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Enhanced Anti-tumor Activity of iC9/CAR-19/IL-15-Transduced CB-NK Cells Combined With PD-1 Inhibitor Treatment in B-Cell Lymphoma

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Background: B-cell lymphoma is a highly prevalent malignancy of the hematopoietic system. Chimeric antigen receptor (CAR)-natural killer (NK) cell therapy is increasingly being used to treat hematologic malignancies. However, relatively few studies have explored the feasibility of combining CAR-NK therapy with PD-1 inhibitors for lymphoma treatment. This study aimed to investigate the feasibility and therapeutic efficacy of combining CAR-NK cells with a programmed death-1 (PD-1) inhibitor for the treatment of B-cell lymphoma.

Methods: Umbilical cord blood (CB)-derived NK cells were transduced with a retroviral vector encoding CAR-19, interleukin-15 (IL-15), and an inducible caspase-9 (*iC9*)-based suicide gene. The impact of iC9/CAR-19/IL-15 CAR-NK cell therapy combined with PD-1 inhibitor treatment on lymphoma cell cytotoxicity was evaluated separately through *in vitro* cell-based assays and *in vivo* animal experiments.

Results: iC9/CAR-19/IL-15 CAR-NK cells combined with PD-1 inhibitors (a combined therapy) selectively killed primary chronic lymphocytic leukemia (CLL) cells *in vitro*. The cytokines CD107a, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) were increased in iC9/CAR-19/IL-15 CAR-NK cells, but were barely changed under the influence of PD-1 inhibitors. Compared to monotherapy, the combined therapy significantly elevated IL-22, IL-6 and IL-15 secretion levels ($p < 0.05$), and more importantly, greatly extended the survival of model mice ($p < 0.001$). Furthermore, a decrease in TNF-α, IL-6 and IL-10 levels was observed in the serum of model mice ($p < 0.05$).

Conclusions: The combined therapy of iC9/CAR-19/IL-15 CAR-NK cells with PD-1 inhibitors exhibits remarkable antitumor activity and prolongs survival, providing a potential novel treatment route for B-cell lymphoma.

Keywords: B-cell lymphoma; CAR-NK cell therapy; PD-1 inhibitors; cellular immunotherapy; antitumor activity; prolong survival

Introduction

B-cell lymphoma, one of the most common hematologic malignancies worldwide, exhibits considerable diversity in morphological, immunophenotypic, and clinical features, largely attributable to underlying genetic variations [1,2]. Conventional therapeutic approaches for patients with relapsed chemosensitive or refractory aggressive lymphoma include high-dose chemotherapy followed by autologous stem cell transplantation (ASCT) [3,4]. However, only a minority of patients achieve long-term recovery through ASCT, and the majority either relapse after extensive pretreatment or develop refractory disease following transplantation, with particularly poor outcomes observed in high-risk individuals [5,6].

Since the approval and clinical introduction of rituximab by the U.S. Food and Drug Administration (FDA), overall survival rates in B-cell lymphoma have improved significantly [7]. Approximately 60–70% of patients achieve complete or partial responses to rituximab-containing treatment regimens [8]. Nevertheless, a substantial proportion of patients still fail to achieve complete remission. In recent years, immunotherapy has garnered increasing attention and has emerged as a promising therapeutic strategy in lymphoma.

Chimeric antigen receptors (CARs) consist of three main components: an extracellular domain, typically derived from antibodies, which specifically recognizes tumor antigens expressed on cancer cells; a transmembrane region; and an intracellular signaling domain responsible for cell activation [9]. Various immune effector cells have been engineered with CARs, and their potential to mediate anticancer responses has been demonstrated in both preclinical studies and clinical trials.

For instance, CAR-T cell therapy has been developed to target various tumor-associated antigens—such as HER2, CEA—in both preclinical and clinical studies of solid tumors [10–12]. In the field of hematologic malig-

nancies, targeted CAR-T therapy has been explored for refractory acute lymphoblastic leukemia [13]. Notably, CD19-directed CAR-T cell therapy has achieved significant success in patients with relapsed or refractory B-cell lymphoma. However, several challenges remain unresolved, including limited CAR-T cell persistence, the immunosuppressive tumor microenvironment (TME), antigen escape, tumor heterogeneity, and the risk of graft-versus-host disease (GVHD), particularly in allogeneic settings [14–16].

In contrast, natural killer (NK) cells represent a promising alternative to T cells for CAR engineering due to their inability to induce GVHD [17]. NK cells can be derived from various sources, including peripheral blood mononuclear cells (PBMCs), the NK-92 cell line, induced pluripotent stem cells (iPSCs), and umbilical cord blood (CB) [18]. CAR-NK cell therapy is an emerging strategy for the treatment of leukemia and is currently under active investigation in multiple clinical trials. However, its therapeutic potential in B-cell lymphoma remains to be fully elucidated.

In addition, programmed death-1 (PD-1), an immune checkpoint molecule belonging to the CD28 family, limits T cell activity during immune responses and mitigates autoimmune damage by binding to its ligands, PD-L1 or PD-L2. This interaction inhibits stimulatory signals from the T cell receptor (TCR) [19]. By competitively binding to PD-1, PD-1 inhibitors disrupt the PD-1/PD-L1 interaction, thereby blocking the transmission of inhibitory signals and enhancing T cell effector function and proliferation [20].

NK cells also participate in the antitumor effects of PD-1/PD-L1 blockade immunotherapy [21]. When combined with PD-1 inhibitors, NK cell-based therapies have been shown to prolong survival and improve treatment efficacy in patients with lung cancer [22]. Moreover, preclinical studies suggest that PD-1 blockade—particularly when used in combination with costimulatory agonists, other immune checkpoint inhibitors, or therapeutic agents such as lenalidomide—may exert synergistic effects in B-cell lymphoma [23].

In this study, umbilical CB-derived NK cells were genetically modified using a retroviral vector to generate inducible caspase-9 (iC9)/CAR-19/interleukin-15 (IL-15) CAR-NK cells. Then, their anti-tumor activity was observed when transduced with IL-15 (cytokines critical for NK cell persistence), anti-CD19 CAR, and iC9. Considering that PD-1 inhibitors can relieve the immune suppression of T cells and NK cells and further enhance their effector functions, we hypothesize that combination treatment may enhance the cytotoxicity of iC9/CAR-19/IL-15 CAR-NK cells.

This study aims to investigate the therapeutic effect of iC9/CAR-19/IL-15 CAR-NK cells combined with PD-1 inhibitors in B-cell lymphoma.

Materials and Methods

Clinical Samples

Between January 2022 and May 2025, in the Department of Hematology at The First Affiliated Hospital of Ningbo University, tumor-involved lymph nodes were obtained from five patients with relapsed/refractory $CD19^+$ B-cell malignancies. In addition, primary chronic lymphocytic leukemia (CLL) cells were isolated from the peripheral blood of one patient with CLL using $CD19^+$ cell sorting. All samples were derived from distinct individuals to ensure biological independence. All procedures were approved and overseen by the Ethics Committee of NBU Health Science Center (Approval No: NBU-2020-140). Samples were collected, immediately cryopreserved, and stored in liquid nitrogen. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Inclusion criteria: (1) Patients must have relapsed or refractory CD19-positive B-cell-derived hematological malignancies; (2) The principal investigator and the patient's attending physician considered that the patient has no other feasible and effective alternative treatment options, such as hematopoietic stem cell transplantation; (3) The expression level of CD19 in tumor cells is detected as over 15% in immunohistochemical detection data or over 30% in flow cytometry detection data; (4) Age: 14 to 70 years old, with no restrictions on gender, race, etc.; (5) Physical condition: Eastern Cooperative Oncology Group (ECOG) score of 0 to 2 points [24]; (6) Cardiac function: Left ventricular ejection fraction is greater than or equal to 40%; (7) Expected survival time >12 weeks; (8) Serum creatinine ≤ 1.6 mg/dL and/or urea nitrogen ≤ 1.5 mg/dL; (9) Serum Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) should be no more than five times the normal value; (10) The patients have the ability to sign the informed consent form by themselves; and the legal guardian of the child patient agrees to sign the informed consent form.

Exclusion criteria: (1) Pregnant or lactating women; (2) Uncontrolled infection; (3) Active hepatitis B virus (HBV) or hepatitis C virus (HCV) infection; (4) Patients who need to use glucocorticoids during cell collection or infusion due to their condition; (5) Those who have undergone allogeneic hematopoietic stem cell transplantation within less than 100 days; (6) Patients with grade 2 to 4 acute or extensive chronic GVHD; (7) Receiving GVHD treatment; (8) Human immunodeficiency virus-infected individuals; (9) Patients with concurrent active central nervous system leukemia or lymphoma invasion; (10) Absolute neutrophil count less than 750/μL or platelet count less than 50,000/μL caused by non-primary diseases; (11) Patients who received systemic chemotherapy two weeks ago or radiotherapy three weeks ago when cells were collected.

Cell Lines and Retrovirus Production

The K562 cells (erythroleukemia) were procured from ATCC (CCL-243, MA, VA, USA) to express membrane-bound IL-21 as feeder cells. Human umbilical cord blood (CB) samples were collected from full-term neonates immediately after delivery, with informed consent obtained from The First Affiliated Hospital of Ningbo University. The collection was performed using sterile techniques with anticoagulant-treated collection bags.

All procedures were approved by the Ethics Committee of NBU Health Science Center (Approve No: NBU-2020-140). As previously mentioned, the retroviral vector encoding CAR-19 and iC9/CAR-19/CD28-zeta-2A-IL-15 was created [25,26], and the retroviral supernatants were produced [26]. Specifically, we cloned the genes encoding a single-chain antibody to target CD19, CD28 intracellular domain, and NK cell receptor complex ζ chain into the SFG retroviral vector to construct CAR-19 retroviral vector.

Subsequently, we developed the second retroviral vector that contained not only the same gene as the CD19-specific CAR but also the human *IL-15* gene and *iC9* suicide gene binding with small molecule dimerizer CID AP20187 to induce cell apoptosis. Using the 2A sequence peptide extracted from foot-and-mouth disease virus to create the iC9/CAR-19/IL-15 retroviral vector, we cloned these three genes together and connected them to the SFG retroviral vector.

Thereafter, 293T cells (SCSP-502, Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were transfected with the retroviral vectors using Fugene6 transfection reagent (11814443001, Roche, Basel, Switzerland). Retrovirus-containing supernatants were obtained 48- and 72-h post-transfection. All cells used in this study underwent short tandem repeat (STR) profiling and Mycoplasma contamination tests, both of which confirmed negative results.

Preparation of CAR-modified NK Cells

CB cells were separated using density centrifugation (Ficoll-Histopaque; Sigma-Aldrich, St. Louis, MO, USA) [27]. $CD56^{+}$NK cells were purified using the NK isolation kit (130-092-657, Miltenyi Biotec, Inc., San Diego, CA, USA), activated on day 0, co-cultured with IL-21-expressing K562 cells (feeder cells:NK = 2:1), and co-stimulated by the stem cell growth medium (SCGM, CellGenix GmbH, Freiburg, Germany) containing serum-free human IL-2 (Proleukin, 200 U/mL; Chiron, Emeryville, CA, USA).

Following transduction with retroviral supernatant on day 4, activated NK cells were seeded onto fibronectin-coated plates (Clontech Laboratories, Inc., Mountain View, CA, USA). Interleukin-2 (IL-2) was re-administered on day 9 to further stimulate NK cell expansion. CAR-transduced NK cells were harvested on day 14.

As a control, NK cells isolated from cord blood (CB) were cultured for 14 days without transduction (non-transduced [NT]), in the presence of IL-2 and feeder K562 cells.

Characterization of CAR Expression and Immune Phenotype in Transduced Cells

All cells were stained using the LIVE/DEAD Fixable Blue Dead Cell Stain Kit (L23105, Invitrogen, Carlsbad, CA, USA) for 15 min at room temperature prior to surface marker staining. To assess CAR expression on NK cell surfaces, biotinylated Protein L (GeneScript, Piscataway, NJ, USA) and allophycocyanin (APC)-conjugated streptavidin (554067, BD Biosciences, San Diego, CA, USA) were employed.

Cells were incubated with biotinylated Protein L (10 µg/mL) for 15 min at 4 °C, washed with phosphate-buffered saline (PBS), and then incubated with APC-conjugated streptavidin (5 µg/mL) for 30 min. Flow cytometry analysis was performed using an LSR II flow cytometer (Becton Dickinson, Sparks, MD, USA) with FACSDiva software (BD Biosciences, Heidelberg, Germany). Data from 10,000 events per sample were collected and analyzed using FlowJo software version 10 (TreeStar, Ashland, OR, USA).

The following antibodies purchased from Invitrogen (Carlsbad, CA, USA) were used for immunophenotypic characterization: CD16 (11-0168-42), CD44 (MA5-13890), KIR (MA1-10111), CXCR3 (17-1831-82), and KLRG-1 (25-5893-82).

Cell Treatment

The CLL cells were incubated with CAR-transduced cells and non-transduced natural killer (NT-NK) cells, namely NT: CLL, CAR-NK: CLL, NT: CLL+PD-1 inhibitor, and CAR-NK: CLL+PD-1 inhibitor. CAR-transduced cells and NT-NK cells were treated with PD-1 inhibitor nivolumab (36/72 µg/mL, OPDIVO, Bristol-Myers Squibb, New York, NY, USA) for 48 h, and co-cultured with CLL cells [28].

NK Cell Cytotoxicity and Cytokine Assays

For cytotoxicity assessment, CAR-transduced and non-transduced NK (NT-NK) cells were tested individually or in combination with a PD-1 inhibitor against ^{51}Cr-labeled CLL cells (positive control) at various effector-to-target (E:T) ratios using a ^{51}Cr release assay [29]. Target cells were prepared at a concentration of 1×10^7 cells/mL in complete culture medium. Sodium chromate ($^{51}Na_2CrO_4$; CR042325, Revvity, Shanghai, China) was added to the cell suspension, which was then incubated at 37 °C for 1 h with gentle agitation every 15 min.

Following incubation, cells were washed three times with ice-cold PBS containing 5% fetal bovine serum (FBS) to remove unincorporated ^{51}Cr. Cells were then resuspended in complete culture medium and counted.

After a 5-h co-culture with effector cells, the mixture was transferred to 96-well Lumaplates (N8145354, PerkinElmer, Waltham, MA, USA). The release of ^{51}Cr from lysed target cells was measured using a liquid scintillation counter (MicroBeta Trilux, PerkinElmer, Waltham, MA, USA). Spontaneous and maximum ^{51}Cr release were determined by incubating target cells alone or with 1% SDS (L4390, Sigma-Aldrich, St. Louis, MO, USA), respectively.

The following formula was used to measure the percentage of specific lysis: 100 × (experimental release c.p.m. – spontaneous release c.p.m.)/(maximum release c.p.m. – spontaneous release c.p.m.).

The measurement of CD107a degranulation and intracellular cytokine production was performed following a previously published protocol [30]. During cell culture, CD107a-PE antibody (555801, BD Biosciences, San Diego, CA, USA), monensin (420701, BioLegend, Koblenz, Germany), and brefeldin A (B7651, Sigma-Aldrich, St. Louis, MO, USA) were added.

Cells were then treated with CAR antibody, CD56-PE (555516, BD Biosciences, San Diego, CA, USA), and CD3-PerCP (552851, BD Biosciences, San Diego, CA, USA), followed by fixation and permeabilization using the Fixation/Permeabilization Kit (554714, BD Biosciences, San Diego, CA, USA). Intracellular staining was conducted using IFN-γ-V450 (560371, BD Biosciences, San Diego, CA, USA) and tumor necrosis factor-alpha (TNF-α)-Alexa 700 monoclonal antibodies (557996, BD Biosciences, San Diego, CA, USA). Cells were analyzed on a BD LSR-Fortessa flow cytometer using the LIVE/DEAD Aqua Dead Cell Stain Kit (L-34965, Life Technologies, Waltham, MA, USA). Data analysis was performed with FlowJo software (FlowJo LLC, Ashland, OR, USA).

IL-15 Cytokine Secretion

Utilizing Human IL-15 Quantikine ELISA Kit (S1500, R&D Systems, Shanghai, China), IL-15 production was determined. Briefly, the collected cell culture supernatants were poured into ELISA plates pre-treated with IL-15-specific antibodies. After incubation indoors for 45 min and subsequent washing, the mixture was further incubated with the substrate solution for 30 min in the dark, followed by the addition of the stop solution. Finally, the optical density was measured at 450 nm using a BioTek microplate reader.

NOD scid gamma(NSG) Mouse Model

The anti-tumor functions of CAR-transduced cord blood-derived natural killer (CB-NK) cells with PD-1 inhibitor were evaluated *in vivo* using the NSG xenograft model. 50 NSG mice (6 weeks old, 26–34 g, Jackson Laboratory, Bar Harbor, ME, USA) were anesthetized by using a precision vaporizer delivering 2% isoflurane (RWD Life Science, Shenzhen, China) in 100% oxygen at a flow rate of 2 L/min [31]. Then, tumor fragments (3 mm × 3 mm) from patients with B-cell lymphoma were implanted into NSG mice using subcutaneous transplantation. For postoperative analgesia, buprenorphine (0.08 mg/kg, B9275, Sigma-Aldrich, St. Louis, MO, USA) was administered subcutaneously every 12 hours for 48 hours following the procedure [32]. Upon tumor growth to 500–1000 mm^3, 50 mice were randomly divided into control, NT-NK, CAR-NK, PD-1 inhibitor, and CAR-NK+PD-1 inhibitor groups (10 mice/group).

According to the established protocol, mice received intravenous injections of saline (5 mL/kg body weight), NT-NK cells (1.5×10^7 cells), CAR-NK cells (1.5×10^7 cells), PD-1 inhibitor (5 mg/kg body weight), or CAR-NK cells (1.5×10^7 cells) combined with PD-1 inhibitor (5 mg/kg body weight), once every 7 days for 4 consecutive weeks, with injections administered on days 1, 8, 15 and 22 [33].

Mouse survival status was monitored and recorded. Finally, mice were euthanized by sodium pentobarbital (150 mg/kg, intraperitoneal injection), followed by cervical dislocation. The study was permitted by the Ethics Committee of Ningbo University (2020-202). All mouse experiments followed the ethical guidelines and protocols of the NIH Institutional Animal Care and Use Committee.

Inflammatory Cytokine Levels

Blood samples were collected from all animals in each group 30 days after the initial injection and were left to clot (4 ℃), followed by centrifugation (1620 ×g, 15 min, 4 ℃) to obtain serum. Subsequently, the serum was collected and frozen at –80 ℃ for cytokine analysis. Enzyme-linked immunosorbent assay (ELISA) for IL-6 (Cat# KMC0061, Invitrogen, Carlsbad, CA, USA), TNF-α (KMC3011, Invitrogen, Carlsbad, CA, USA), IL-10 (88-7105-88, Invitrogen, Carlsbad, CA, USA), and IL-2 (88-7024-88, Invitrogen, Carlsbad, CA, USA) was conducted as instructed. Absorbance (450 nm) was measured using a spectrophotometer (Jenway Spectrophotometer, London, UK).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA). The Shapiro-Wilk test and Brown-Forsythe test were used to assess normality and homogeneity of variance, respectively. Multiple group comparisons were conducted using one-way Analysis of variance (ANOVA), followed by Dunnett's and Tukey's post hoc tests where appropriate. Survival analysis of mice was performed using the log-rank test. A p-value of less than 0.05 was considered statistically significant.

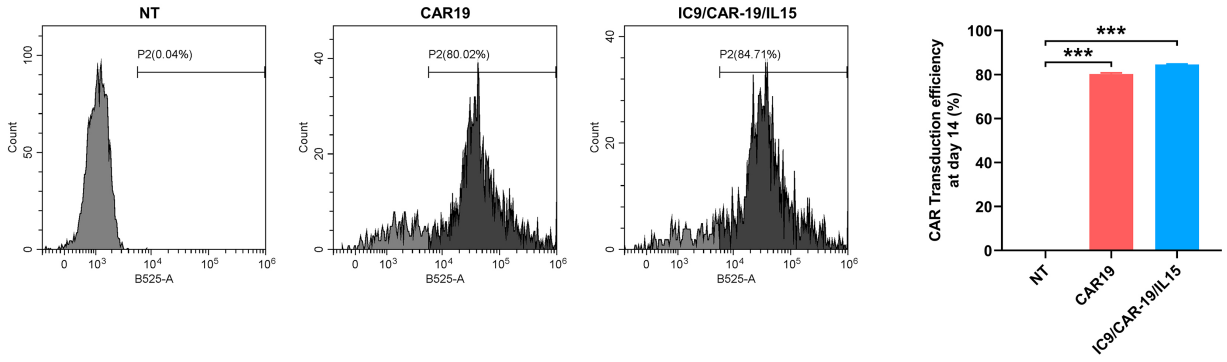

Fig. 1. Stable transduction of cord blood-derived natural killer (CB-NK) cells with inducible caspase-9/chimeric antigen receptor (CAR) targeting the CD19 antigen/interleukin-15 (iC9/CAR-19/IL-15) using retroviral vectors for expression. The transduction efficiency of iC9/CAR-19/IL-15 vectors in CB-NK cells (flow cytometry). $^{***}p < 0.001$. Each experiment was replicated thrice (mean $\pm$ standard deviation (SD)). Multi-group comparisons were performed by one-way Analysis of variance (ANOVA), and Dunnett post hoc tests were used. NT, non-transduced; CAR-19, chimeric antigen receptor (CAR) targeting the CD19 antigen; iC9/CAR-19/IL-15, inducible caspase-9/chimeric antigen receptor (CAR) targeting the CD19 antigen/interleukin-15.

Results

Transduction and Expression of CB-NK Cells

NK cells were extracted from CB and co-cultured for 14 days (non-transduced [NT]) with IL-2 and K562 cells as controls, and they were transduced with retroviral vectors to express CAR-19 and iC9/CAR-19/IL-15, respectively.

Flow cytometry data revealed highly expressed CAR on day 14 post-transduction, implying the efficacy of transduction ($p < 0.001$, Fig. 1). No statistical significance was found in CAR expression levels between CAR-19 (80.28 $\pm$ 0.49) and iC9/CAR-19/IL-15 (84.57 $\pm$ 0.21) groups.

Potentiated Tumor Suppression of iC9/CAR-19/IL-15 Transduction Combined With PD-1 Inhibitor Treatment

^{51}Cr release assays were conducted to explore the cytotoxicity of iC9/CAR-19/IL-15 on CD19-expressing tumors. iC9/CAR-19/IL-15 CB-NK (CAR-NK) cells were cultured (14 days), and cultivated with CLL cells exposed to PD-1 inhibitor at different effector-to-target cell ratios. PD-1 inhibitor treatment enhanced the cytotoxicity of NT cells and CAR-NK cells ($p < 0.01$, Fig. 2A). Importantly, the cytotoxicity of CAR-NK cells combined with PD-1 inhibitor treatment was superior and statistically significant at all effector-to-target cell ratios ($p < 0.01$, Fig. 2A).

To delineate the molecular basis of potentiated cytotoxic activity against CD19 targets, we assessed the levels of IFN-γ, CD107a, and TNF-α. Results revealed that compared to NT cells, there was no change in cytokine levels in PD-1 inhibitor-treated cells, whereas TNF-α, IFN-γ, and CD107a levels were increased in CAR-NK cells (Fig. 2B). NT-NK or CAR-NK cells were exposed to PD-1 inhibitor and then co-cultured with CLL cells. The test results showed that at 24, 48 and 72 h, the IL-2 levels in the supernatants of the co-culture systems of NT-NK or CAR-NK cells treated with the PD-1 inhibitor were significantly elevated ($p < 0.01$, Fig. 2C), and meanwhile, PD-1 inhibitor treatment also augmented IL-6 levels in the co-culture of CAR-NK cells ($p < 0.001$, Fig. 2D). Additionally, at 24 and 72 h, PD-1 inhibitor treatment increased IL-6 levels in the co-culture of NT cells, while the increase was more evident with additional treatment of CAR-NK cells ($p < 0.05$, Fig. 2D).

ELISA analysis data demonstrated that CAR-NK cells co-treated with PD-1 inhibitor expressed substantially higher IL-15 ($p < 0.001$, Fig. 2E). Moreover, the NK cell phenotype was determined employing multiparameter flow cytometry. Relative to CB-NK, NT and CAR-NK treatments resulted in upregulated CD16, CD44, KIR, as well as CXCR3, and downregulated KLRG-1 ($p < 0.001$, Fig. 3A–F). PD-1 inhibitor treatment increased CD16, CD44, and CXCR3 levels in the co-culture of NT cells ($p < 0.01$, Fig. 3A–C,E), while PD-1 inhibitor treatment decreased CXCR3 levels in the co-culture of CAR-NK cells ($p < 0.01$, Fig. 3A,E). PD-1 inhibitor treatment promoted KIR and inhibited KLRG-1 levels in the co-culture of NT cells ($p < 0.05$, Fig. 3A,D,F).

Tumor Suppression of iC9/CAR-19/IL-15-transduced CB-NK Cells Combined With PD-1 Inhibitor Treatment In Vivo

To further validate the efficiency of iC9/CAR-19/IL-15 in animals, we established a xenograft model. Mice were randomly assigned to control, NT, CAR-NK, PD-1 inhibitor, and CAR-NK+PD-1 inhibitor groups. In contrast with control mice or mice with NT cells, mice injected with CAR-NK cells presented substantially prolonged survival time.

CAR-NK cells combined with PD-1 inhibitor treatment on prolonging survival outperformed CAR-NK cells

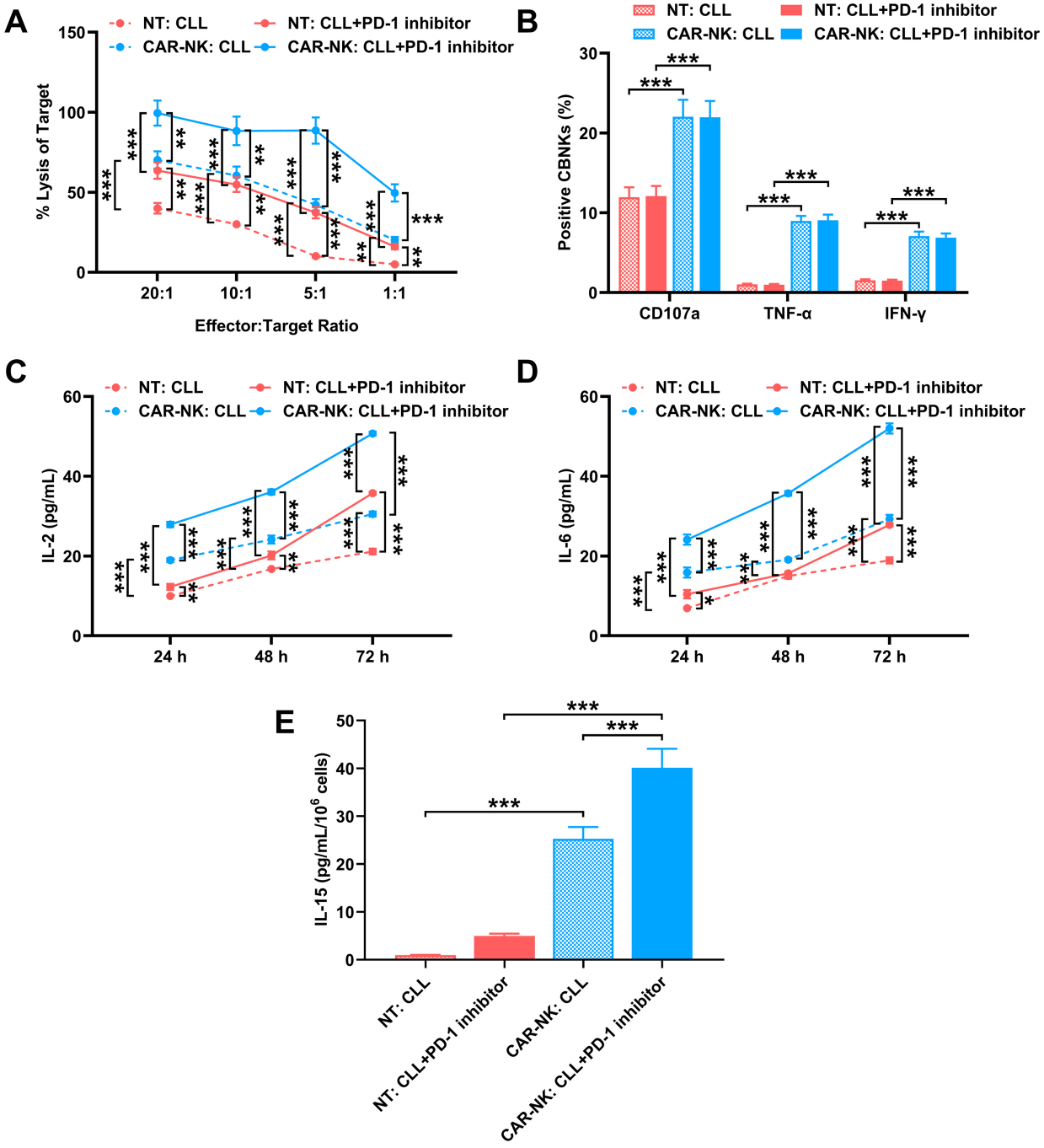

Fig. 2. ***In vitro*** **analysis of the antitumor function of iC9/CAR-19/IL-15-transduced CB-NK cells combined with programmed death-1 (PD-1) inhibitor treatment.** (A) Cytotoxicity (chromium-51 (^{51}Cr) release assay). (B) CD107a and effector cytokines (interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α)) (Flow cytometry analysis). (C–E) Interleukin-2 (IL-2) and IL-6 levels at different timepoints (enzyme-linked immunosorbent assay (ELISA)). NT: CLL, CLL cells co-cultured with NT cells; NT: CLL+PD-1 inhibitor, CLL cells co-cultured with NT cells with PD-1 inhibitor; CAR-NK: CLL, CLL cells co-cultured with CAR-NK cells; CAR-NK: CLL+PD-1 inhibitor, CLL cells co-cultured with CAR-NK cells with PD-1 inhibitor.$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Each experiment was replicated thrice (mean $\pm$ SD).

alone ($p < 0.001$, Fig. 4A). ELISA data on inflammatory cytokines in mouse serum unveiled lower IL-6, TNF-α, and IL-10 levels in the CAR-NK and PD-1 inhibitor groups compared to the NT group ($p < 0.001$, Fig. 4B). CAR-NK combined with PD-1 inhibitor treatment was superior to single treatment in inhibiting inflammatory cytokines ($p < 0.05$, Fig. 4B).

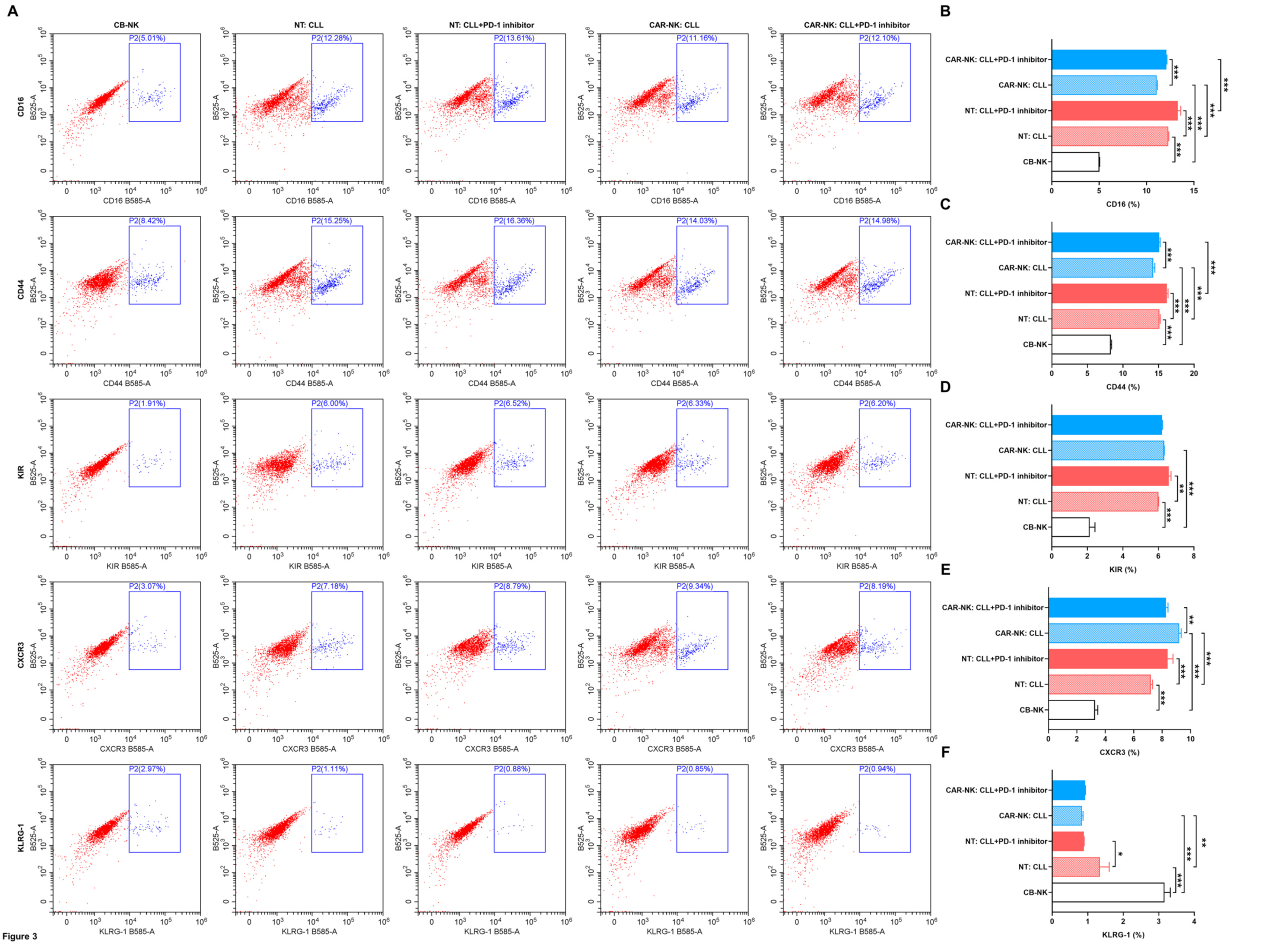

Fig. 3. iC9/CAR-19/IL-15-Transduced CB-NK cell phenotype was comparable to NT cells. (A) The NK cell phenotypes (CD16, CD44, KIR, CXCR3, and KLRG-1) (multiparameter flow cytometry). (B–F) Quantitative result of CD16 (B), CD44 (C), KIR (D), CXCR3 (E), and KLRG-1 (F). The group was the same as that of Fig. 2. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Each experiment was replicated thrice (mean ± SD).

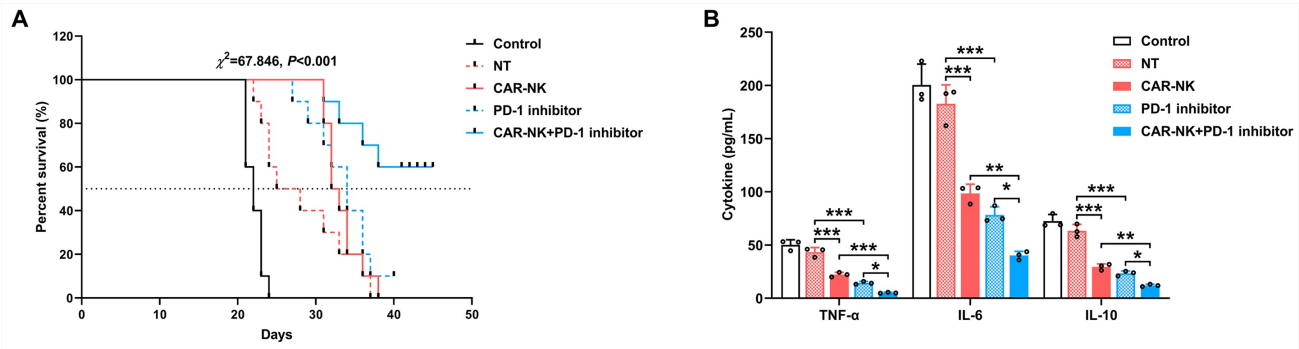

Fig. 4. Antitumor activity and anti-inflammatory effects of iC9/CAR-19/IL-15-transduced CB-NK cells combined with PD-1 inhibitor treatment in NOD scid gamma (NSG) chronic lymphocytic leukemia (CLL) mouse model. (A) Kaplan-Meier curve showing the survival probability of mice in the control, NT, CAR-NK, PD-1 inhibitor, and CAR-NK+PD-1 inhibitor groups (10 mice/group). (B) Levels of inflammatory cytokines (TNF-α, IL-6, and IL-10) in the serum of mice in the control, NT, CAR-NK, PD-1 inhibitor, and CAR-NK+PD-1 inhibitor groups (ELISA). Control, intravenous injections of saline; NT, intravenous injections of NT-NK cells (1.5×10^7 cells); CAR-NK, intravenous injections of CAR-NK cells (1.5×10^7 cells); PD-1 inhibitor, intravenous injections of PD-1 inhibitor (5 mg/kg body weight); CAR-NK+PD-1 inhibitor, intravenous injections of CAR-NK cells (1.5×10^7 cells) combined with PD-1 inhibitor. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Each experiment was replicated thrice (mean ± SD).

Discussion

Cell therapy harnesses the immune system for cancer treatment [34]. The CD19-targeted CAR-T cell therapy marks a prominent progress in adoptive cell treatment for cancer [35]. NK cells have gained attention as a genetically modified immune cell treatment due to their distinct biological characteristics [36]. The advantages of NK cells include both CAR-dependent and CAR-independent targeting mechanisms, minimal impact from tumor-expressed PD-L1, and the ability to be formulated into products and cryopreserved for long-term time [37].

CAR-NK cell therapy has demonstrated efficacy in preclinical and clinical trials against various hematologic diseases (including those targeting antigens like CD19, CD22, and CS1), and solid tumor cells (like ladder and lung cancer cells) [38]. While CAR-NK cells targeting other antigens (e.g., CD22 in esophageal squamous cell carcinoma [39], CD44v6 in Head and neck squamous cell carcinoma [40]) result in promising results, we focused on CD19 due to its established role in B-cell malignancies and directly compared it with CD19-CAR-T therapies. Therefore, CAR-NK cell immunotherapy holds promise for advancing the treatment of lymphoma.

IL-15 plays a vital role in regulating NK cell immunity, including NK cell survival, activation, cytotoxicity, and antitumor activity [41]. In melanoma, IL-15 enhances NK cell-mediated cytotoxicity and reduces tumor metastasis [42]. Studies have also identified IL-15 as essential for maintaining NK cell viability and promoting their expansion [43], leading to strategies that engineer NK cells to express membrane-bound or secreted IL-15. Furthermore, recent research has demonstrated that targeting checkpoints within IL-15 signaling can enhance NK cell-mediated cancer immunotherapy [41]. Additionally, the upregulation of PD-1 on CAR-NK cells may be associated with their cytotoxic activity [44]. It has been reported that blocking the PD-1/PD-L1 interaction enhances the anti-cancer efficacy of CAR-NK therapies, such as those using HER2-targeted CAR-NK cells [45].

Dampening the PD-1/PD-L1 checkpoint increases the toxic effect of CAR-NK cells to IFN-γ-stimulated ovarian cancer cells (PD-L1$^+$ cells) [46]. Beyond PD-1/PD-L1, other immune checkpoints like CTLA-4 can enhance CAR-NK or other immunotherapies. While CTLA-4 blockade shows efficacy, particularly in T-cell-mediated responses [47], its role and impact specifically in CAR-NK cell therapy, especially compared to PD-1 blockade, warrant further investigation. In B-cell lymphoma, PD-1 expression is frequently elevated in T lymphocytes that have invaded tumors [48]. The previous study has shown that while PD-1 inhibition moderately improves T cell function, the anticancer efficacy cannot be restored, primarily due to damaged T cell metabolism, cytoskeletal synapse formation defects, and T cell malfunction [49]. Currently, FDA-approved PD-1 antibodies include nivolumab and pembrolizumab, and nivolumab therapy improves antigen-specific T cell responses, such as effector T cell proliferation and cytokine generation [50].

Our results showed that IL-2 and IL-6 levels in the supernatant of the co-culture system of PD-1 inhibitor-treated NT-NK or CAR-NK cells were significantly increased *in vitro*, which might mainly be attributed to the direct effect of PD-1 inhibitors on NK cells. By blocking the PD-1 signaling pathway on the surface of NK cells, PD-1 inhibitors may directly relieve their intrinsic functional inhibitory state, thereby enhancing NK cell activation and cytokine secretion, including IL-2. However, in *in vivo* experiments, PD-1 inhibitors dampened the IL-6 level, indicating PD-1 inhibitors mainly activated T cells, and CAR-NK cells were highly effective in controlling tumors. The reduction of tumor burden and improvement of the microenvironment are the main drivers for decreased inflammatory factors.

In this study, we successfully constructed iC9/CAR-19/IL-15 CAR-NK cells and evaluated their antitumor activity in lymphoma. Our results demonstrated that iC9/CAR-19/IL-15 CAR-NK cells effectively killed primary CLL cells, consistent with previous findings [30], further supporting the therapeutic potential of CD19-targeted CAR-NK approaches. However, a prior study reported that CAR/NK-92 cell therapy may induce sustained immunosuppression within the tumor microenvironment (TME), thereby dampening antitumor responses [51].

NK cells are key producers of IFN-γ within the TME [52,53]. Upon binding to target cells, CAR-NK cells secrete substantial amounts of IFN-γ, which tumor cells rapidly respond to. IFN-γ interacts with interferon receptors, activating JAK1, JAK2, and STAT1 signaling pathways, leading to the upregulation of PD-L1 on tumor cells [46]. Moreover, all CAR-NK cell populations exhibit upregulation of exhaustion-related molecules, including PD-1, following prolonged antigen exposure. As an immune checkpoint receptor, PD-1 is associated with NK cell exhaustion. This provides a rationale for combining CAR-NK cell therapy with immune checkpoint inhibitors targeting PD-1 to enhance clinical efficacy [54].

Consistently, our results showed that combining iC9/CAR-19/IL-15 CAR-NK cells with PD-1 inhibitor treatment significantly enhanced cytotoxicity against tumor cells, suggesting that blocking the PD-1/PD-L1 axis strengthens CAR-NK cell-mediated cytotoxicity. However, this finding requires further experimental validation.

However, in this study, we observed no significant changes in levels of TNF-α, IFN-γ, and CD107a after nivolumab treatment, possibly owing to the involvement of nivolumab in regulating other cytokine levels [33]. CD107a, associated with lysosomal membranes, serves as a marker for the degranulation of NK and CD8$^+$ T cells, and hence functions as an activation indicator [55]. NK cell-derived IFN-γ and TNF-α can promote tumor cell apop-

tosis, cytotoxic activity of CD8+ cells, and production of antitumor antibodies by B cells [56].

Our study suggested TNF-α, IFN-γ, and CD107a responded to $CD19^+$ targets mostly derived from iC9/CAR-19/IL-15 CAR-NK cells, thereby enhancing their antitumor activity. The lack of further increase in these markers with nivolumab implies that the enhanced cytotoxicity in the combination group might stem from mechanisms other than boosted degranulation or cytokine secretion per cell, such as improved survival, persistence, or relief from inhibition within the TME.

TNF-α time-dependently induces nuclear export of IL-15Rα and IL-15, and facilitates exocytosis of IL-15 [57]. In our research, we observed upregulated IL-15 in iC9/CAR-19/IL-15 CAR-NK cells, and a higher IL-15 level after PD-1 inhibitor treatment. This indicated that the treatment of iC9/CAR-19/IL-15 transduction and PD-1 inhibitor may enhance NK cell activation and persistence via increasing IL-15 levels, thereby enhancing their antitumor activity, which was further confirmed *in vivo*. This finding highlighted a potential synergistic mechanism unique to combining IL-15-engineered CAR-NK cells with PD-1 blockade. The responses of functional NK cells to IL-15, including IFN-gamma secretion and CD107a expression, exhibit a similar pattern, confirming the importance of cytokine receptor switch in physiological processes [58]. The receptor complexes for IFN-γ signal via the JAK-STAT pathway are at the crossroad of NK cell development and maturation [59,60]. The JAK inhibitor ruxolitinib with anti-PD-1 antibody nivolumab enhances the efficacy of immune checkpoint blockade in preclinical solid tumor and lymphoma models, manifesting that PD-1 inhibitors may modulate IL-15 levels via JAK-STAT signaling to enhance anti-tumor activity, a hypothesis that requires additional experimental verification.

Although this study confirmed that the combination of iC9/CAR-19/IL-15 CAR-NK cells and PD-1 inhibitor exerts potent antitumor effects, several challenges remain. First, only one cell line was used; future studies should include at least two different cell lines to rule out cell-specific discrepancies. Second, the precise antitumor mechanisms underlying the combination therapy remain unclear. Further investigations—such as improved controls using mock-transduced or irrelevant CAR-expressing cells—are needed to eliminate potential interference from exogenous vectors, confirm gene-editing effects, and verify target specificity. Third, the expression levels of PD-1 and PD-L1 on both NK and CLL cells should be directly assessed in future work. Fourth, the safety profile of this combination therapy in NSG mouse models requires thorough evaluation, including monitoring body weight, organ histology, and cytokine release markers, to enhance robustness and facilitate clinical translation. Finally, CAR-NK cell therapy is still in its infancy. To successfully implement this approach clinically, it will be essential to optimize CAR-NK cell design and address issues such as limited *in vivo* proliferation, activation, and persistence. Overcoming these challenges will be critical for advancing CAR-NK therapy as a promising new option for cancer treatment.

Conclusions

The combination of iC9/CAR-19/IL-15 CAR-NK cells and PD-1 inhibitor exerts antitumor effects, evidenced by increased cytotoxicity and IL-2/IL-6/IL-15 levels, decreased secretion of inflammatory cytokines and ultimately prolonged survival of mice, offering a novel view for immunotherapy in B-cell lymphoma.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

Substantial contributions to conception and design: YLL. Data acquisition, data analysis and interpretation: LXS, JPW, GFO, SSY, ZNL. Drafting the article or critically revising it for important intellectual content: All authors. Final approval of the version to be published: All authors. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: All authors.

Ethics Approval and Consent to Participate

This research on humans was approved and instructed by the Ethics Committee of NBU Health Science Center (Approve No: NBU-2020-140) and conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all human samples. The study on animals was permitted by the Ethics Committee of Ningbo University (2020-202).

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Conflict of Interest

The authors declare no conflict of interest.

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