBMCs

The numbers indicate different donors. Note that ^{Allo}15MCAR-NKT cells displayed a CD4⁺CD8⁺ phenotype, therefore the CD4⁺ populations of PBMC-NKT and PBMC-αβTc cells were used in this study. Note that the sequencing data for certain cell samples have been incorporated from our previous studies.⁵³ Cell samples were sorted using a FACSARIA™ III Sorter (BD Biosciences). Total RNAs were isolated from each cell sample using a miRNeasy Mini Kit (QIAGEN) and delivered to the UCLA TCGB Core to perform Bulk RNA-seq using an Illumina HiSeq3000, following the manufacturer's instructions and the TCGB Core's standard protocols. cDNAs were synthesised using an iScript cDNA Synthesis Kit (BioRad). Libraries were constructed using an Illumina TruSeq Stranded Total RNA Sample Prep kit and sequenced with 50 bp single-end reads (targeting 20 x 10⁶ reads per sample) on an Illumina HiSeq3000. The raw sequencing data underwent quality control and adapter trimming using fastp (version 0.23.2), and trimmed reads were mapped to the human genome (hg38) using with STAR 2.7.9a with default parameters. The read counts for each gene were obtained using featureCounts from Subread package (version 2.0.3). Sequencing depth normalized counts (cpm, counts per million) were obtained using edgeR (version 3.38.4). Top 2,000 most variable genes were identified, and the log transformation with pseudocount 1 was used for principal-component analysis (PCA).

Single cell RNA sequencing (scRNA-seq)

In one study, scRNA-seq was utilized to examine the gene expression profiles of primary ovarian cancer patient-derived tumor cells, as shown in Figure 1A. Two in-house OC patient samples, comprising matched chemo-naïve (C) and recurrent (R) samples, were included. The tumor cells were isolated using a Human Tumor Cell Isolation Kit (Miltenyi Biotec) following the manufacturer's instructions, and then subjected to scRNA-seq. The scRNA-seq analyses of primary OC samples also incorporated data from publicly available datasets (GSE165897, GSE184880, and PRJCA005422).

In another study, scRNA-seq was utilized to examine the *in vivo* gene expression profile of therapeutic cells, tumor cells, and mouse immune cells in an OVCAR8-FG human OC xenograft NSG mouse model as shown in Figure 5A. Two types of therapeutic cells (^{Allo}15MCAR-NKT and MCAR-T cells) were collected at four time points (Week 0, Week 4, Week 8, and Week 9). Week 0 samples were collected prior to infusion, Week 4 and 8 samples were collected from the peritoneal cavity tumor sites of the experimental mice, and Week 9 samples were generated by stimulating Week 8 samples *in vitro* with MSLN-expressing aAPCs for one week. The tumor cells (OVCAR8-FG) and the mouse immune cells (mouse CD45⁺ cells) were collected on Week 4. Cells isolated from 5-10 mice of each experimental group were combined for scRNA-seq.

Freshly collected samples were immediately delivered to the UCLA TCGB Core for library construction and scRNA-seq. Cells were quantified using a Cell Countess II automated cell counter (Invitrogen/Thermo Fisher Scientific). A total of 10,000 cells from each experimental group were loaded on the Chromium platform (10X Genomics), and libraries were constructed using the Chromium Next GEM Single Cell 3' Kit v3.1 and the Chromium Next GEM Chip G Single Cell Kit (10X Genomics), according to the manufacturer's instructions. Library quality was assessed using the D1000 ScreenTape on a 4200 TapeStation System (Agilent Technologies). Libraries were sequenced on an Illumina NovaSeq using the NovaSeq S4 Reagent Kit (100 cycles; Illumina).

For cell clustering and annotation, the merged digital expression matrix generated by Cellranger was analyzed using an R package Seurat (v.4.0.0) following the official website guidelines.¹⁷⁵⁻¹⁷⁷ Briefly, after filtering the low-quality cells, the expression matrix was normalized using NormalizeData function, followed by selecting top 2,000 most variable genes across datasets using FindVariableFeatures and SelectIntegrationFeatures functions. To correct the batch effect, FindIntegrationAnchors and IntegrateData functions were used based on the selected feature genes. The corrected dataset was subjected to standard Seurat workflow for dimension reduction and clustering. In this study, clusters of therapeutic cells were manually merged and annotated based on gene signatures reported from Human Protein Atlas (proteintatlas.org) and previous studies,⁹⁶⁻¹⁰³ and clusters of mouse immune cells were merged and annotated based on the immune lineage markers. AddModuleScore was used to calculate module scores of each list of gene signatures, and FeaturePlot function was used to visualize the expression of each signature in the UMAP plots. For gene set enrichment analysis (GSEA), clusterProfiler packages^{178,179} were used to calculate the enrichment scores of each cluster in the signature gene list.

For RNA-velocity analysis, the LOOM files containing spliced/unspliced expression matrices were generated for each sample. The further analysis was conducted using the Velocity.R-package (v0.6).¹⁸⁰ After loading LOOM files information through the ReadVelocity function, databases were merged and the RunVelocity function was executed to obtain the velocity vectors. Finally,

the velocities were projected into a lower-dimensional embedding using the `velocity_graph` function and visualized on the UMAP embedding in each intended cell cluster using the `show.velocity.on.embedding.cor` function. All `velocity` functions were utilized with default parameters.

For pathway analysis, differentially expressed genes in OVCAR8-FG tumor cells were analyzed using ShinyGO 0.82 (<https://bioinformatics.sdstate.edu/go/>), with either GO Biological Process or KEGG pathways used for the analyses. A low threshold for differential expression was applied due to the subtle differences observed between sample groups. For cell-cell communication, CellChat¹⁸¹ was utilized for ligand-receptor analysis. CellChat objects for the scRNA-seq data of MCAR-T cells, Allo¹⁵MCAR-NKT cells, MCAR-T cell-treated tumor cells, and Allo¹⁵MCAR-NKT cell-treated tumor cells were created using the `createCellChat` function, respectively, followed by identifying the overexpressed ligand-receptor interactions using `identifyOverExpressedGenes` function and `identifyOverExpressedInteractions` function. The gene expression data were also projected onto protein-protein interaction network using `projectData` function. To infer the biologically significant cell-cell communication by assigning each interaction with a probability value, `computeCommunProb` and `computeCommunProbPathway` functions were used to assign each interaction with a probability value. Database CellChatDB in human was used in this study. To perform integrated analysis, `mergeCellChat` function was used to merge all CellChat objects. To compare number of interactions and interaction strength among different cell populations, `netVisual_diffInteraction` function was utilized to visualize the number of ligand-receptor interactions of among therapeutic cells (MCAR-T or Allo¹⁵MCAR-NKT) and OVCAR8-FG tumor cells. To discover major signaling changes in response to MCAR-T cell or Allo¹⁵MCAR-NKT cell treatment, the `rankNet` function was utilized to compare the information flow change of each signaling pathway within the network. To show interactions sending from therapeutic cells to tumor cells, `netVisual_chord_gene` function was used to visualize ligand-receptor pairs behind each signaling pathways.

Methylation sequencing (Methyl-seq)

6 samples including 3 Allo¹⁵MCAR-NKT and 3 MCAR-T cell samples were analyzed using Methyl-seq. Genomic DNA was isolated from experimental samples using a QIAGEN DNeasy Blood & Tissue kit, then sonicated using a Covaris M220 Focused-ultrasonicator, following the manufacturers' instructions. DNA fragments of around 250 bp were enriched using the Ampure XP beads (Beckman-Coulter), then subjected to DNA library preparation using an NEBNext Ultra II DNA library prep Kit (Cat#E7645) following the manufacturers' instructions. The DNA libraries were then subjected to sequencing on Illumina NovaSeq sequencer with 2 x 150 bp configuration (Azenta). The raw bisulfite sequencing reads were trimmed by cutadapt to remove sequencing adapters. The trimmed reads were then aligned to hg19 reference genome by Bismark.¹⁸² Then duplicated reads from PCR amplification were identified and removed by Bismark. The deduplicated reads were then sorted and indexed using Samtools.¹⁸³ After that, the methylated and unmethylated cytosines were counted at every CpG site by Bismark. The methylation at a gene promoter region was quantified as the beta value, i.e., the ratio between the number of methylated cytosines and the total number of cytosines mapped to the region. The promoter region of a gene was identified by GeneHancer with the highest confidence score. The beta values were then used for heatmap visualization.

Histological analysis

Tissues were collected from mice and fixed in 10% Neutral Buffered Formalin for 72 hours, then embedded in paraffin. Tissues were sectioned to 5 μ m thickness and stained with Hematoxylin and Eosin (H&E) by the UCLA Translational Pathology Core Laboratory, per the Core's standard protocols. The sections were imaged on an Olympus BX51 upright microscope equipped with an Optronics Macrofire CCD camera (AU Optronics) at 20 x magnifications. The images were analyzed using Optronics PictureFrame software (AU Optronics).

Western blot (WB)

WB was used to analyze the IFN- γ signaling events in Allo¹⁵MCAR-NKT cells. Conventional MCAR-T cells and quiescent T cells prepared from the same healthy donor PBMCs were included as controls. Quiescent T cells were MACS-sorted from PBMCs using mixed anti-human CD4 and CD8 magnetic beads (Miltenyi Biotec) to avoid the potential CD3 stimulation, following the manufacturer's instructions.

Cells were stimulated with IFN- γ (10 ng/ml) for 15 minutes (for blotting p-STAT1/STAT1), 18 hours (for blotting IRF-1), or 48 hours (for blotting CIITA/SP1). Total proteins were extracted using a RIPA lysis buffer (Thermo Fisher Scientific) containing 20 mM HEPES (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, and protease/phosphatase inhibitor cocktail (Cell Signaling Technology). Protein concentration was measured using a Bicinchoninic Acid (BCA) Assay Kit (Thermo Fisher Scientific). Equal amounts of total protein were resolved on a 4–15% Mini-PROTEAN® TGX™ Precast Protein Gel (BIO-RAD) and then transferred to a polyvinylidene difluoride (PVDF) membrane by electrophoresis. The following antibodies were used to blot for the proteins of interest: anti-human CIITA (Invitrogen), anti-human p-STAT1(Y701) (clone 58D6, Cell Signaling Technology, CST), anti-human STAT1 (clone D1K9Y, CST), anti-human IRF-1 (clone D5E4, CST), anti-human SP1 (clone D4C3, CST), and secondary anti-rabbit IgG (CST). β -Actin (clone D6A8, CST) and GAPDH (clone D16H11, CST) were used as internal controls. Signals were visualized using a ChemiDoc™ Imaging Systems (BIO-RAD). The data were analyzed using ImageJ (Version 1.53s).

Generation and analysis of ^{Allo15}MCAR-NKT cells overexpressing a constitutively active STAT1

^{Allo15}MCAR-NKT cells overexpressing a constitutively active STAT1¹⁷⁴ and an EGFP reporter (denoted as ^{Allo15}MCAR-NKT-STAT1C-EGFP cell)s were generated by further engineering the ^{Allo15}MCAR-NKT cells. The Lenti/STAT1C-EGFP vector or a mock control Lenti/Fluc-EGFP vector was added into the ^{Allo15}MCAR-NKT cell culture during Stage 4 (on day 3 of Stage 4), followed by the continuation and completion of Stage 4 culture. The resulting ^{Allo15}MCAR-NKT-STAT1C-EGFP cells, as well as their mock control ^{Allo15}MCAR-NKT-FG cells, were then analyzed using flow cytometry. The overexpression of transgenic STAT1C was reflected by EGFP co-expression in ^{Allo15}MCAR-NKT-STAT1C-EGFP cells. The rescue effect of STAT1C overexpression in ^{Allo15}MCAR-NKT-STAT1C-EGFP cells was assessed by comparing these cells with the control ^{Allo15}MCAR-NKT-FG cells for cell surface expression of HLA-I/II molecules and NK ligands (i.e., ULBP-1 and MIC-A/B).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software). Student's two-tailed t test was used for pairwise comparisons. Ordinary 1-way ANOVA followed by Tukey's or Dunnett's multiple comparisons test was used for multiple comparisons. Log rank (Mantel-Cox) test adjusted for multiple comparisons was used for Meier survival curves analysis. Data are presented as the mean ± SEM, unless otherwise indicated. In all figures and figure legends, "N" represents the number of samples or animals utilized in the indicated experiments. A p value of less than 0.05 was considered significant. ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. For scRNA-seq analysis, p values of violin plots were determined by Wilcoxon rank sum test.

Supplemental information

Overcoming ovarian cancer resistance and evasion to CAR-T cell therapy by harnessing allogeneic CAR-NKT cells

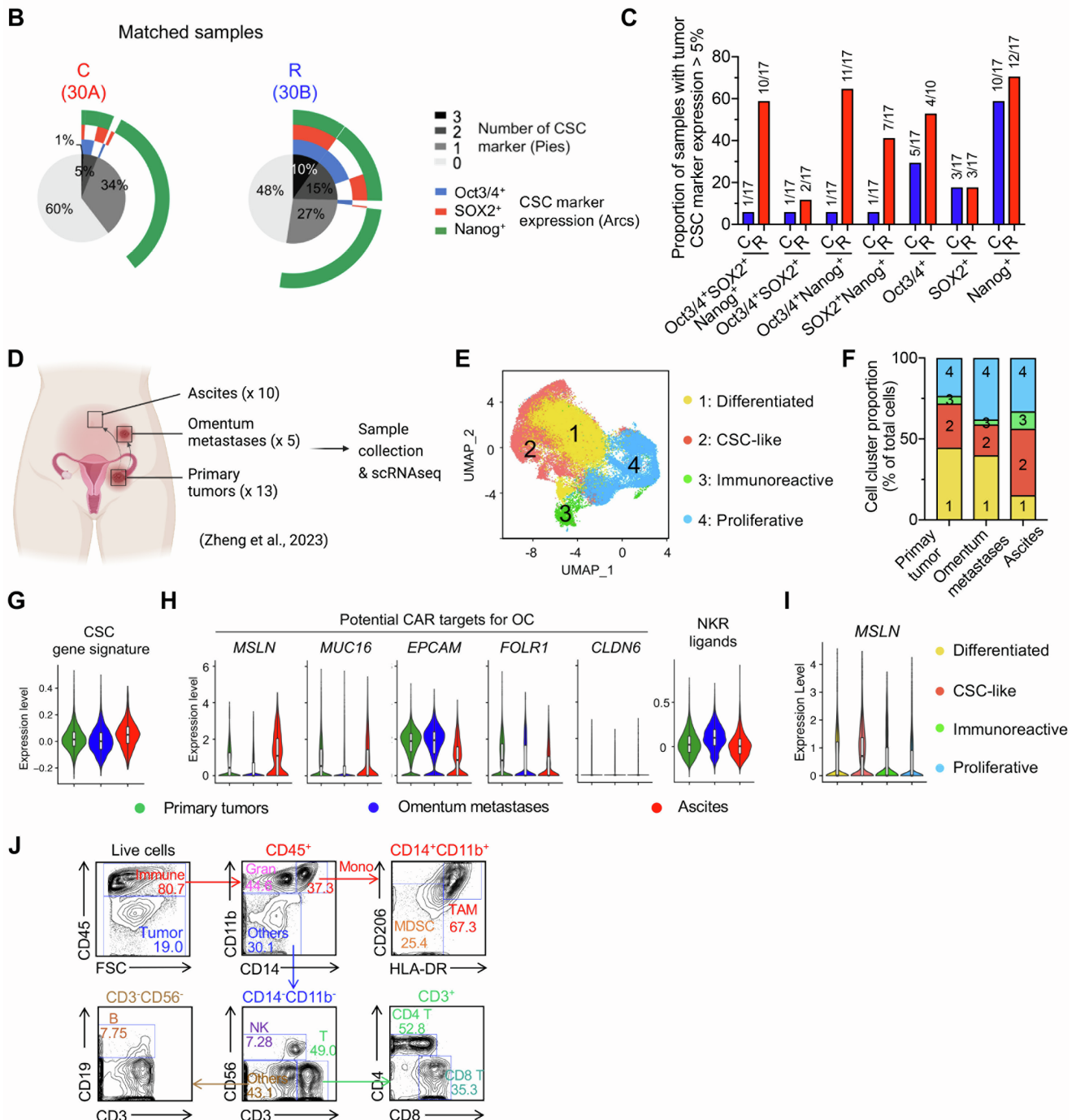
Yan-Ruide Li, Zhe Li, Yichen Zhu, Miao Li, Yuning Chen, Derek Lee, Christopher J. Ochoa, Tanya Singh, Gabriella DiBernardo, Wenbin Guo, Shuo Li, Yang Zhou, Yanqi Yu, Adam Kramer, Matthew Wilson, Kuangyi Zhou, Zachary Spencer Dunn, Ying Fang, Jiaji Yu, Yu Jeong Kim, Jie Huang, Xinyuan Shen, Yu-Chen Wang, Ryan Hon, Taylor Basso, Evelyn Chen, Enbo Zhu, Siyu Lin, Adam Neal, Neda A. Moatamed, Scott S. Oh, Saeed Sadeghi, Calvin Pan, Aijun Wang, Aldons J. Lulis, Xianghong Jasmine Zhou, Jin J. Zhou, Matteo Pellegrini, Pin Wang, Sanaz Memarzadeh, and Lili Yang

Supplemental information

Overcoming ovarian cancer resistance and evasion to CAR-T cell therapy by harnessing allogeneic CAR-NKT cells

Yan-Ruide Li, Zhe Li, Yichen Zhu, Miao Li, Yuning Chen, Derek Lee, Christopher J. Ochoa, Tanya Singh, Gabriella DiBernardo, Wenbin Guo, Shuo Li, Yang Zhou, Yanqi Yu, Adam Kramer, Matthew Wilson, Kuangyi Zhou, Zachary Spencer Dunn, Ying Fang, Jiaji Yu, Yu Jeong Kim, Jie Huang, Xinyuan Shen, Yu-Chen Wang, Ryan Hon, Taylor Basso, Evelyn Chen, Enbo Zhu, Siyu Lin, Adam Neal, Neda Moatamed, Scott S. Oh, Saeed Sadeghi, Calvin Pan, Aijun Wang, Aldons J. Lysis, Xianghong Jasmine Zhou, Jin J. Zhou, Matteo Pellegrini, Pin Wang, Sanaz Memarzadeh, and Lili Yang

A	Dataset	Number of OC samples	Number of OC patients	Characterization of OC samples	Reference
	A	2	1	Matched chemonaive and recurrent samples	In-house samples
	B	20	10	Matched chemonaive and post-NACT samples	GSE165897
	C	7	7	Chemonaive samples	GSE184880



samples. Pie charts represent proportions of the indicated tumor cell groups expressing the indicated numbers (0-3) of CSC markers. Colored arcs indicate the specific combinations of CSC markers expressed. (C) Bar graphs summarizing the comparison between C (n = 17) and R samples (n = 17) for their CSC marker expression.

(D-I) Transcriptome profiling of primary OC tumors collected from three disease sites, including primary tumors, omentum metastases, and ascites. A public dataset (GSA-Human, PRJCA005422) was analyzed. (D) Schematics showing the experimental design. (E) Combined UMAP plot showing the formation of four major cell clusters. (F) Bar graphs showing the cell cluster proportions of the indicated OC tumor samples. (G) Violin plots showing the expression distribution of CSC gene signature in the indicated OC tumor samples. (H) Violin plots showing the expression distribution of genes encoding CAR targets and NKR ligands in the indicated OC tumor samples. (I) Violin plots showing the expression distribution of *MSLN* in the four cell clusters.

(J) FACS gating strategies for tumor cells and immune cells in the OC TME. Gran, granulocyte; mono, monocyte; TAM, tumor-associated macrophage; MDSC, myeloid-derived suppressor cell; NK, natural killer cell. Other T cells indicate CD4⁻CD8⁻CD3⁺ T cell populations, including gamma delta (γδ) T cells, NKT cells, mucosal-associated invariant T (MAIT) cells, and double-negative conventional αβ T cells.

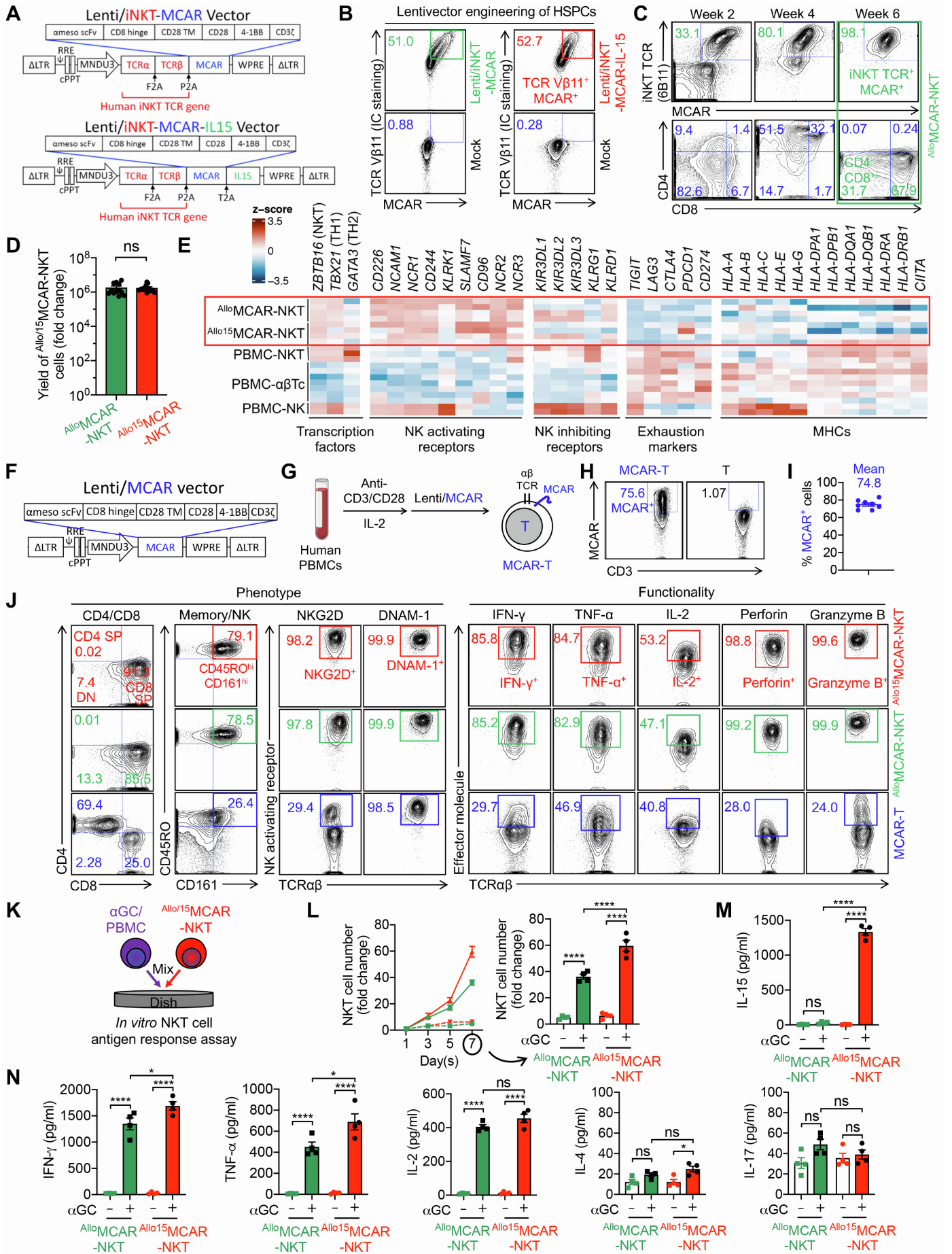


Figure S2. Generation and characterization of allogeneic CAR-NKT cells; related to Figure 2.

(A) Schematics showing the design of the indicated lentivectors. iNKT, a pair of human iNKT TCR α and β chains; MCAR, MSLN-targeting CAR; LTR, long terminal repeats; MNDU3, internal promoter derived from the MND retroviral LTR U3 region; Ψ , packaging sequence; RRE, rev-responsive element; cPPT, central polypurine tract; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

(B) FACS detection of intracellular expression of iNKT TCR (identified as TCR V β 11⁺) and surface expression of MCAR in CD34⁺ HSPCs at 72 h after lentiviral transduction.

(C) FACS monitoring of the generation of ^{Allo}MCAR-NKT cells over the 6-week culture. A 6B11 monoclonal antibody was used to stain iNKT TCR.

(D) Yields of ^{Allo}/¹⁵MCAR-NKT cell products generated from multiple CB donors (n = 12; n indicates different donors).

(E) Heatmap showing the expression levels of the indicated genes in the indicated cell samples. Each row represents an individual sample. Each column represents an indicated gene.

(F-I) Generation of MCAR-T cells from healthy donor PBMCs. (F) Schematics showing the design of the Lenti/MCAR vector. (G) Schematics showing the generation of conventional MCAR-T cells from healthy donor PBMCs. (H) FACS analyses of MCAR expression on MCAR-T cells. (I) Quantification of (H) (n = 8; n indicates different PBMC donors.)

(J) FACS analyses of surface markers, intracellular cytokines, and intracellular cytotoxic molecules in the ^{Allo}/¹⁵MCAR-NKT cell products. Conventional MCAR-T cell product was included as a control.

(K-N) Antigen responses of ^{Allo}/¹⁵MCAR-NKT cells. ^{Allo}/¹⁵MCAR-NKT cells were stimulated with/out alpha-galactosylceramide (α GC)-loaded PBMCs (α GC/PBMCs) for one week. (K) Experimental design. (L) Growth curve of ^{Allo}/¹⁵MCAR-NKT cells (n = 4). (M) ELISA measurements of IL-15 levels in the culture supernatants collected at 48 hours (n = 4). Note the successful incorporation and expression of *IL-15* transgene in ^{Allo}/¹⁵MCAR-NKT cells. Also note that *IL-15* transgene is regulated by a constitutive promoter and therefore should be constitutively expressed; the seemingly no production of IL-15 from ^{Allo}/¹⁵MCAR-NKT cells without α GC stimulation is likely due to a combination of low cell number and the short half-life time of IL-15 in supernatants, resulting in measurements below the ELISA detection level. (N) ELISA measurements of effector cytokine (IFN- γ , TNF- α , IL-2, IL-4, and IL-17a) levels in the culture supernatants collected on day 7 (n = 4).

Representative of 3 experiments. Data are presented as the mean $\pm$ SEM. ns, not significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by Student's *t* test (D) or one-way ANOVA (L-N).

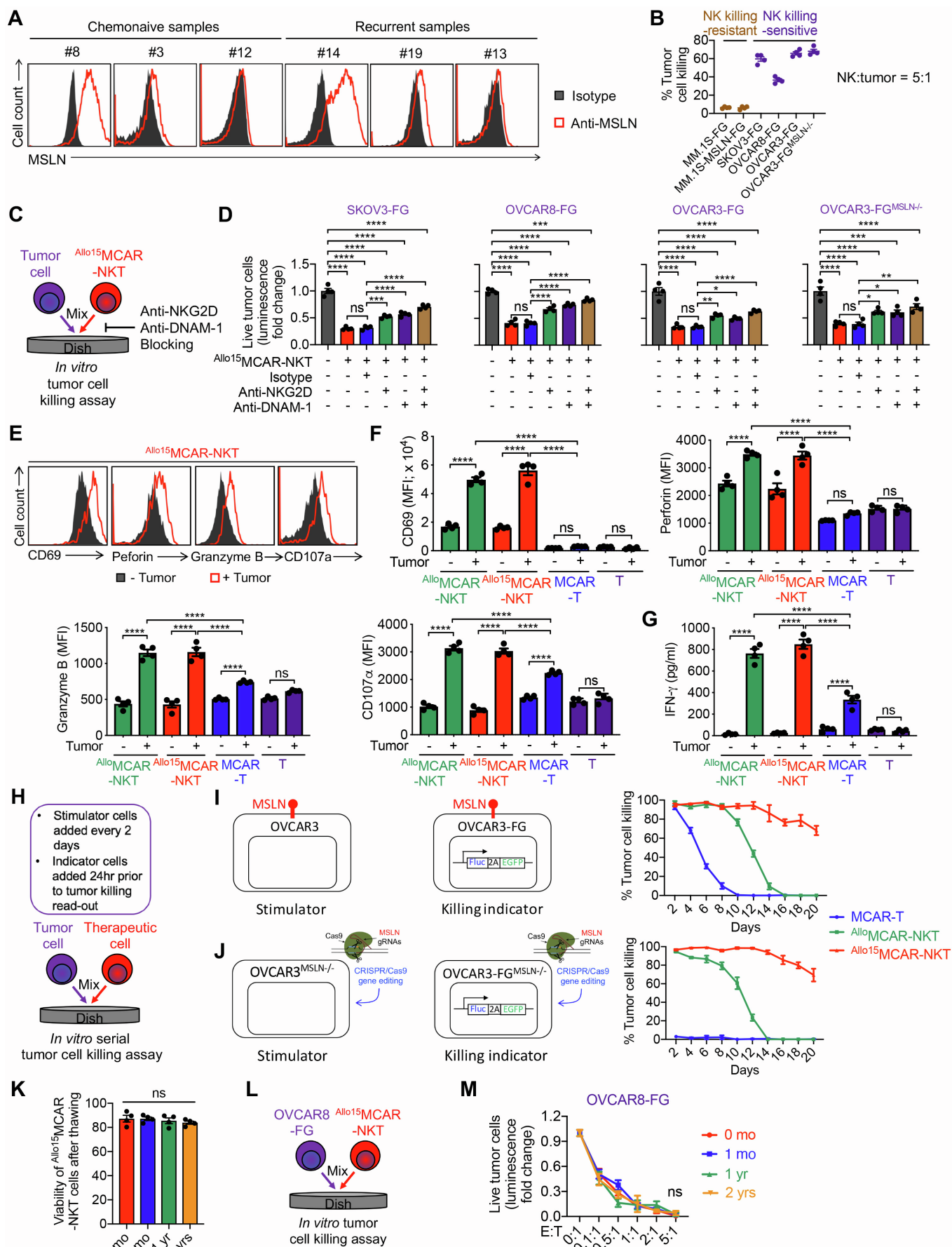


Figure S3. Studying the OC tumor cell killing capacity of allogeneic CAR-NKT cells; related to Figure 2.

(A) FACS detection of MSLN expression on primary OC tumor cells. Three chemo-naïve and three recurrent OC samples expressing different levels of MSLN were shown. Sample information are described in Table S1. Data are related to Figures 2E and 2F.

(B) *In vitro* tumor cell killing data by primary PBMC-NK cells at 24 h (n = 4). Freshly isolated PBMC-derived NK cells were co-cultured with the indicated tumor cell lines at an E:T ratio of 5:1. The two MM cell lines exhibited resistance to NK cell-mediated cytotoxicity, whereas the four OC cell lines were sensitive to NK cell killing.

(C and D) Studying the direct tumor cell killing mechanism of $Allo^{15}$ MCAR-NKT cells mediated by NKR (i.e., NKG2D and DNAM-1). (B) Experimental design. (C) Tumor cell killing data at 24 h (n = 4). SKOV3-FG and OVCAR3-FG^{MSLN^{-/-}}, E:T ratio = 2:1; OVCAR8-FG and OVCAR3-FG, E:T ratio = 1:1.

(E-G) FACS and ELISA characterizations of $Allo^{15}$ MCAR-NKT cells 24 hours after coculturing with OVCAR3-FG cells. Healthy donor PBMC-derived T and MCAR-T cells were included as therapeutic cell controls. (E) FACS plots showing the expression of activation marker (i.e., CD69), cytotoxic molecules (i.e., Perforin and Granzyme B), and lysosomal-associated membrane protein (i.e., CD107a) of the therapeutic cells. Data of $Allo^{15}$ MCAR-NKT cells are presented. (F) Quantification of E (n = 4). MFI, mean fluorescence intensity. (G) ELISA measurements of IFN- γ production by the indicated therapeutic cells (n = 4).

(H-J) Studying the anti-OC efficacy of $Allo^{15}$ MCAR-NKT cells using an *in vitro* serial tumor cell killing assay. (H) Experimental design. Note the stimulator cells were not labeled with FG while the indicator cells were labeled with FG. (I) OVCAR3-FG tumor cell killing data (n = 4). (J) OVCAR3-FG^{MSLN^{-/-}} tumor cell killing data (n = 4).

(K-M) Stability study of allogeneic CAR-NKT cell products. (K) FACS analyses of the viability of indicated samples (n = 4). mo, month; yr, year. (L) Schematics showing the *in vitro* tumor cell killing assay used to test cytotoxicity. (M) Tumor cell killing data at 24 h (n = 4).

Representative of 3 experiments. Data are presented as the mean $\pm$ SEM. ns, not significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by one-way ANOVA (D, F, G, K, and M).

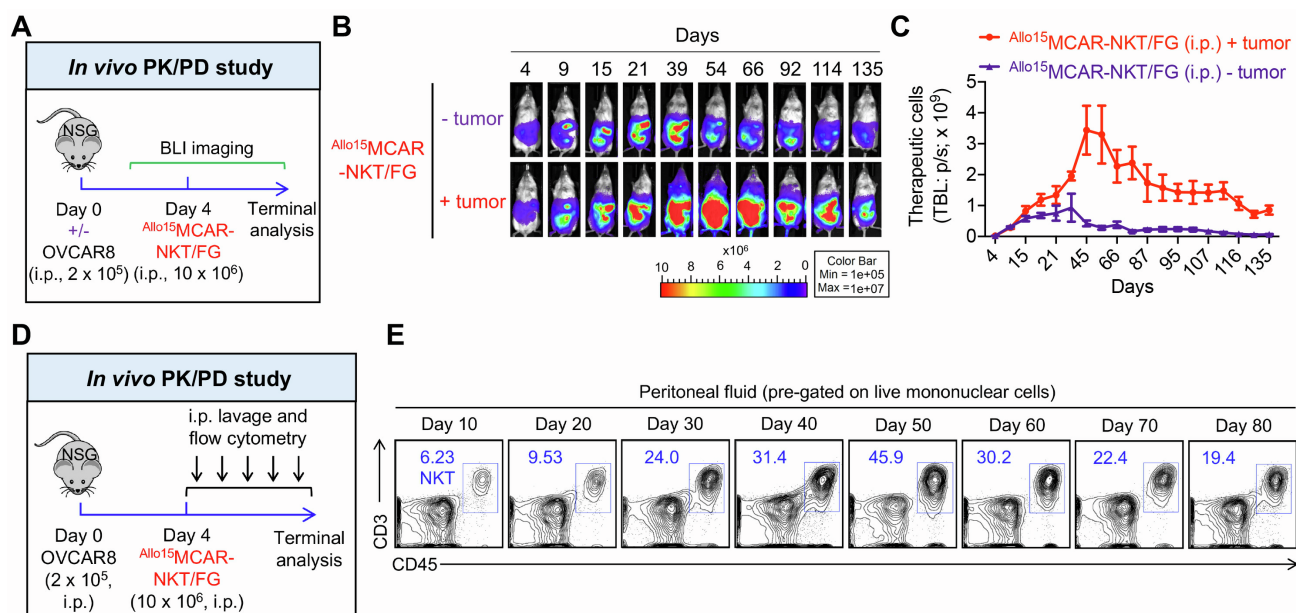
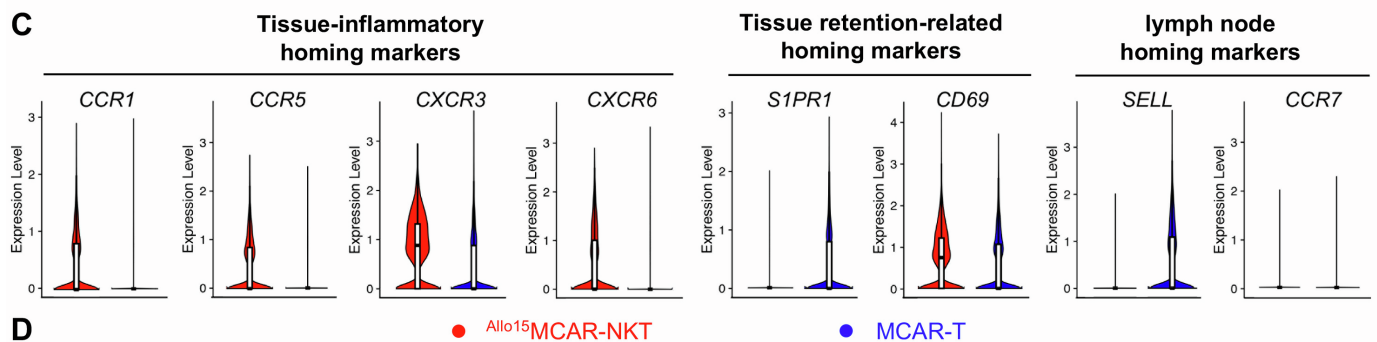
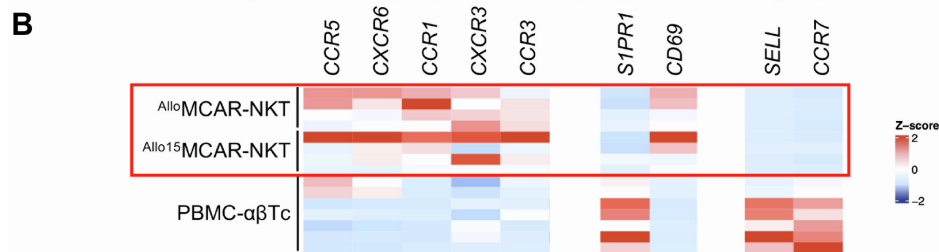
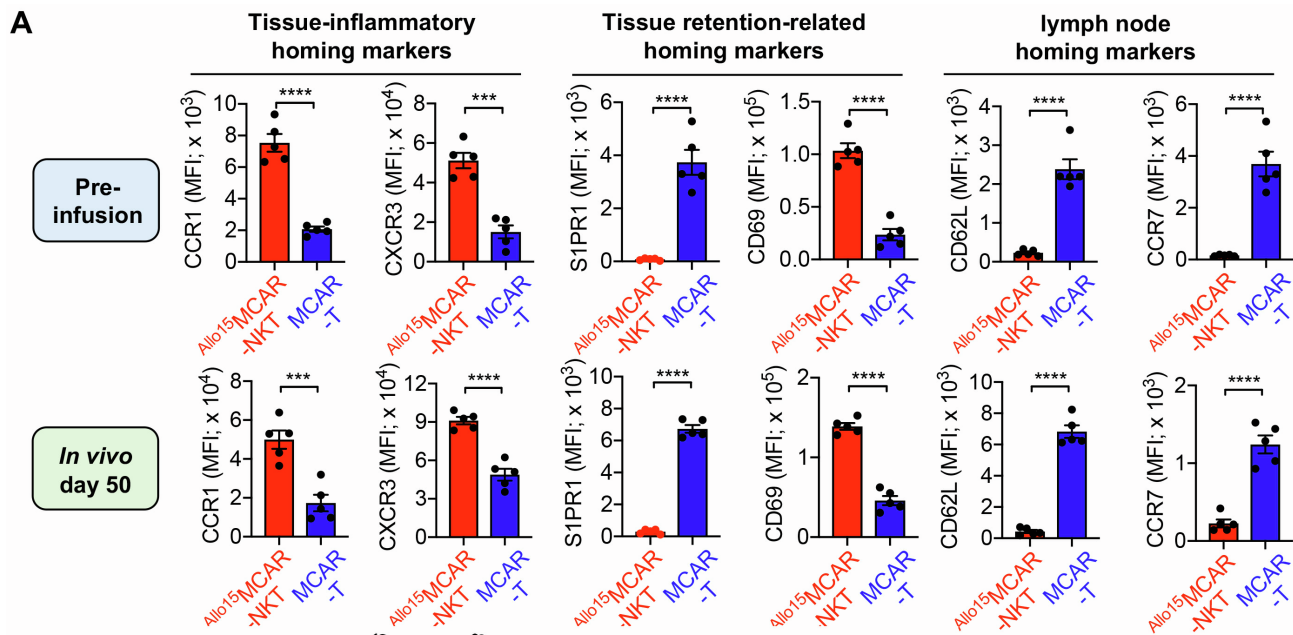


Figure S4. Studying the *in vivo* PK/PD of allogeneic CAR-NKT cells; related to Figure 3.

(A-C) Studying the *in vivo* PK/PD of $Allo^{15}MCAR-NKT$ cells with or without OVCAR8 tumor cell inoculation. (A) Experimental design. $Allo^{15}MCAR-NKT/FG$ cells were i.p. injected into experimental mice either bearing or not bearing OVCAR8 tumor cells. (B) BLI images showing the presence of therapeutic cells in experimental mice over time. (C) Quantification of (B) ($n = 3-5$). Note that the data for the $Allo^{15}MCAR-NKT + tumor$ groups were also presented in the main Figures 3I and 3J.

(D-E) Studying the *in vivo* PK/PD of $Allo^{15}MCAR-NKT$ cells using i.p. lavage and flow cytometry. (D) Experimental design. (E) FACS plots showing the presence of $Allo^{15}MCAR-NKT/FG$ cells in the peritoneal fluid of experimental mice.

Representative of 3 experiments.



D

In vivo tumor homing capacity of Allo15MCAR-NKT cells and their distinct pattern of homing markers	
Potent tumor homing capacity	High expression of tissue-inflammatory homing markers (e.g., CCR1, CCR3, CCR5, CXCR3, and CXCR6)
Improved tissue retention and reduced blood circulation	S1PR1 ^{low} CD69 ^{high}
Reduced lymph node-homing	CD62L ^{low} CCR7 ^{low}

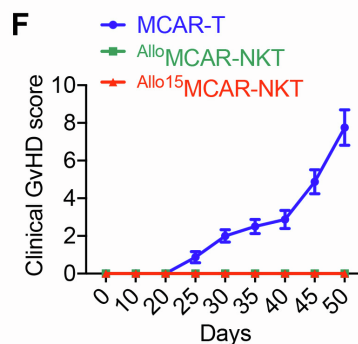
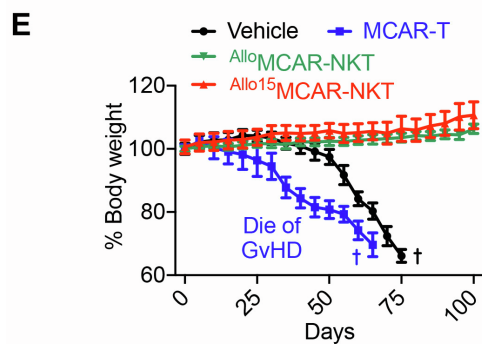


Figure S5. Distinct homing marker pattern of allogeneic CAR-NKT cells, and GvHD risk of allogeneic CAR-NKT cells; related to Figures 3 and 4

(A-D) Studying the distinct homing marker pattern of allogeneic CAR-NKT cells. (A) FACS measurements of surface homing markers on $^{Allo^{15}}$ MCAR-NKT cells before infusion (upper panels; n = 5) and post *in vivo* anti-OC response as described in Figures 3A and 3B (lower panels; n = 5). (B) Bulk RNA-seq analyses of the indicated therapeutic cells before infusion. Heatmap is presented showing the expression levels of the indicated homing marker genes in the $^{Allo^{15}}$ MCAR-NKT and PBMC- $\alpha\beta$ Tc cell samples. Each row represents an individual sample. Each column represents an indicated gene. (C) scRNA-seq analyses of the indicated therapeutic cells collected from the OVCAR8-FG xenograft mice at week 8 as described in Figure 5A. Violin plots are presented showing the expression distribution of the indicated homing marker genes in $^{Allo^{15}}$ MCAR-NKT and MCAR-T cells. (D) Table summarizing the *in vivo* tumor homing capacity of $^{Allo^{15}}$ MCAR-NKT cells and their distinct pattern of homing markers.

(E-F) Studying the GvHD risk of allogeneic CAR-NKT cells. Data is related to main Figures 4A-4D. (E) Body weight measured over time (n = 8). (F) Clinical GvHD score recorded over time (n = 8). The score was calculated as the sum of individual scores of 6 categories (body weight, activity, posture, skin thickening, diarrhea, and dishevelment; score 0-2 for each category).

Representative of 3 (A, E, and F) and 1 (B and C) experiments. Data are presented as the mean $\pm$ SEM. ***p < 0.001, ****p < 0.0001 by Student's *t* test (A).

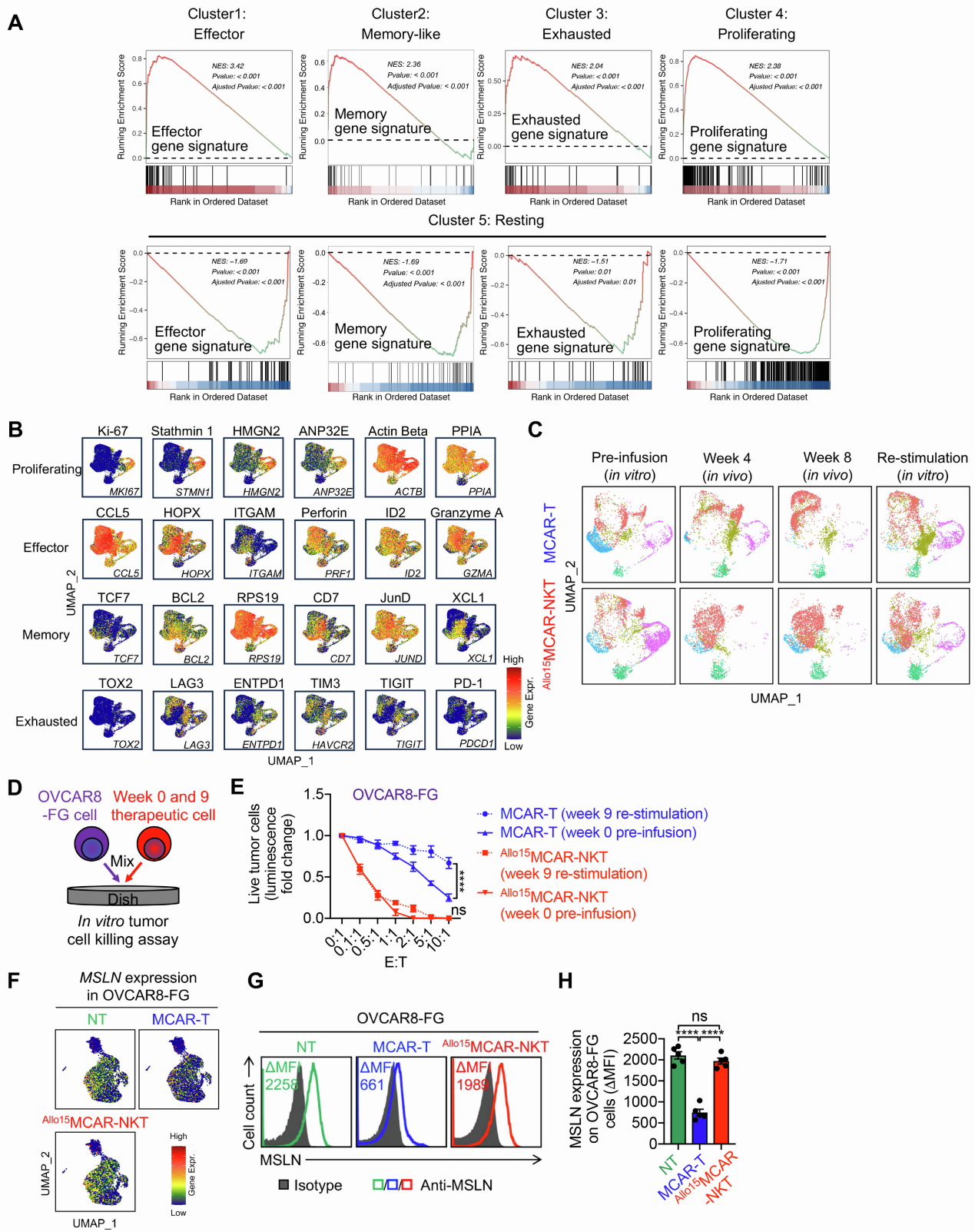


Figure S6. Studying the *in vivo* gene profile of $\text{Allo}^{15}\text{MCAR-NKT}$ cells using scRNA-seq analyses and the functional validations; related to Figure 5.

(A) Gene Set Enrichment Analysis (GSEA) plots showing the enrichment of gene signatures in the indicated five cell clusters.

(B) UMAP plots showing the expression distribution of the indicated signature genes.

(C) Individual UMAP plots showing cell cluster composition of the indicated samples. Each dot represents a single cell and is colored according to its cell cluster assignment. Data are related to Figures 5B and 5C.

(D and E) Studying the OC tumor cell killing capacity of Week 0 and 9 therapeutic cells. Peritoneal fluids were collected from the experimental mice at week 8, followed by *in vitro* re-stimulation with MSLN-expressing artificial antigen-presenting cells (MSLN-aAPCs) for one week to generate the Week 9 therapeutic cells, as shown in Figure 5A. (D) Experimental design. (E) Tumor cell killing data at 24 h (n = 4). Note that the data for the Week 0 therapeutic cell groups were also presented in the main Figure 2K.

(F) UMAP plots showing the expression distribution of *MSLN* gene in the indicated samples. Data are related to Figure 5H.

(G and H) Studying the CAR antigen escape of OC tumor cells. Peritoneal fluids were collected from the experimental mice at week 4, and the OVCAR8-FG tumor cells (identified as GFP⁺ cells) were analyzed using flow cytometry. (G) FACS detection of MSLN expression on OVCAR8-FG tumor cells collected from the indicated experimental mice. (H) Quantification of G (n = 5).

Representative of 1 (A-C) and 3 (D-H) experiments. Data are presented as the mean $\pm$ SEM. ns, not significant, ****p < 0.0001 by one-way ANOVA (E and H).

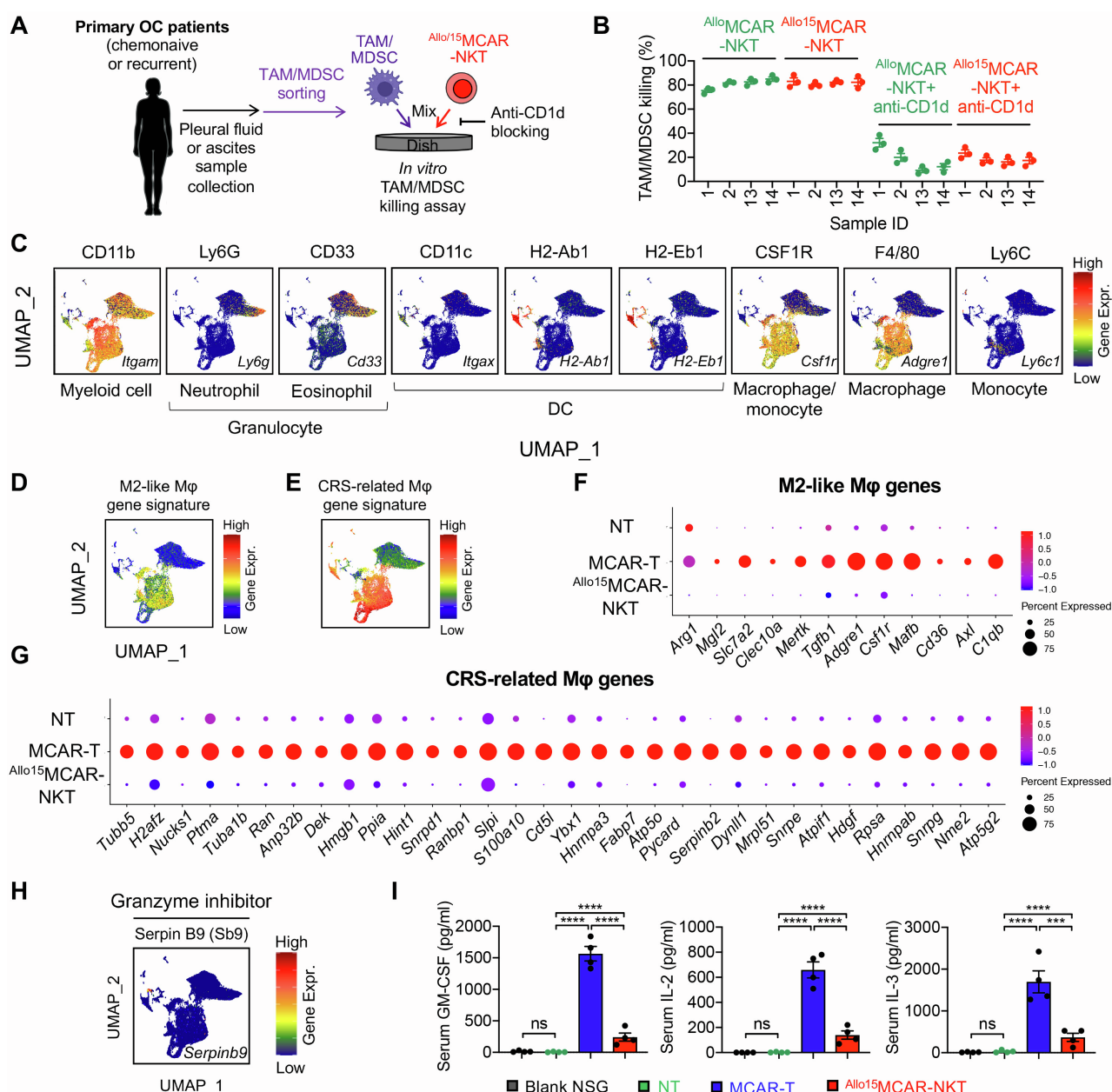


Figure S7. Allogeneic CAR-NKT cells dramatically alter the OC TME; related to Figure 6.

(A and B) Studying Allo¹⁵MCAR-NKT cells targeting of OC TME using primary OC patient samples. A total of 4 primary OC samples were analyzed. (A) Experimental design. (B) TAM/MDSC killing data at 24 h (n = 3).

(C) UMAP plots showing the gene expression distribution of the indicated mouse immune cell lineage markers, that were used to define cell clusters in Figure 7J.

(D) UMAP plot showing the expression distribution of M2-like macrophage gene signature.

(E) UMAP plot showing the expression distribution of CRS-related macrophage gene signature.

(F and G) Dot plots showing the expression levels of the indicated M2-like (F) and CRS-related (G) macrophage genes. The dot color represents the gene expression level, with a gradient from low (blue) to high (red). The dot size indicates the percentage of cells expressing the corresponding gene.

(H) UMAP plot showing the expression distribution of *Serpinb9* gene which encodes the Sb9 Granzyme inhibitor.

(I) ELISA analyses of human CRS-related cytokines in the serum samples collected from the indicated experimental mice on Day 3 (n = 4).

Representative of 1 (C-H) and 3 (A, B, and I) experiments. Data are presented as the mean $\pm$ SEM. ns, not significant, ***p < 0.001, ****p < 0.0001 by one-way ANOVA (I).

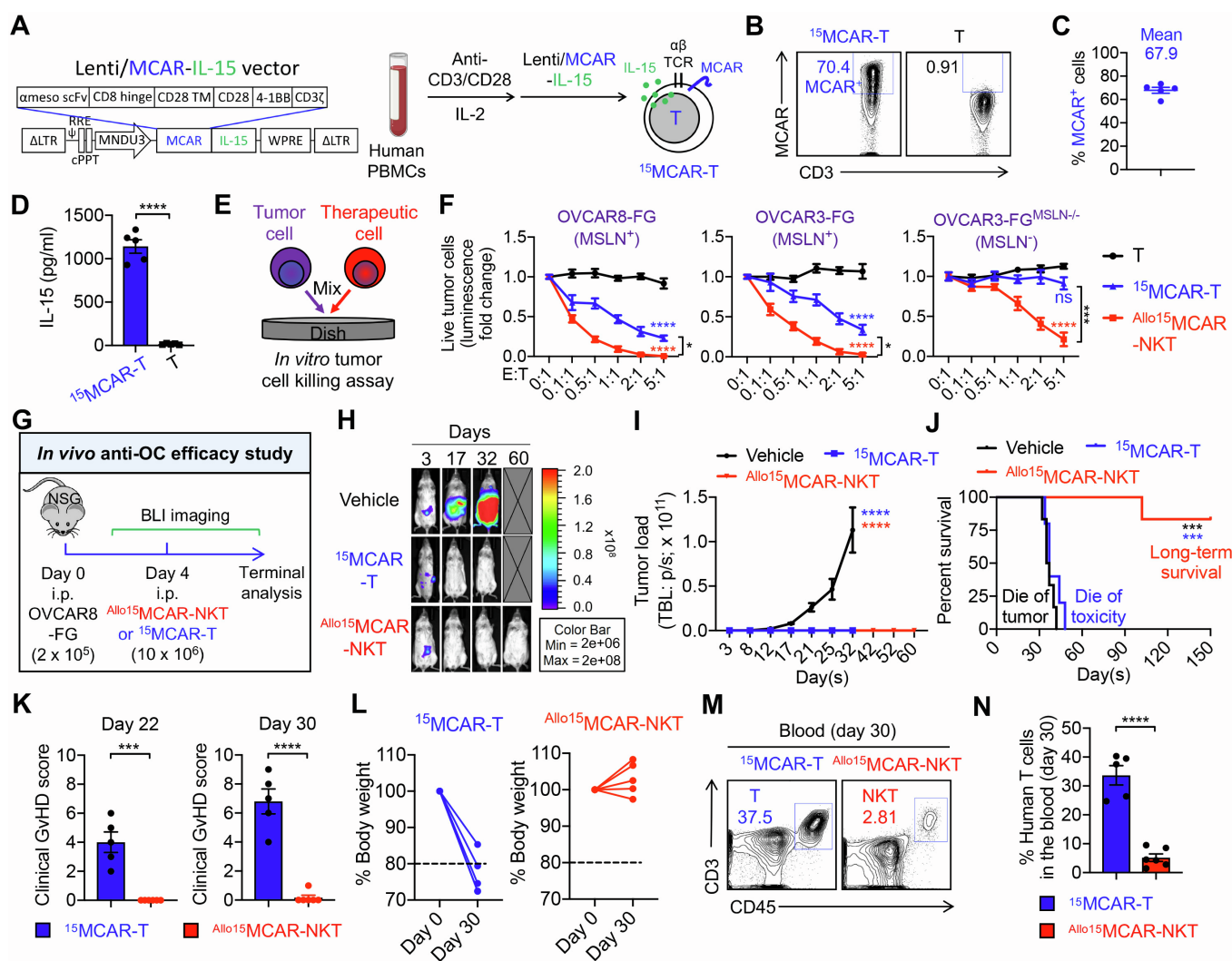


Figure S8. Allogeneic CAR-NKT cells exhibit a superior safety profile compared to IL-15-enhanced conventional MCAR-T ($^{15}\text{MCAR-T}$) cells; related to Figures 2, 3, and 7.

(A-D) Generation of $^{15}\text{MCAR-T}$ cells. (A) Diagram showing the design of Lenti/MCAR-IL-15 lentivector, and the generation of $^{15}\text{MCAR-T}$ cells from healthy donor PBMCs. (B) FACS detection of the MCAR expression on T and $^{15}\text{MCAR-T}$ cells. (C) Quantification of (B) (n = 5; n indicates different PBMC donors). (D) ELISA analyses of IL-15 production by T and $^{15}\text{MCAR-T}$ cells cultured *in vitro* for 24 h (n = 5; n indicates different PBMC donors).

(E and F) Studying the *in vitro* antitumor efficacy of $\text{Allo}^{15}\text{MCAR-NKT}$ and $^{15}\text{MCAR-T}$ cells against human OC cell lines. (E) Experimental design. (F) Tumor cell killing data at 24 h (n = 4).

(G-J) Studying the *in vivo* antitumor efficacy of $\text{Allo}^{15}\text{MCAR-NKT}$ and $^{15}\text{MCAR-T}$ cells in an OVCAR8-FG human OC xenograft NSG mouse model. (G) Experimental design. (H) BLI images showing tumor loads in experimental mice over time. (I) Quantification of H (n = 5-6). (J) Survival curve (n = 5-6). Note that the data for the Vehicle and $\text{Allo}^{15}\text{MCAR-NKT}$ groups were also presented in the main Figures 3A-3D.

(K-N) Studying the *in vivo* safety of $\text{Allo}^{15}\text{MCAR-NKT}$ and $^{15}\text{MCAR-T}$ cells in an OVCAR8-FG human OC xenograft NSG mouse model. (K) Clinical GvHD score of experimental mice on days 22 and 30 (n = 5-6). A clinical GvHD score was calculated as the sum of individual scores of 6 categories (body weight, activity, posture, skin thickening, diarrhea, and dishevelment; score 0-2 for each category). (L) Body weight of experimental mice on days 0 and 30 (n = 5-6).

6). For each mouse, body weight on day 0 was normalized to 100%. (M) FACS detection of Allo¹⁵MCAR-NKT and ¹⁵MCAR-T cell percentage among total mononuclear cells in mouse blood collected on day 30. (N) Quantification of (M) (n = 5-6).

Representative of 3 experiments. Data are presented as the mean $\pm$ SEM. ns, not significant, *p < 0.05, ****p < 0.0001, by Student's *t* test (K and N), one-way ANOVA (F and I), or log rank (Mantel-Cox) test adjusted for multiple comparisons (J).

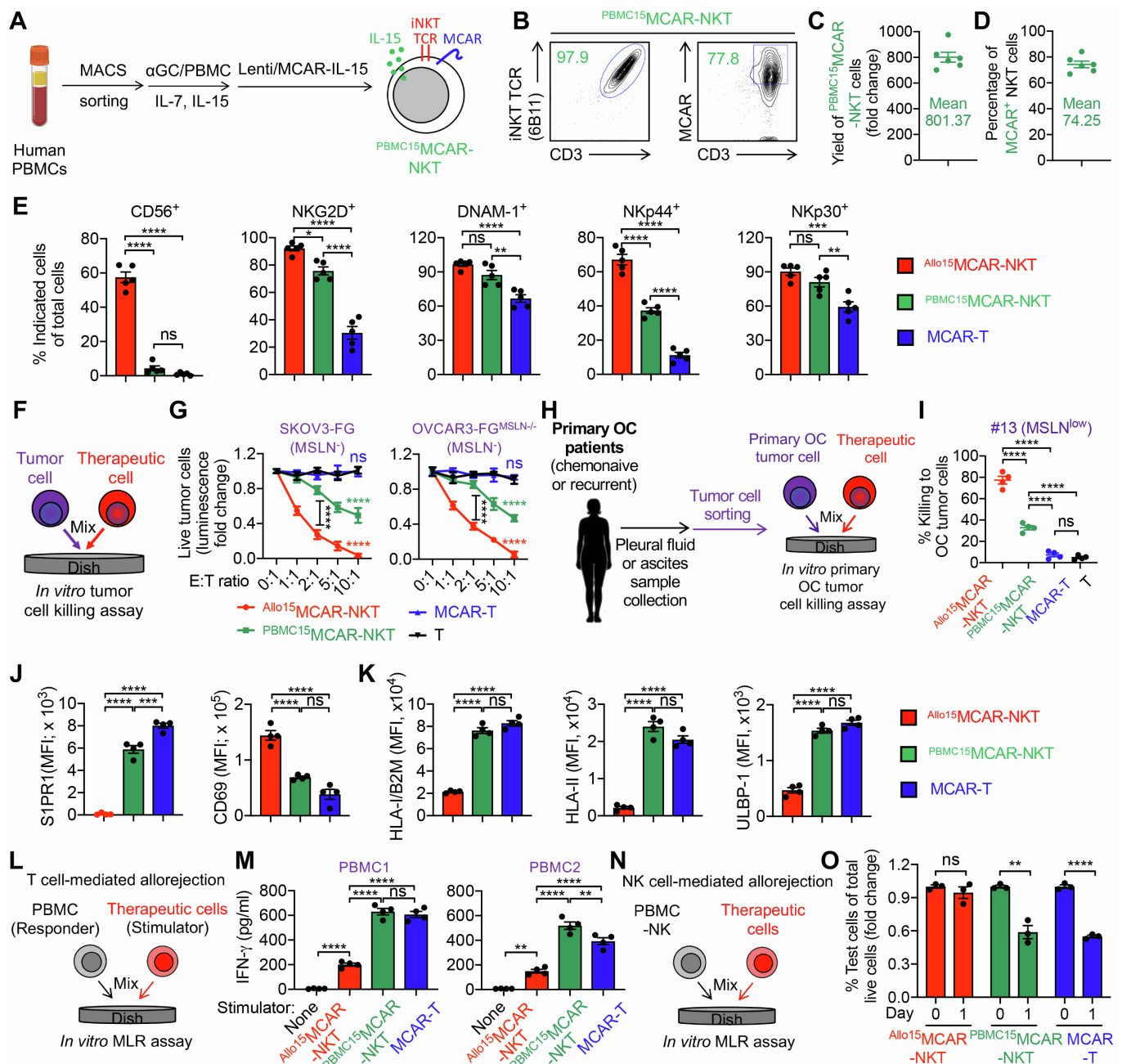


Figure S9. Allogeneic CAR-NKT cells exhibit unique features distinct from PBMC-derived CAR-NKT cells; related to Figures 2, 3, and 7.

(A-D) Generation of healthy donor PBMC-derived IL-15-enhanced MCAR-NKT (PBMC15 MCAR-NKT) cells. (A) Schematics showing the generation of PBMC15 MCAR-NKT cells. (B) FACS plots showing the purity and MCAR expression of PBMC15 MCAR-NKT cells. (C) Yield of PBMC15 MCAR-NKT cells (n = 6 from 6 different healthy donors). (D) MCAR expression of PBMC15 MCAR-NKT cells (n = 6 from 6 different healthy donors).

(E-I) Comparing NKR expression and NKR-mediated tumor cell killing between Allo15 MCAR-NKT and PBMC15 MCAR-NKT cells. (E) FACS analyses of surface NK marker and NKRs on the indicated therapeutic cells. (F) Experimental design to study the *in vitro* tumor cell killing of the three therapeutic cells using human tumor cell lines. (G) Tumor cell killing data at 24 h (n = 4). (H) Experimental design to study the *in vitro* tumor cell killing of the three therapeutic cells

using primary OC patient samples. (I) Tumor cell killing data at 24 h (n = 4). MSLN^{low} indicates OC tumor samples expressing low levels of MSLN.

(J) FACS analyses of surface homing markers (i.e., S1PR1 and CD69) on the three therapeutic cells (n = 4).

(K) FACS analyses of surface HLA-I/II and ULBP-1 on the three therapeutic cells (n = 4).

(L and M) Studying the T cell-mediated allojection against the three therapeutic cells using an *in vitro* MLR assay. PBMCs from over five random mismatched healthy donors were used as responder cells. Data from two representative donor are presented. (L) Experimental design. (M) ELISA analyses of IFN- γ production on day 4 (n = 4).

(N and O) Studying the NK cell-mediated allojection against the three therapeutic cells using an *in vitro* MLR assay. PBMC-NK cells isolated from over five random mismatched healthy donors were used. Data from one representative donor are presented. (N) Experimental design. (O) FACS quantification of the indicated live cells on day 0 and day 1 (n = 3).

Representative of 3 experiments. Data are presented as the mean $\pm$ SEM. ns, not significant, **p < 0.01, ****p < 0.0001, by Student's *t* test (O), one-way ANOVA (E, I-K, and M), or two-way ANOVA (G).

Table S1. Clinical ovarian cancer (OC) patient samples; related to Figure 1.

	Sample ID	Diagnosis	Type	Stage	Treatment Status	Platinum Sensitivity
CHEMONAIVE n = 12	1	HGSOC	Ascites	3C	Chemonaive	Sensitive
	2	HGSOC	Ascites	3C	Chemonaive	Sensitive
	3	HGSOC	Ascites	3C	Chemonaive	Sensitive
	4	HGSOC	Ascites	3C	Chemonaive	Sensitive
	5	HGSOC (80%) with sarcomatous elements (20%)	Ascites	2B	Chemonaive	Sensitive
	6	HGSOC (90%), endometrioid (10%)	Ascites	3C	Chemonaive	Sensitive
	7	HGSOC	Ascites	4B	Chemonaive	Resistant
	8	HGSOC	Ascites	4A	Chemonaive	Resistant
	9	HGSOC	Ascites	3C	Chemonaive	Resistant
	10	HGSOC	Pleural Fluid	4B	Chemonaive	Resistant
	11	HGSOC & clear cell carcinoma	Ascites	3C	Chemonaive	Resistant
	12	HGSOC	Ascites	3C	Chemonaive	Resistant
RECURRENT OR POST-NACT n = 13	13	HGSOC	Ascites	3C	Recurrent	Resistant
	14	HGSOC	Pleural Fluid	3C	Recurrent	Resistant
	15	HGSOC	Pleural Fluid	2A	Recurrent	Resistant
	16	HGSOC	Pleural Fluid	3C	Recurrent	Resistant
	17	HGSOC	Ascites	2A	Recurrent	Sensitive
	18	HGSOC	Pleural Fluid	4A	Recurrent	Resistant
	19	HGSOC	Pleural Fluid	4B	Recurrent	Resistant
	20	HGSOC	Pleural Fluid	3C	Recurrent	Resistant
	21	HGSOC	Ascites	4	Recurrent	Resistant
	22	HGSOC	Pleural Fluid	4B	Recurrent	Resistant
	23	HGSOC	Pleural Fluid	4B	Recurrent	Resistant
	24	HGSOC	Pleural Fluid	3C	Recurrent	Sensitive
	25	HGSOC	Ascites	3C	Post-neoadjuvant chemotherapy (NACT)	Resistant
MATCHED n = 10	26A	HGSOC	Ascites	3C	Chemonaive	Sensitive
	26B	HGSOC	Ascites	3C	Recurrent	Resistant
	27A	HGSOC	Ascites	3C	Chemonaive	Sensitive
	27B	HGSOC	Pleural Fluid	3C	Recurrent	Resistant
	28A	HGSOC	Ascites	3C	Chemonaive	Sensitive
	28B	HGSOC	Ascites	3C	Recurrent	Resistant
	29A	HGSOC	Ascites	3C	Chemonaive	Resistant
	29B	HGSOC	Ascites	3C	Recurrent	Resistant
	30A	HGSOC	Ascites	3C	Chemonaive	Sensitive
	30B	HGSOC	Ascites	3C	Post-NACT	Sensitive

Table S2. MSLN expression on the primary OC tumor cells; related to Figure 1.

Sample ID	MSLN expression level	
1	++	
2	-	
3	++	
4	++	
5	-	
6	+	
7	+	
8	+++++	
9	-	
10	-	
11	-	
12	-	
13	-	
14	+++++	
15	-	
16	-	
17	+	
18	+++	
19	+++	
20	++	
21	+	
23	++	
24	-	
25	+	
26A	+	
26B	+	
27A	++	
27B	++	
28A	+	
28B	+	
29A	+	
29B	+	
30A	+	
30B	+	

+++++ Expression > 80%
++++ Expression 60-80%
+++ Expression 40-60%
++ Expression 20-40%
+ Expression 5-20%
- Expression < 5%

Table S3. Purity data of allogeneic MCAR-NKT cell products generated from multiple cord blood (CB) donors; related to Figure 2.

CB donors	Purity of ^{Allo} MCAR-NKT cells (identified as iNKT TCR ⁺ CD3 ⁺ cells)	Purity of ^{Allo15} MCAR-NKT cells (identified as iNKT TCR ⁺ CD3 ⁺ cells)
1	98.5% (Data represent the average of three independent cell culture batches; the same applies to the data presented below)	99.6%
2	98.4%	99.3%
3	99.2%	99.7%
4	97.8%	96.8%
5	99.2%	97.5%
6	98.2%	98.4%
7	98.0%	99.2%
8	99.0%	99.0%
9	98.2%	98.0%
10	97.2%	99.2%