

Development of Tumor-Primed CD8⁺ NKT-Like Cells as a Promising New Cell-Based Therapy for Relapsed/Refractory Multiple Myeloma

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Abstract

Relapsed and refractory multiple myeloma (RRMM) represents a persistent and difficult clinical problem. The majority of patients ultimately experience disease recurrence after standard therapies and have very few remaining treatment alternatives. Although BCMA CAR-T cell therapy has delivered impressive clinical responses in selected cases, its broader application is constrained by dependence on patient-derived cells, lengthy manufacturing periods, elevated costs, and significant immune-mediated toxicities. These barriers have generated a strong need for alternative immunotherapies that are safer, easier to obtain, and can be administered more promptly. Umbilical cord blood mononuclear cells (CBMC) were grown in co-culture with X-ray-irradiated bone marrow mononuclear cells (BMMC) sourced from RRMM patients, supplemented with a specific set of cytokines, to create a new immune effector cell product suitable for RRMM treatment. Phenotypic profiling and functional properties were examined, with particular focus on MHC-restricted and MHC-unrestricted killing pathways, using flow cytometric marker analysis, cytokine quantification, in vitro tumor lysis assays, and receptor-blocking studies. In vivo antitumor potency was tested in immunodeficient mouse xenograft models bearing MM.1S and RPMI-8226 human myeloma cells. Through this tumor-priming approach, we reliably expanded CD8⁺ NKT-like cells that displayed potent tumor-killing activity and markedly increased cytokine release when challenged with myeloma cell lines and freshly isolated RRMM patient cells. These tumor-primed CD8⁺ NKT-like cells (TPNC) mediated cytotoxicity via dual mechanisms: MHC-independent pathways dependent on LFA-1 and DNAM-1, as well as MHC-restricted, TCR-driven target recognition. TPNC efficiently assembled immunological synapses, rapidly redirected cytotoxic granules toward targets, and was capable of sequential killing of multiple tumor cells. In xenograft models, administration of TPNC led to a substantial reduction in tumor burden, markedly extended survival times, and detectable persistence in peripheral blood without apparent adverse effects. Applying the identical priming protocol to acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) also yielded functional TPNC with strong lytic activity. Notably, TPNC killed primary ALL cells at levels comparable to anti-CD19 CAR-NK cells. TPNC constitute an innovative cytotoxic lymphocyte population generated by tumor antigen-driven priming. Their capacity for both innate-like and adaptive-like target recognition, combined with versatile effector functions and an encouraging safety profile, suggests they could serve as a practical, scalable, and patient-specific platform for immunotherapy across multiple hematologic malignancies.

Keywords: Cellular immunotherapy, Relapsed/refractory multiple myeloma, NKT-like cells, Hematologic malignancies

Introduction

Multiple myeloma (MM) is a cancer of plasma cells that, despite major improvements in available treatments, is

still considered largely incurable [1, 2]. Although proteasome inhibitors, immunomodulatory agents, monoclonal antibodies, and autologous stem cell transplantation have extended survival, disease relapse or resistance eventually develops in nearly all patients [3-5]. For those with relapsed/refractory multiple myeloma (RRMM), particularly after multiple prior lines of therapy, median overall survival typically ranges from only 2 to 3 years [6-8].

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BCMA-targeted CAR-T cell products, including idecabtagene vicleucel and ciltacabtagene autoleucel, have achieved high overall response rates and deep remissions (including MRD negativity) in RRMM [9-11]. However, responses are often transient, with the majority of patients progressing within 1–2 years [12, 13]. These therapies are also linked to serious toxicities such as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS). Furthermore, their personalized autologous manufacturing process results in long production times and limited accessibility, especially problematic for patients with rapidly advancing disease [14-16]. These issues emphasize the demand for next-generation immune cell therapies that are safer, more readily manufactured, and immediately usable.

Recent evidence shows that certain innate immune cells, especially NK cells, can acquire adaptive, memory-like characteristics after sustained exposure to antigens. These changes include amplified cytotoxic capacity, elevated proinflammatory cytokine output, and prolonged in vivo survival [17-19]. Such adaptive NK cells undergo clonal proliferation, epigenetic reprogramming, and enhanced recall responses to previously encountered viral or tumor antigens [20, 21], positioning them as attractive candidates for advanced cancer immunotherapy.

Inspired by these observations, we developed a tumor-priming culture system in which umbilical cord blood mononuclear cells (CBMC) were incubated with irradiated RRMM patient-derived cells under defined cytokine support. Instead of generating classical memory-like NK cells as initially anticipated, the cultures were dominated by CD8⁺ NKT-like cells. Comprehensive characterization revealed that these tumor-priming CD8⁺ NKT-like cells (TPNC) possess potent lytic activity against both established myeloma lines and primary RRMM samples, high levels of IFN- γ and TNF- α secretion, and efficient formation of immune synapses with polarized degranulation. Mechanistically, they integrate both MHC-restricted TCR signaling and MHC-independent pathways, reflecting a hybrid innate-adaptive phenotype.

When infused into xenograft mouse models, TPNC potently suppressed myeloma progression, significantly prolonged host survival, and demonstrated in vivo persistence without detectable toxicity. These encouraging results prompted extension of the priming strategy to AML and ALL, where similarly functional

TPNC were obtained. The approach therefore offers a promising non-gene-modified, off-the-shelf-compatible platform for treating hematologic malignancies.

Materials and Methods

Human materials and isolation

This research was approved by the Institutional Review Board of Dong-A University (approval number: 2-1040709-AB-N-01-202303-BR-002-02) and the ethics committee of the collaborating hospital (DAUHIRB-23-029) in compliance with the Declaration of Helsinki. Written informed consent was secured from every participant. Bone marrow aspirates were obtained during routine diagnostic or monitoring procedures from patients with RRMM (to evaluate clonal plasma cells) or with ALL/AML (to assess leukemic blasts). CBMC and BMMC were purified from the mononuclear cell layer by density gradient centrifugation using Lymphoprep™ (STEMCELL Technologies, Canada).

CIK cells and TPNC culture

Both cytokine-induced killer (CIK) cells and TPNC were expanded from CBMCs isolated by mechanical processing and density gradient separation. All cultures were performed in CTS™ NK-Xpander™ medium (Gibco, USA) supplemented with 5% human platelet lysate (HPL; DYNE BIO, Korea) and 1% penicillin/streptomycin (Capricorn, Germany) at 37 °C in a 5% CO₂ atmosphere.

CIK cells culture

3×10^6 CBMC were seeded per well in 24-well plates. On day 0, 1000 U/mL recombinant human IFN- γ (Peprotech, USA) was introduced. On day 1, 50 ng/mL anti-CD3 monoclonal antibody (Invitrogen, USA) and 20 ng/mL IL-2 (Peprotech) were added. Culture medium was exchanged every 2–3 days, each time with fresh medium containing 20 ng/mL IL-2.

TPNC culture

For TPNC generation, 3×10^6 CBMC were co-cultured with 5×10^5 X-ray-irradiated autologous or patient-matched BMMC (derived from RRMM, ALL, or AML cases) in 24-well plates. Feeder cells were irradiated to inhibit proliferation and prevent any potential transfer of viable tumor cells, in line with established protocols for adoptive cell therapy expansion. Stimulation on day 0 included 20 ng/mL IL-2, 5 ng/mL IL-21 (PeproTech), and 25 ng/mL IL-18 (R&D Systems). On days 3 and 5,

medium was replaced with fresh medium containing IL-2 (20 ng/mL). On day 7, 1×10^6 TPNC were re-challenged with 5×10^5 freshly irradiated BMMC in medium supplemented with 20 ng/mL IL-2 and 5 ng/mL IL-21, with further medium changes every 2–3 days thereafter.

Cell lines

Multiple myeloma (IM-9, RPMI-8226), acute lymphoblastic leukemia (CCRF-CEM, MOLT4), and acute myeloid leukemia (HL-60, THP-1) human cell lines were obtained from the Korean Cell Line Bank. They were routinely propagated in RPMI 1640 medium (Welgene, Korea) with 10% fetal bovine serum (FBS; Welgene, Korea) and 1% penicillin/streptomycin (Capricorn, Germany). Luciferase-tagged MM.1S (MM.1S-luc) and U266 lines were provided as a kind gift by Professor Sunghoon Jung at Chonnam National University Hwasun Hospital, Korea. Regular mycoplasma screening was performed on all cell lines.

Flow cytometry

Flow cytometry was used to monitor temporal changes in CD3 and CD56 levels throughout the culture and to characterize the surface phenotype and intracellular cytotoxic effector molecules in both CIK and TPNC populations. Surface marker assessments involved collecting cells at weekly intervals (days 0–21) and staining them with anti-CD3 and anti-CD56 antibodies. For terminal phenotyping, 1×10^5 cells were washed and incubated with antibodies for 30 minutes at 4 °C, protected from light, before acquisition. Intracellular detection of perforin and granzyme B was followed by surface staining using a fixation/permeabilization kit. Data acquisition was performed on an Attune NxT flow cytometer (Thermo Fisher Scientific, USA), and analysis was conducted in FlowJo version 10.9.0 (FlowJo, LLC, USA). Gating was primarily performed on the CD3⁺CD56⁺ fraction except where indicated otherwise.

Cytotoxicity assay

Killing capacity was determined by employing CIK cells and TPNC as effectors against a panel of targets that included MM cell lines (IM-9, RPMI-8226, U266), ALL lines (CCRF-CEM, MOLT4), AML lines (HL-60, THP-1), and primary malignant cells from patients diagnosed with RMM, ALL, or AML. Targets were pre-stained with CFSE and mixed with effectors at multiple effector-to-target (E: T) ratios in 96-well plates for a 4-hour

incubation at 37 °C under 5% CO₂. Post-co-culture, fixable viability dye (FVD) was added to distinguish dead cells. Lysis was quantified as the proportion of CFSE⁺FVD⁺ events measured on an Attune NxT flow cytometer (Thermo Fisher Scientific, USA) and processed with FlowJo v10.9.0 (FlowJo, LLC, USA).

Degranulation assay

Assessment of degranulation relied on detection of CD107a upregulation on the cell surface. Effector and target cells were combined at a 1:1 E: T ratio in 96-well plates and incubated for 4 hours at 37 °C with 5% CO₂ in the presence of anti-CD107a antibody and GolgiStop (Monensin; BD Biosciences, USA). Following incubation, effector cells were identified by CD3 and CD56 staining. Samples were then fixed and permeabilized with Cytfix/Cytoperm solutions, followed by washing in Perm/Wash buffer (BD Biosciences, USA). CD107a levels within the CD3⁺CD56⁺ population were evaluated using an Attune NxT flow cytometer (Thermo Fisher Scientific, USA) and FlowJo software v10.9.0 (FlowJo, LLC, USA).

ELISA

CIK cells or TPNC were incubated with target cells at a 1:1 E: T ratio for 24 hours in 96-well plates. Harvested culture supernatants were assayed for IFN- γ and TNF- α concentrations with ELISA kits (Abcam, UK) following the supplier's recommended procedures.

TCR sequencing and analysis

RNA extracted from TPNC was used to prepare a TCR β repertoire library. cDNA synthesis began with a TCR-specific reverse primer, followed by multiplex PCR (PCR-1) incorporating V β primers carrying UMIs and constant-region primers, and then a second PCR (PCR-2) to add indices and Illumina adapters. Library quantification was performed by qPCR, and fragment analysis was performed on an Agilent TapeStation. Sequencing was performed on an Illumina NovaSeq X Plus instrument using 150 bp paired-end reads. Paired reads were merged (minimum 60 bp overlap) with UMI error correction applied. Alignment against the NCBI IgBLAST TR database was performed with IgBLAST v3.6, retaining only in-frame VJ rearrangements that met identity cutoffs (V $\geq$ 80%, J $\geq$ 80%). Sequences were clustered at 95% identity via CD-HIT. Abundant clonotypes were selected, normalized, and visualized with VDJtools and Immunarch packages. CDR3 amino

acid sequences were annotated using the VDJdb database.

Live cell imaging

Imaging was conducted on a modified Olympus IX 83 epifluorescence microscope equipped with a 40× (UPlanFLN, NA = 1.30) objective and ANDOR Zyla 4.2 sCMOS camera, with automated control via Micro-manager software.

Animal studies

Six-week-old female NOD-Prkdc em1Baek Il2rg em1Baek (NSGA) mice were sourced from GEM Biosciences Inc. (Cheongju, Republic of Korea). All procedures received approval from the Institutional Animal Care and Use Committee (DIACUC-23-49). In the systemic tumor model, 5×10^6 MM.1S-luc cells were injected intravenously into NSGA mice ($n = 6$ per group). Five days later, 5×10^6 CIK cells or TPNC in 100 μ L PBS were infused intravenously, while control mice received PBS. Weekly bioluminescence imaging was performed using the IVIS Spectrum system (Xenogen, Caliper Life Sciences, USA) to quantify tumor burden via total flux (photons/sec). On day 40, blood samples were taken to assess human cell persistence by flow cytometry. For localized tumors, 1×10^6 RPMI-8226 cells were subcutaneously injected into the flank ($n = 5$ /group). Seven days later, 5×10^6 CIK or TPNC were administered intravenously. Tumor size was monitored biweekly with calipers, and volume computed as $(\text{width})^2 \times \text{length} \times 0.5$. Animals were euthanized on day 21 for tumor weighing and ex vivo imaging. Survival experiments involved intravenous injection of 1×10^6 RPMI-8226 cells, with mice followed until humane endpoints; data were plotted as Kaplan–Meier curves. Housing and experiments occurred at the Dong-A University Animal Facility in accordance with applicable guidelines.

Statistical analysis

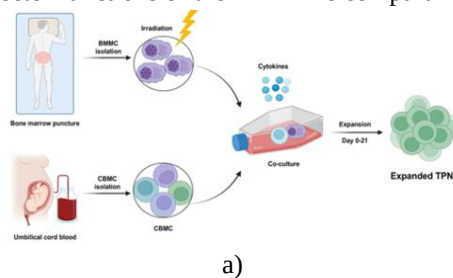
All assays were independently replicated at least 3 times—two-group comparisons employed two-tailed unpaired Student's t-tests. Multi-group analyses used one-way ANOVA with appropriate post-hoc corrections. Survival was evaluated using the Kaplan–Meier method. GraphPad Prism 9 (GraphPad Software, USA) was used for all statistical computations. Results reaching $P < 0.05$ were deemed significant. Test details for each experiment appear in the relevant figures and legends.

Results and Discussion

Development of tumor-priming CD8⁺ NKT-like cells (TPNC) capable of targeting patient-derived RRMM cells

To create a robust adoptive cell therapy option for RRMM, initial experiments evaluated the lytic potential of cord blood-derived NK (CB-NK) cells. These CB-NK cells effectively eliminated established myeloma cell lines, including IM-9 and RPMI-8226, validating their basic anticancer activity. However, exposure to freshly isolated primary RRMM tumor cells resulted in considerably weaker responses. This finding reveals a substantial limitation when moving from cell line-based testing to patient-relevant material, thereby constraining conventional NK cell strategies for clinical use in RRMM. To bridge this gap, a tumor-priming method was implemented using patient-derived tumor cells to foster the development of enhanced, memory-like effector cells. In this setup, CBMC were combined with irradiated (50 Gy) BMMC from RRMM patients along with a tailored cytokine mixture.

At culture initiation (day 0), CBMC were placed with RRMM patient BMMC in medium containing IL-2 (20 ng/mL), IL-18 (25 ng/mL), and IL-21 (5 ng/mL). Further IL-2 (20 ng/mL) was added on days 3 and 5. On day 7, a second dose of the same irradiated patient BMMC was administered, and cultures were maintained through day 21 under sustained IL-2 and IL-21 support (**Figure 1a**). Contrary to expectations, the system generated a diverse immune cell mixture rather than a predominant NK population. Notably, CD3⁺CD56⁺ NKT-like cells underwent marked preferential outgrowth, reaching about 85% of the total cells by day 21 and expanding, on average, roughly 43,000-fold (**Figures 1b–1d**). Subsequent assays demonstrated superior tumor-killing ability of these NKT-like cells compared with NK cells or conventional CD3⁺ T cells from parallel cultures, indicating that tumor priming preferentially augmented the effector functions of the NKT-like compartment.



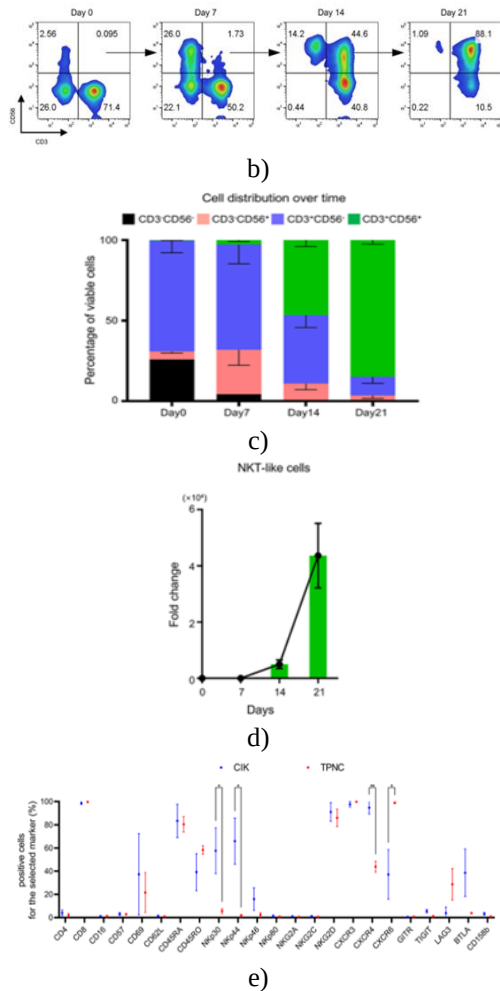


Figure 1. Generation and surface marker profiling of tumor-priming CD8⁺ NKT-like cells (TPNC).

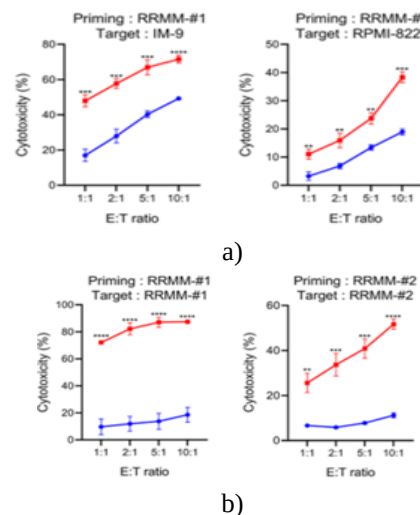
(a) Overview of the TPNC manufacturing protocol involving co-culture between CBMC and X-ray-irradiated (50 Gy) BMMC collected from individuals with RRMM. (b) Example flow cytometry dot plots illustrating changes in the frequencies of CD3-CD56⁻, CD3-CD56⁺, CD3⁺CD56⁻, and CD3⁺CD56⁺ subsets throughout the 21-day expansion culture. (c) Graphical summary of the percentages of each of the four subpopulations shown in panel (b). (d) Calculation of the expansion rate for CD3⁺CD56⁺ NKT-like cells by comparing absolute cell numbers at later time points with the initial count on day 0. (e) Flow cytometric comparison of key surface antigen expression between TPNC and standard CIK cells. Values are shown as mean $\pm$ SD derived from a minimum of three independent experiments. *P < 0.05; **P < 0.01 using Student's t-test.

Additional detailed phenotyping of these NKT-like cells involved analysis of multiple surface receptors. Conventional CD3⁺CD56⁺ cytokine-induced killer (CIK) cells acted as the benchmark population for comparison. Both cell types displayed broadly overlapping expression patterns for most lineage and activation markers; however, noticeable distinctions emerged specifically in NKp30, NKp44, CXCR4, and CXCR6 levels (**Figure 1e**).

Overall, the data reveal that although the experimental setup was originally intended to drive expansion of cytotoxic NK cells, the tumor-priming approach instead resulted in CD8⁺ NKT-like cells (TPNC) becoming the main expanded population.

Superior tumor-killing ability and effector functions of TPNC directed at RRMM targets in vitro

The lytic effectiveness of TPNC was tested on both commercial MM cell lines and freshly obtained primary RRMM specimens. Compared with controls, TPNC demonstrated substantially greater killing efficiency across a range of effector-to-target (E: T) ratios when challenged with IM-9 and RPMI-8226 lines (**Figure 2a**), autologous RRMM cells from the patients whose samples were used for priming (RRMM #1 and #2); (**Figure 2b**), and allogeneic RRMM primary cells from other patients (RRMM #3 and #4); (**Figure 2c**).



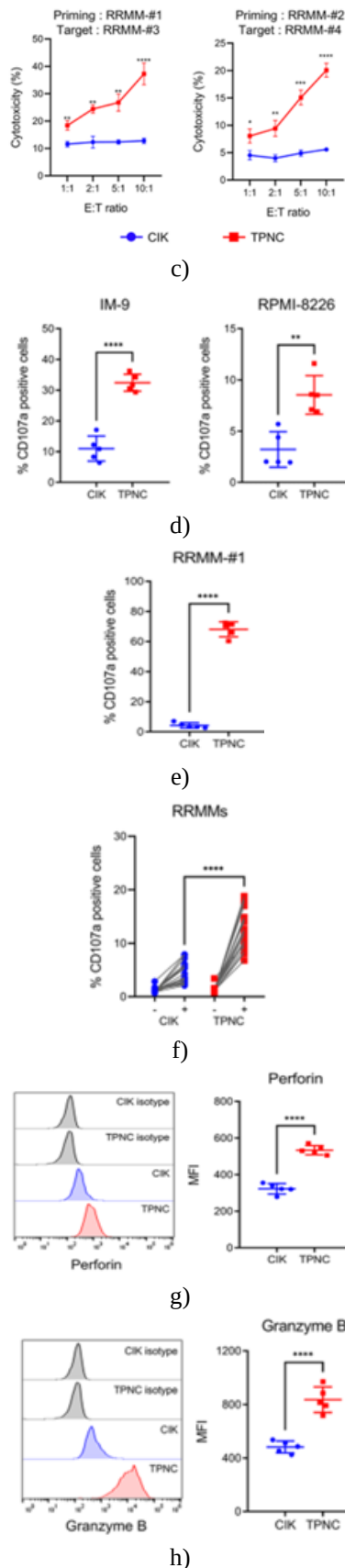


Figure 2. Functional cytotoxicity and effector activities displayed by TPNC in vitro.

(a–c) Tumor cell lysis mediated by TPNC and CIK populations tested at differing effector-to-target (E: T) ratios on MM cell lines (A), RRMM cells matched to the priming donors (B), and RRMM specimens originating from patients outside the priming cohort (C). (D–F) Evaluation of degranulation through detection of CD107a on the cell surface after incubation with MM cell lines (D), RRMM-#1 (E), and primary samples from eight independent RRMM patients not involved in the original priming (F). (G–H) Flow cytometric measurement of intracellular perforin (G) and granzyme B (H) content in TPNC relative to CIK cells. (I–J) Quantification of secreted IFN- γ (I) and TNF- α (J) by ELISA following exposure of TPNC and CIK cells to MM cell lines and primary RRMM targets. All data shown as mean $\pm$ SD compiled from a minimum of three independent experiments. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ according to Student's t-test.

TPNC also demonstrated upregulated CD107a on the surface and elevated intracellular stores of perforin and granzyme B compared with CIK cells, reflecting superior degranulation (**Figures 2d–2h**). Moreover, exposure to MM cell lines and patient-derived RRMM cells triggered substantially greater release of IFN- γ and TNF- α from TPNC than from CIK cells (**Figures 2i and 2j**).

In summary, these data highlight the markedly improved functional profile of TPNC compared with conventional CIK cells, characterized by greater lytic potency, more efficient degranulation, and enhanced secretion of key proinflammatory cytokines against both laboratory MM lines and authentic patient RRMM samples.

TPNC exert cytotoxicity through combined MHC-independent and MHC-dependent recognition pathways. Exploration of the killing mechanisms used by TPNC began with analysis of MHC-unrestricted routes, which are already well documented for CIK cells [22]. Levels of NKG2D, an important receptor for MHC-independent target recognition, proved comparable between TPNC and CIK populations (**Figure 1e**). Yet both MM cell lines and primary RRMM cells displayed only minimal expression of NKG2D ligands, indicating that this particular axis contributes little in the current setting. Subsequent investigation therefore centered on alternative MHC-unrestricted mechanisms mediated by LFA-1 and DNAM-1 [21, 23, 24], molecules previously linked to CIK cell function [25]. Surface expression of LFA-1 was notably higher on TPNC than on CIK cells, while DNAM-1 appeared strongly on both cell types (**Figures 3a–3d**). The ligands for these receptors, CD112 and CD155, were readily detectable on the majority of MM cell lines and patient-derived RRMM cells (**Figure 3e**). In functional assays, antibody-mediated blockade of either LFA-1 or DNAM-1 produced clear reductions in TPNC-driven killing of several MM cell lines (IM-9, RPMI8226, U266) and the autologous RRMM-#10 specimen (**Figures 3f–3i**). This demonstrates that MHC-independent pathways are critical to TPNC's cytotoxic activity.

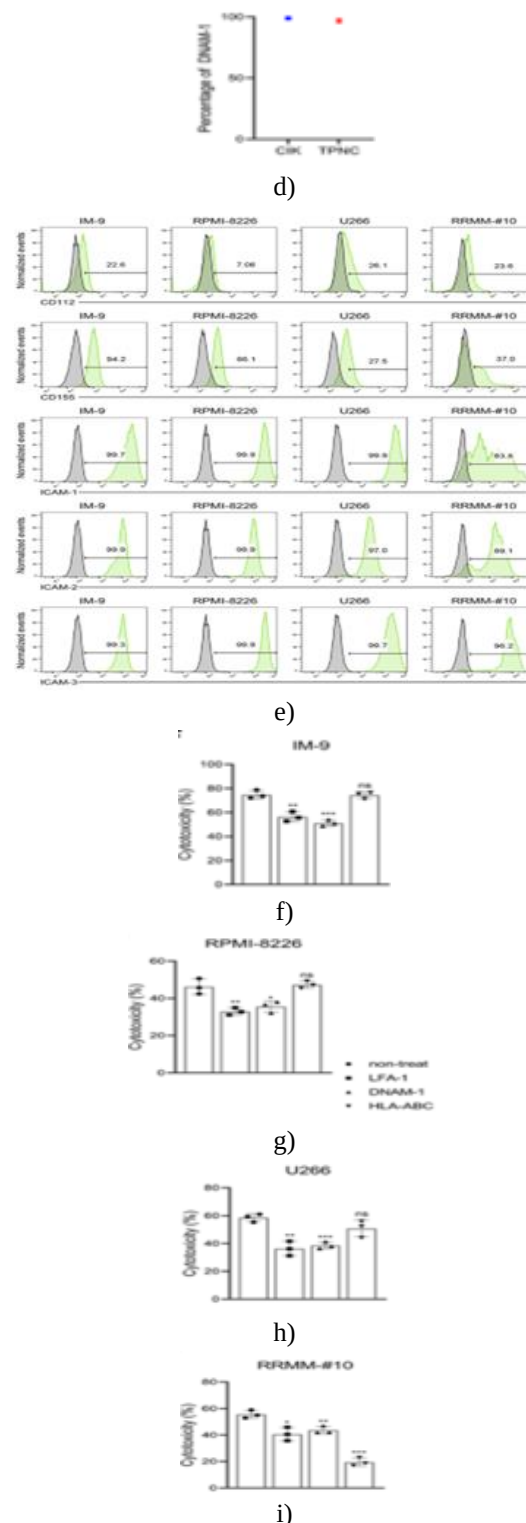
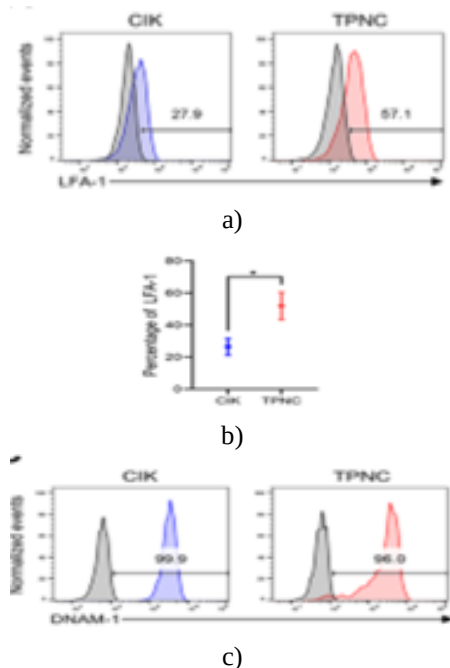


Figure 3. Mechanistic investigation of TPNC-mediated killing.

(a–d) Flow cytometric evaluation of LFA-1 and DNAM-1 surface expression on CIK cells and TPNC. (e) Levels

of the matching ligands (CD112, CD155, and ICAM-1/2/3) present on MM cell lines (IM-9, RPMI-8226, U266) and one primary RRMM specimen (RRMM-#10). (F–H) Impact of antibody blockade targeting LFA-1, DNAM-1, or HLA-ABC on TPNC cytotoxicity against MM cell lines. I Effect of the same blocking antibodies on TPNC killing of the autologous RRMM-#10 sample used during priming. Data shown as mean $\pm$ SD from at least three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by Student's t-test.

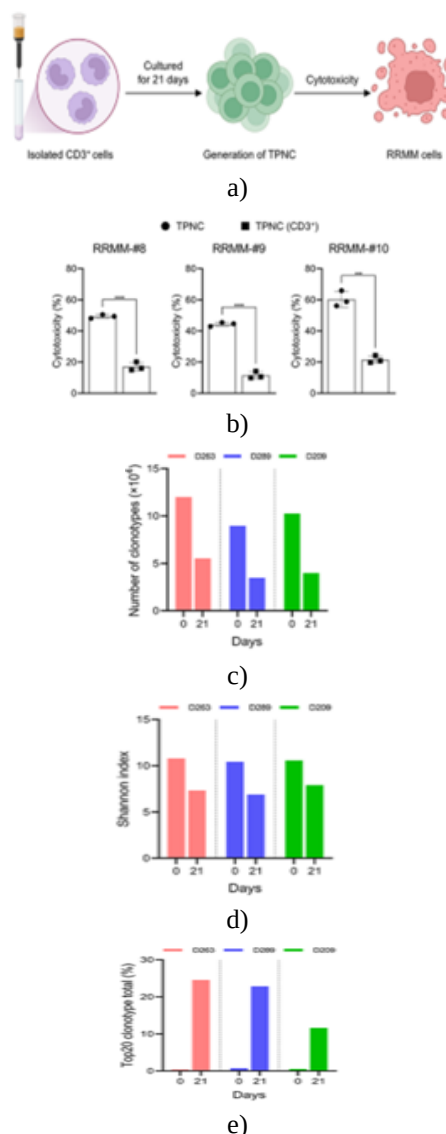
Since NKT-like cells have previously been shown to support MHC-restricted killing [26, 27], we evaluated this possibility through additional blocking studies. Interference with HLA-ABC produced no change in TPNC lysis of MM cell lines (**Figures 3f–3h**). In contrast, the same blockade caused a clear decrease in cytotoxicity toward the autologous RRMM-#10 target that had been used for priming (**Figure 3i**). Similar outcomes were obtained with other autologous samples (RRMM-#8 and #9), consistent with TPNC possessing the ability to perform antigen-specific, TCR-driven cytotoxicity, especially when confronting autologous tumor cells.

To determine whether MHC-restricted killing is restricted to autologous targets or can also occur against unrelated patient samples of the same cancer type, TPNC generated using RRMM-#10 were tested against unrelated RRMM-#8 and RRMM-#9 cells in the presence of HLA-ABC blockade. The blockade resulted in a significant reduction in killing of these unrelated targets. This finding suggests that TPNC can engage MHC class I-dependent mechanisms even when interacting with tumor cells from different patients sharing the same disease.

In aggregate, these experiments show that TPNC predominantly depend on MHC-unrestricted pathways driven by LFA-1 and DNAM-1, while retaining the capacity for MHC-restricted antigen-specific cytotoxicity. The observation that HLA-I blockade impaired killing in three separate donor–target pairings (patients #8, #9, and #10), including against unrelated RRMM samples, points toward recognition of shared tumor-associated antigens across this malignancy. Additional studies involving a wider array of RRMM models will be needed to establish how broadly this mechanism applies.

TCR repertoire dynamics during TPNC expansion indicate antigen-driven selection by antigen-presenting cells within CBMC

Because antigen-presenting cells (APCs) were both present and functionally activated in the CBMC culture environment, we proposed that these APCs could support MHC-dependent priming of TPNC. To test the importance of APCs in shaping TPNC effector functions, CD3⁺ T cells were purified from CBMC and subjected to the identical tumor-priming protocol but without APCs (**Figure 4a**). In the absence of APCs, the resulting cells displayed substantially weaker cytotoxicity against patient-derived RRMM targets (**Figure 4b**). This result underscores that APCs play a critical role in enabling full cytotoxic potential, most likely through TCR-mediated antigen presentation and priming.



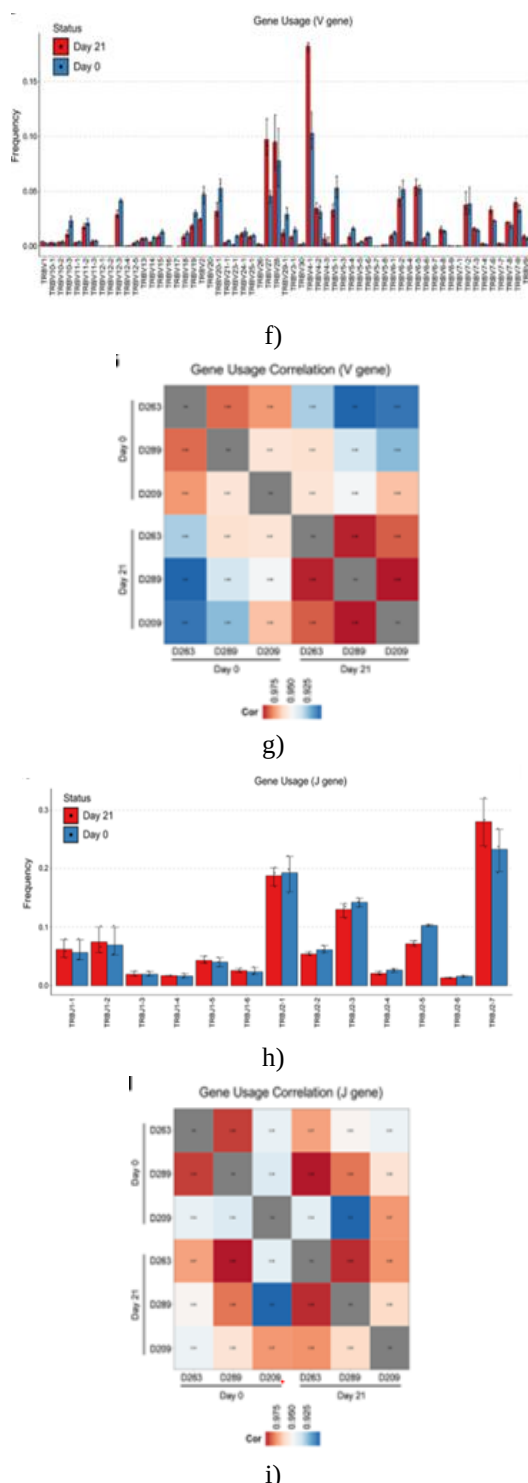


Figure 4. APCs in CBMC drive TCR clonal selection during TPNC expansion.

(a) Experimental design for generating TPNC either with the complete CBMC population or using only isolated CD3⁺ cells (APCs removed). (b) Comparison of killing activity at a 5:1 effector-to-target (E: T) ratio against

three different primary RRMM samples, showing impaired cytotoxicity when TPNC-like cells were generated from CD3⁺ cells alone without APCs. (c–e) TCR repertoire profiling demonstrating reduced clonal diversity and increased clonal dominance in day 21 TPNC relative to starting CBMC, as measured by total number of unique clonotypes (c), Shannon diversity index (d), and proportion of the top 20 most abundant clonotypes (e). (f) Relative usage frequencies of TRBV gene segments across three donors at day 0 versus day 21. (g) Heatmap illustrating pairwise similarity in TRBV gene usage patterns, with samples clustered according to time point. (h) Relative frequencies of TRBJ gene segment usage across the same three donors at day 0 and day 21. (i) Heatmap of TRBJ usage correlations, grouped by sampling day. Data represent mean $\pm$ SD from at least three independent experiments. *** $P < 0.001$; **** $P < 0.0001$ by Student's t-test.

To determine whether antigen-driven clonal selection occurs during priming culture, TCR β sequencing was performed on TPNC generated from three separate CBMC donors. By day 21, TPNC showed a clear contraction in overall TCR diversity compared with the initial CBMC, as evidenced by fewer distinct clonotypes, lower Shannon entropy, and marked overrepresentation of specific TCR clonotypes (**Figures 4c–4e**). This pattern is indicative of antigen-driven clonal outgrowth. Examination of V(D)J segment usage further showed consistent overrepresentation of TRBV4-1, TRBV27, TRBV28, and TRBJ2-7 gene segments at day 21 (**Figures 4f–4i**), reinforcing the occurrence of antigen-specific TCR repertoire remodeling throughout the tumor-priming process.

Although the precise tumor antigens involved have not yet been identified, the combined results strongly suggest that APCs within the CBMC fraction present tumor-derived antigens via MHC class I molecules, thereby driving antigen-specific selection and expansion of CD8⁺ NKT-like cells during TPNC production.

Efficient immune synapse assembly supports the rapid cytotoxic action of TPNC

Elevated surface expression of LFA-1 (**Figure 3a**), a critical adhesion molecule for immune synapse stabilization [28, 29], raised the possibility that improved synapse formation could underlie the enhanced killing efficiency of TPNC. To directly test this idea, live-cell imaging experiments were performed to monitor and measure the dynamics of immune synapse interactions

between effector cells (TPNC or CIK) and MM target cells at single-cell resolution. IM-9 target cells were placed in microwell arrays and co-incubated with either TPNC or CIK populations (**Figure 5a**). Targets were pre-labeled with CFSE. In contrast, effector cell cytotoxic granules were visualized using LysoTracker dye. Four key sequential stages of the killing process were monitored: (i) firm conjugate formation, (ii) polarization of cytotoxic granules, (iii) target cell membrane rupture, and (iv) effector detachment (**Figure 5b**). Representative time-lapse sequences captured the complete progression of TPNC-mediated target elimination (**Figure 5c**), with an accompanying video available.

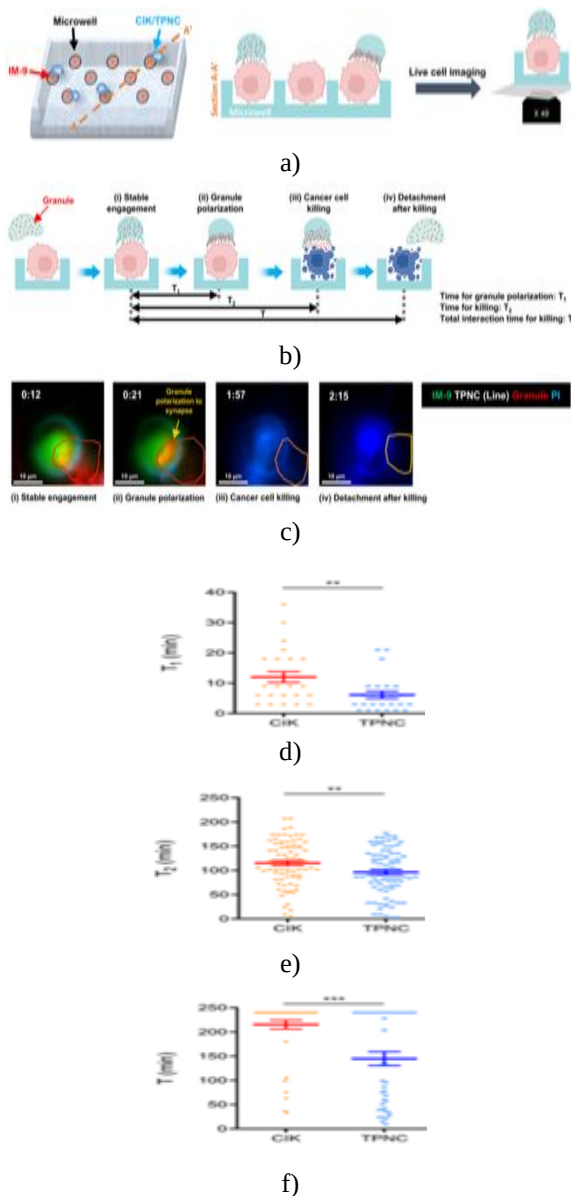


Figure 5. Live-cell imaging demonstrates rapid and effective immune synapse assembly along with swift target destruction by TPNC.

(a) Illustration of the microwell-based single-cell assay setup. Red-labeled IM-9 target cells were positioned individually in each well before addition of blue-labeled CIK or TPNC effector cells to monitor conjugate formation. (b) Defined phases of the effector-target engagement process: initial stable attachment, directed movement of cytotoxic granules toward the contact site, destruction of the target cell, and final separation. T_1 indicates duration until granule reorientation, T_2 duration until lysis, and T the complete length of interaction. (c) Selected time-lapse frames depicting a TPNC effector destroying an IM-9 cell. Timestamps shown in hh:mm format. Green: IM-9 targets; red: lytic granules; blue: PI staining; orange outline: effector cells (CIK or TPNC). (d–f) Measured durations for granule polarization (T_1), target cell killing (T_2), and overall contact time (T). (g) Assessment of serial killing potential, recorded as the average number of distinct tumor targets engaged and eliminated by each effector cell during a 5-hour imaging session. Results expressed as mean $\pm$ SD across at least three independent experiments. ** $P < 0.01$; *** $P < 0.001$ using Student's t-test.

A detailed quantitative evaluation showed that TPNC formed immune synapses faster and with greater reliability than CIK cells. They consistently required shorter periods to polarize cytotoxic granules (T_1), complete target lysis (T_2), and conclude each interaction cycle (T) (**Figures 5d–5f**). TPNC further outperformed CIK cells in killing multiple targets sequentially (**Figure 5g**).

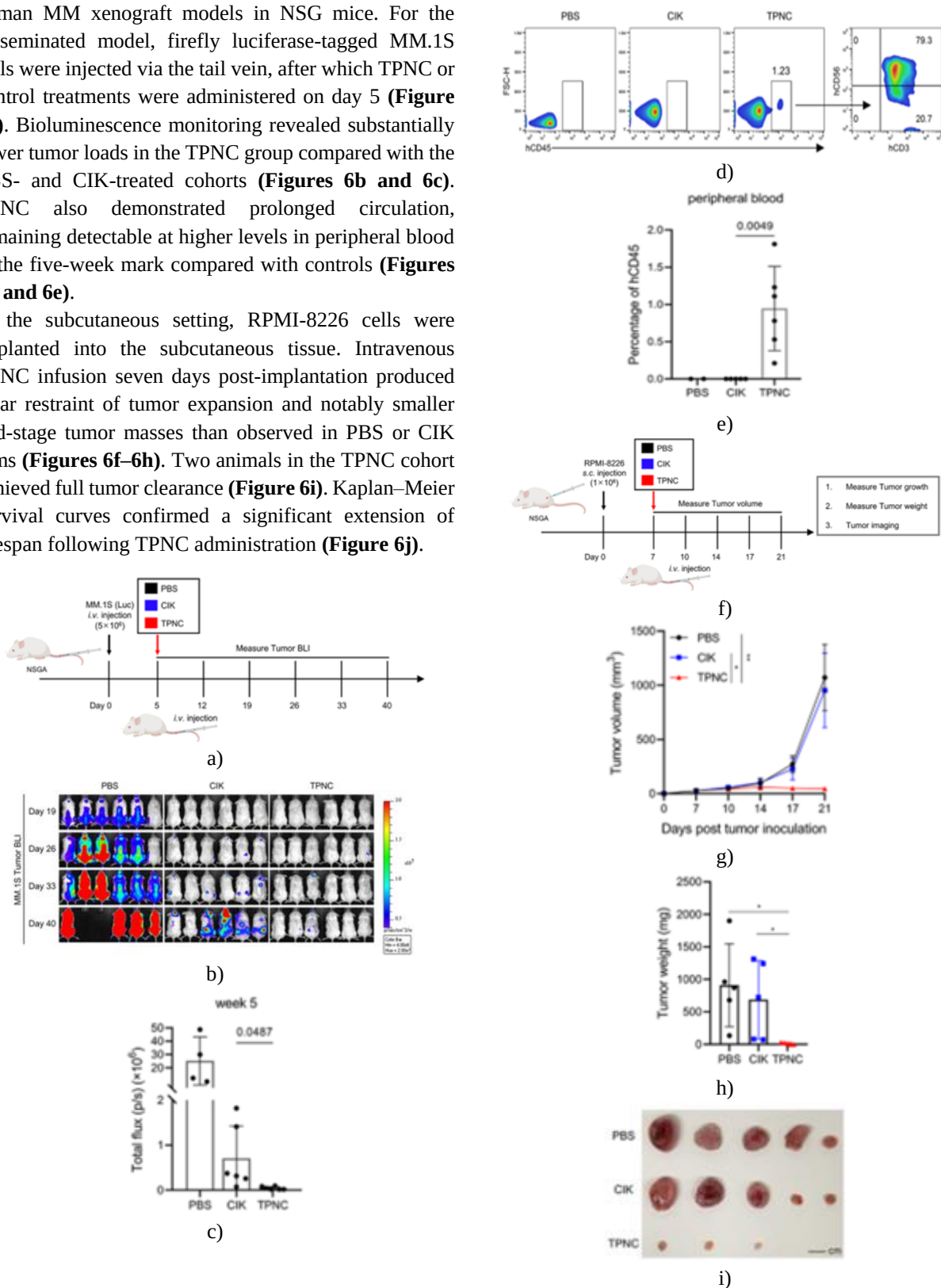
Overall, the imaging data establish that TPNC create more functional and timely immune synapses than CIK cells, which facilitates faster execution of cytotoxicity and greater capacity for serial tumor cell elimination.

TPNC display robust in vivo tumor control with an excellent safety profile

The therapeutic efficacy of TPNC was examined in living animals using both disseminated and focal subcutaneous

human MM xenograft models in NSG mice. For the disseminated model, firefly luciferase-tagged MM.1S cells were injected via the tail vein, after which TPNC or control treatments were administered on day 5 (**Figure 6a**). Bioluminescence monitoring revealed substantially lower tumor loads in the TPNC group compared with the PBS- and CIK-treated cohorts (**Figures 6b and 6c**). TPNC also demonstrated prolonged circulation, remaining detectable at higher levels in peripheral blood at the five-week mark compared with controls (**Figures 6d and 6e**).

In the subcutaneous setting, RPMI-8226 cells were implanted into the subcutaneous tissue. Intravenous TPNC infusion seven days post-implantation produced clear restraint of tumor expansion and notably smaller end-stage tumor masses than observed in PBS or CIK arms (**Figures 6f–6h**). Two animals in the TPNC cohort achieved full tumor clearance (**Figure 6i**). Kaplan–Meier survival curves confirmed a significant extension of lifespan following TPNC administration (**Figure 6j**).



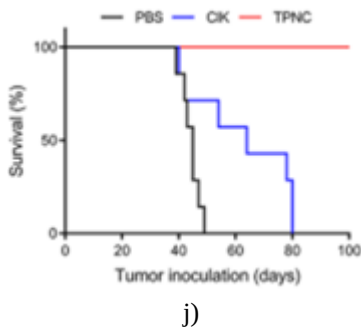


Figure 6. In vivo performance and long-term survival of TPNC in preclinical multiple myeloma mouse models.

(a) Experimental outline for the systemic model: NSGA mice received intravenous injection of luciferase-tagged MM.1S cells on day 0, followed by infusion of PBS, CIK cells, or TPNC on day 5. (b) Serial bioluminescence images capturing tumor progression at selected intervals (days 19, 26, 33, and 40). (c) Summary of total photon flux measured at the five-week time point. (d) Flow cytometry examples highlighting the presence of human CD45⁺ cells in blood samples collected on day 40. Detectable human cells were primarily found in the TPNC group and were subsequently examined for CD3 and CD56 markers. (e) Proportions of circulating human CD45⁺ cells in the various treatment cohorts. (f) Design of the localized subcutaneous model employing RPMI-8226 cells. Tumor establishment was performed via subcutaneous inoculation on day 0, with therapeutic cell administration on day 7. (g) Changes in tumor volume tracked over the course of the experiment. (h) Final tumor mass determined at the conclusion of the study (day 21). (i) Visual examples of tumors removed from mice in each experimental arm. (j) Survival curves generated by the Kaplan–Meier method for animals challenged intravenously with RPMI-8226 cells and subsequently treated with PBS, CIK cells, or TPNC. Results are mean $\pm$ SD compiled from a minimum of three independent studies. * $P < 0.05$; ** $P < 0.01$ via one-way ANOVA and appropriate post hoc comparisons.

These in vivo experiments collectively confirm that TPNC exerts powerful antitumor effects and maintains robust persistence in MM xenograft models, reinforcing its promise as a development candidate for an allogeneic therapeutic. Safety profiling involved weekly intravenous delivery of 1×10^8 TPNC or CIK cells for 3 weeks in NSG mice, with follow-up monitoring extending to 8 weeks. No meaningful alterations in body weight occurred, and microscopic review of key organs

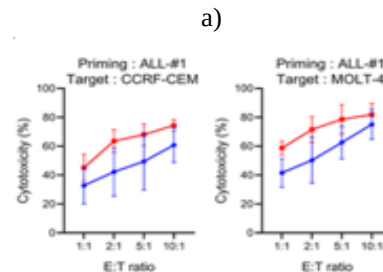
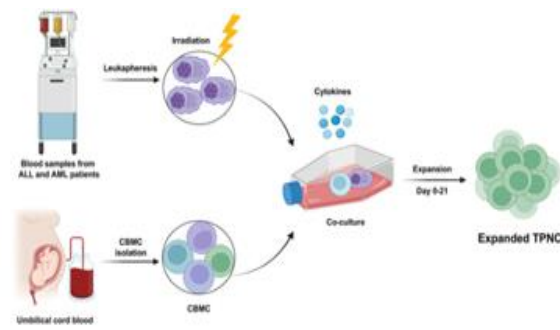
showed no pathological changes or inflammatory responses, consistent with the absence of graft-versus-host disease (GvHD)-related complications.

Off-target reactivity was additionally probed by incubating TPNC with autologous donor PBMC and with unrelated allogeneic CBMC. TPNC failed to induce any notable lysis of these normal cell types.

In conclusion, TPNC displays selective cytotoxicity toward cancerous cells while sparing healthy immune populations, supporting its favorable safety profile and suitability as a broadly applicable allogeneic cell therapy platform.

Broad cytotoxic activity of TPNC across multiple hematologic cancers

The versatility of the TPNC strategy was examined by producing these cells from leukapheresis material collected from ALL and AML patients and testing their tumor-killing capacity (**Figure 7a**). TPNC showed comparable lytic potency to standard CIK cells when confronting established leukemia lines including CCRF-CEM, MOLT-4, HL-60, and THP-1 (**Figures 7b and 7c**). However, against primary leukemic blasts freshly obtained from ALL and AML patients, TPNC showed considerably higher cytotoxicity (**Figures 7d and 7e**), demonstrating clear advantages in more physiologically relevant, patient-specific contexts.



b)

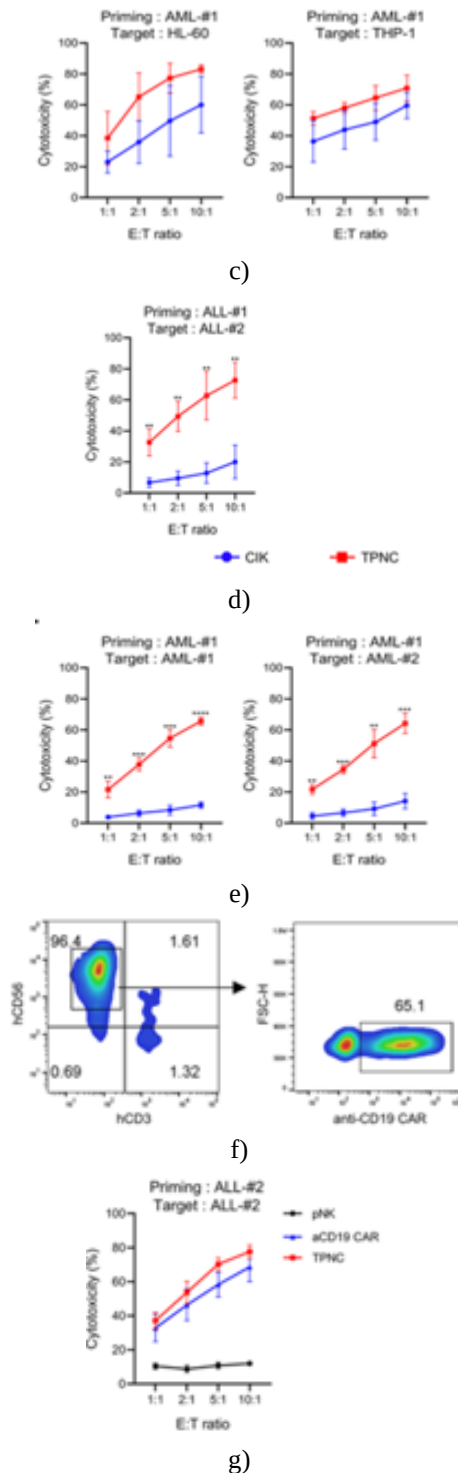


Figure 7. Cytotoxicity of TPNC against leukemia cell lines and primary samples from ALL and AML patients.

(a) Overview of the protocol for generating TPNC through co-culture of CBMC with leukapheresis-derived cells from patients diagnosed with ALL or AML. (b) and

(c) Lytic activity of TPNC compared with CIK cells against established leukemia lines, specifically CCRF-CEM and MOLT-4 (b) as well as HL-60 and THP-1 (c). (d-e) Killing efficiency of TPNC versus CIK cells when exposed to primary leukemic blasts isolated from ALL patients (d) and AML patients (e). Testing included cells from both the original priming donor and from unrelated patients. (f-g) Head-to-head comparison of TPNC cytotoxicity against primary ALL cells with that of primary NK (pNK) cells and anti-CD19 CAR-NK cells. Data shown as mean $\pm$ SD from at least three independent experiments. **P < 0.01; ***P < 0.001; ****P < 0.0001 by Student's t-test.

Notably, TPNC efficiently lysed leukemia cells, regardless of whether the targets were from the same patient used for priming or from different individuals, demonstrating significant cross-reactivity. These observations suggest broad antitumor activity of TPNC in hematologic cancers and justify additional studies on donor compatibility and practical clinical implementation.

Direct side-by-side testing further validated TPNC's strength by comparing its performance with patient-derived ALL blasts, anti-CD19 CAR-NK cells, and primary NK (pNK) cells. TPNC achieved killing levels that matched or surpassed those of CAR-NK cells in several cases (**Figures 7f and 7g**). This is particularly noteworthy given that TPNC require no genetic engineering.

Taken together, these findings reinforce the value of TPNC as a versatile allogeneic cell therapy platform that can address multiple hematologic malignancies without relying on antigen-specific gene editing.

In the present work, we established a new type of cytotoxic lymphocyte termed TPNC by incubating cord blood mononuclear cells with irradiated patient-derived tumor cells in the presence of selected cytokines. These cells, produced entirely without genetic modification, demonstrated potent antitumor activity against RRMM, AML, and ALL. TPNC displayed strong tumor lysis, robust cytokine secretion, and effective disease control in animal models, positioning them as a promising general-purpose cellular immunotherapy approach.

Although recent progress with adoptive therapies such as CAR-T cells, CIK cells, and memory-like NK cells has shown encouraging results in blood cancers [30-33], each modality has notable drawbacks. CAR-T therapies provide highly specific targeting but depend on patient-specific cell collection and gene editing, which pose

challenges in production, cost, and toxicity [34-37]. CIK and NK cells provide immediate MHC-unrestricted killing but often exhibit insufficient tumor specificity and short-lived activity [38-40]. Memory-like NK cells exhibit enhanced recall responses and non-MHC killing. Still, they are primarily directed against viral or stress ligands and lack the broad clonal diversity typical of adaptive T-cell responses [20, 41].

TPNCs address several of these shortcomings by merging the rapid, flexible responses characteristic of innate NK cells with elements of antigen-driven specificity from adaptive immunity. They mediate tumor destruction through two complementary routes: MHC-independent killing supported by LFA-1 and DNAM-1, and MHC-restricted recognition via TCR engagement. MHC-blocking studies confirmed this duality. While HLA class I blockade had minimal impact on lysis of MM cell lines, it substantially weakened activity against primary autologous RRMM cells. This pattern suggests that TCR signaling becomes more important when confronting primary tumors that may evade innate recognition. TCR sequencing data further supported antigen-driven selection by revealing reduced clonal diversity and biased TRBV gene usage. Moreover, removal of APCs during culture severely compromised cytotoxic function, confirming the necessity of antigen presentation for proper effector development.

Antigen-specific priming also shaped broader effector characteristics. Compared with CIK cells, TPNC showed higher expression of perforin, granzyme B, and LFA-1 despite sharing a similar CD3⁺CD8⁺CD56⁺ surface profile. This functional superiority likely stems from differences in their expansion methods. CIK cells rely on nonspecific anti-CD3 stimulation [42, 43], whereas TPNC are exposed to physiologic tumor-derived antigens presented by patient cells. Engagement of the TCR complex triggers key signaling cascades, including MAPK, PI3K–mTOR, and NFAT pathways [44], which, in turn, promote cytotoxic granule production, granule reorientation, and LFA-1-mediated adhesion, resulting in more effective immune synapse assembly and target destruction [45-47]. These TPNC enhancements are likely reinforced by the specific cytokines included during culture. IL-2 drives proliferation and cytolytic gene expression [48, 49], IL-18 boosts IFN- γ release and degranulation [50, 51], and IL-21 supports sustained granule formation and metabolic fitness through STAT3 and mTOR signaling [52-54].

The cytokine combination may have additionally contributed to the distinctive NKT-like phenotype observed in TPNC, characterized by simultaneous expression of CD3, CD8, and CD56. Earlier reports indicate that IL-18, especially when combined with IL-12 or IL-15, can promote trained immunity features in CD3⁺CD56⁺ cells, including heightened cytotoxicity and memory-like traits [55]. IL-21 has similarly been linked to the development of CD8⁺ T cells that acquire innate-like properties such as rapid degranulation and upregulation of NK markers including CD56 and NKG2D [27]. Thus, the interplay between IL-18 and IL-21, in the context of tumor antigen presentation by APCs, likely steered conventional CD8⁺ T cells toward this hybrid NKT-like state.

The integration of antigen-driven TCR signaling and supportive cytokine conditions therefore appears to have generated a unique transcriptional and functional program optimized for potent tumor elimination. These insights also help explain the preferential outgrowth of CD8⁺ NKT-like cells rather than classical NK cells under the described culture conditions.

The antigenic specificity of TPNC was dictated by the tumor type used for priming. Cells generated against RRMM, ALL, or AML failed to lyse tumor cells from unrelated cancer types, indicating that the antigenic profile of the original disease shapes the TCR repertoires of these cells. Within the same malignancy category, however, TPNC showed effective cross-reactivity toward samples from different patients. This pattern suggests the presence of shared tumor-associated antigens across individuals with the same cancer subtype. It opens the door to semi-personalized applications based on common neoantigens or mutation signatures.

In vivo experiments further bolster the clinical outlook for TPNC. Treatment in xenograft models resulted in clear tumor control and sustained circulation of human cells without detectable off-target toxicity or GvHD-like effects, as evidenced by stable body weight and normal organ histology. Derived from cord blood, manufactured without gene editing, and amenable to pre-preparation, TPNC represent a potentially off-the-shelf option for patients with relapsed or refractory hematologic cancers. Nevertheless, important limitations persist. The exact antigens recognized by TPNC remain unknown; their identification will be essential for optimizing donor selection and treatment precision. The exclusive use of immunodeficient mice limits assessment of memory responses, long-term engraftment, and interplay with

host immunity. Additionally, the scarcity of primary RRMM cells restricted development of patient-derived xenograft (PDX) models concurrent with TPNC generation. Early attempts yielded poor engraftment, likely due to inadequate tumor cell numbers. Finally, dependence on patient-derived feeder cells complicates future GMP-scale manufacturing. Safety and activity in other blood cancers beyond RRMM also require deeper evaluation. Future efforts to replace primary feeders with engineered antigen-presenting systems could streamline production and accelerate clinical development.

Conclusion

TPNC represent non-genetically modified cytotoxic lymphocytes that blend innate and adaptive immune features. Through dual recognition strategies, strong functional performance, good safety, and straightforward scalability, they constitute a practical and effective platform for cellular immunotherapy against relapsed and refractory hematologic malignancies.

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