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Adoptive cell transfer therapy with ex vivo primed peripheral lymphocytes in combination with chemotherapy in locally advanced or metastatic triple-negative breast cancer: the ImmunoBreast phase Ib clinical trial

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ABSTRACT

Background Adoptive cell transfer-based immunotherapy holds promise for treating advanced cancer. However, a key challenge remains: generating sufficient numbers of lymphocytes capable of recognizing and targeting a broad range of cancer antigens. We recently developed Autologous Lymphoid Effector Cells Specific Against Tumor (ALECSAT), a novel procedure for selecting, expanding and maturing polyclonal lymphocytes from peripheral blood with the capacity to target cancer cells. In this single-center phase Ib trial, we evaluated the safety, tolerability, and preliminary efficacy of ALECSAT in combination with standard carboplatin and gemcitabine in patients with locally advanced or metastatic triple-negative breast cancer (mTNBC).

Methods This clinical study enrolled 15 patients with mTNBC. The patients received three ALECSAT doses, administered every 28 days. Subsequently, ALECSAT doses were given at 6-week intervals. Carboplatin and gemcitabine were administered on days 1 and 8 in 3-week cycles. The cell composition of ALECSAT preparations was analyzed using flow cytometry. Additionally, patient-derived xenograft (PDX) mouse models were generated and treated with ALECSAT to assess treatment responses.

Results 14 patients with mTNBC, who had received one to four prior treatment lines, were treated with 1–10 doses of ALECSAT. The combination of ALECSAT with carboplatin and gemcitabine was well tolerated and demonstrated a favorable safety profile. Common adverse events (AEs), including fatigue, nausea, and hematological abnormalities, were consistent with the known toxicity profiles of carboplatin and gemcitabine. Notably, grade ≥3 AEs were predominantly hematological, with manageable durations of neutropenia and thrombocytopenia. Among treated patients, one achieved a complete response, four had partial responses, five had stable disease, and four had progressive disease. The objective response rate was 36% (95% CI 12.8% to 64.9%). Median progression-free survival was 4.3 months (95% CI 1.6 to 7.0), while median overall survival was 8.7 months (95% CI 5.1 to 12.4).

A positive correlation was observed between the total number of administered ALECSAT cells (particularly CD8+T cells) and time to progression. Additionally, ALECSAT

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Triple-negative breast cancer (TNBC) is a highly heterogeneous disease with varying levels of tumor mutational burden (TMB) and immune infiltration. While some TNBC subtypes exhibit high programmed death-ligand 1 (PD-L1) expression and high TMB, which may contribute to an immunogenic tumor microenvironment, others show limited immune activation. Consequently, immune checkpoint blockade targeting programmed death-1/PD-L1 has been incorporated into the standard treatment for metastatic TNBC (mTNBC). However, its efficacy remains limited, with response rates generally around 20%, which may reflect the proportion of patients with truly immunogenic tumors. As an alternative immunotherapeutic approach, adoptive transfer of tumor-reactive immune cells has shown promise in other cancer types, but its potential in mTNBC remains largely unexplored.

WHAT THIS STUDY ADDS

⇒ This study demonstrates that Autologous Lymphoid Effector Cells Specific Against Tumor (ALECSAT) products are consistently tumor-reactive across patient preparations. We investigated the combination of ALECSAT and chemotherapy in patients with mTNBC and showed that this treatment is feasible, safe, and showed signs of response in some patients with mTNBC. A correlation between higher ALECSAT cell doses—particularly CD8+T cells—and improved clinical outcomes was observed. ALECSAT treatment outcomes in patients correlated with responses observed in their corresponding patient-derived xenograft models.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study shows that ALECSAT may have a role in cancer therapy including TNBC. Based on our recent discovery in patient-derived xenograft models that combining ALECSAT and anti-PD-L1 therapy enhances the therapeutic effects—and given the observed predictive value of these models in this study—a clinical trial evaluating the safety and signs of efficacy of combined ALECSAT and anti-PD-L1 therapy should be pursued.

treatment outcomes in patients correlated with responses observed in their corresponding PDX models.

Conclusion ALECSAT, in combination with carboplatin and gemcitabine, was safe, well tolerated, and demonstrated promising antitumor activity in mTNBC. These findings support further investigation in larger clinical trials.

Trial registration number NCT00891345.

INTRODUCTION

Triple-negative breast cancer (TNBC), defined by the absence of hormone receptors and human epidermal growth factor receptor 2 (HER2), is an aggressive subtype accounting for 10–15% of all breast cancers. Patients with unresectable locally advanced or metastatic TNBC (mTNBC) have a poor prognosis, with a median overall survival (OS) of approximately 12–18 months.[1 2] Chemotherapy has traditionally been the primary systemic treatment of mTNBC, with taxanes or anthracyclines commonly used as first-line therapy.[3] However, responses are typically short-lived. Additionally, since most patients have already received anthracycline and taxanes in the (neo)adjuvant setting, these regimens may not be optimal in the metastatic setting. The combination of gemcitabine and carboplatin is included in the European Society for Medical Oncology guidelines and is established first-line treatment for mTNBC.[3 4] Clinical studies have reported an objective response rate (ORR) of 30.2%, a median progression-free survival (PFS) of 4.6 months, and a median OS of 12.6 months with this regimen.[5]

Certain TNBC subtypes exhibit features of immunogenicity, such as moderate to high tumor mutational burden and increased amounts of tumor-infiltrating lymphocytes (TILs); however, the disease often progresses, leading to poor clinical outcomes.[6–8] A dysfunctional tumor immune microenvironment is consistent with the high programmed death-ligand 1 (PD-L1) expression commonly seen in TNBC tumors. Immune checkpoint blockade targeting programmed death-1 (PD-1) or PD-L1 has shown promising, but limited efficacy in mTNBC, with overall response rates of less than 20% in unselected patients. However, in PD-L1+tumors with a combined positive score of at least 10, the anti-PD-1 antibody pembrolizumab, in combination with standard chemotherapy, has shown statistically significant and clinically meaningful improvements in PFS and OS in patients with mTNBC (OS: 23.0 vs 16.1 months, HR, 0.73; 95% CI 0.55 to 0.95; two-sided p=0.0185).[9 10] These findings have led to regulatory approval by both the Food and Drug Administration (FDA) and European Medicines Agency (EMA). The anti-PD-L1 antibody atezolizumab, in combination with nab-paclitaxel, has also been approved by FDA and EMA for the treatment of PD-L1+mTNBC; however, this trial failed to demonstrate significant OS benefit in the intention-to-treat (ITT) population, and FDA approval was later withdrawn due to lack of confirmatory data.[11] Furthermore, based on the positive results from the KEYNOTE-522 phase III trial, pembrolizumab in combination with chemotherapy is now also approved in the neoadjuvant setting for stage II and III early TNBC, followed by adjuvant pembrolizumab.

Several targeted therapies have recently been approved for TNBC. The antibody-drug conjugate (ADC) sacituzumab govitecan, which consists of an anti-Trop2 antibody conjugated to SN-38 (the active metabolite of irinotecan) as well as the HER2 ADC, trastuzumab deruxtecan, has been approved by the FDA and EMA for mTNBC following promising results from the phase III ASCENT trial and DESTINY-Breast04.[12 13] Additionally, poly ADP-ribose polymerase inhibitors have been approved for patients with germline BRCA1 and BRCA2 mutations, which are present in approximately 20% of patients with TNBC.[14]

Adoptive cell transfer (ACT) therapy is an approach that harnesses the patient's immune system to target and eliminate cancer cells.[15 16] Effector cells, such as cytotoxic T lymphocytes (CTLs), recognize tumor antigens and induce tumor cells lysis or cytokine secretion on antigen encounter. Although tumor-specific CTLs are present in many patients with cancer, their activity is often suppressed by the tumor microenvironment.[17] To overcome this, in vitro activation and expansion of CTLs for reinfusion into patients represents a promising strategy. ACT therapy encompasses several approaches, including TILs and genetically engineered T cells expressing novel T-cell receptors or chimeric antigen receptors (CARs).[18–21] In recent clinical studies, TIL therapy has demonstrated high efficacy.[22] However, melanoma remains the only cancer type that consistently yields cancer-recognizing TIL cultures.[23] This emphasizes the critical need to accelerate the development of cellular therapies to optimize their therapeutic potential and expand their applicability to a broader patient population. Such advancements are particularly essential for malignancies with limited therapeutic options, including mTNBC.

ALECSAT (Autologous Lymphoid Effector Cells Specific Against Tumor) is a personalized ACT approach generated from the patient's own lymphocytes. Instead of relying on pre-selection or genetic engineering of antigen-specific T cells, ALECSAT employs stimulation with epigenetically modulated antigen-presenting cells, which drives the expansion of heterogeneous T-cell populations with tumor reactivity. Preclinical studies have demonstrated activity against multiple breast cancer cell lines and induction of interferon-γ responses to cancer-testis antigens such as MAGE-A, GAGE, and CTAG1.[24 25]

In this phase Ib clinical trial, we evaluated ALECSAT as an add-on therapy to carboplatin and gemcitabine chemotherapy in patients with mTNBC. The primary objective was to assess the safety and feasibility of this combination therapy. Secondary objectives included trends of efficacy, such as ORR, duration of response (DOR), disease control rate (DCR), PFS, and OS. Additionally, we analyzed the administered ALECSAT cell product to correlate immune cell phenotypes with patient outcomes. Finally, patient-derived xenograft (PDX) mouse models were generated from mTNBC biopsies to investigate their potential as predictive tools for therapeutic response.

PATIENTS AND METHODS

Trial design

This phase Ib, single-center, open-label, single-arm, investigator-initiated clinical study was conducted at the Department of Oncology, Odense University Hospital, Odense, Denmark, between May 15, 2020, and December 29, 2023 (ClinicalTrials.gov identifier: NCT00891345).

The primary objective was to determine the safety and tolerability of ALECSAT in combination with standard carboplatin and gemcitabine in female patients with histologically confirmed, inoperable, locally advanced or mTNBC who had received no more than four prior systemic therapies for mTNBC. A schematic representation of the treatment schedule is depicted in figure 1A. The secondary objectives included trends of efficacy through evaluation of ORR, DOR, DCR, PFS, and OS based on Response Evaluation Criteria In Solid Tumors, V.1.1 (RECIST V.1.1).[26] Exploratory analysis included ORR, DOR, and PFS using modified RECIST 1.1 for immune-based therapeutics, alongside standard RECIST V.1.1, with both measures assessed via local radiologic review. Additionally, we evaluated potentially predictive biomarkers of response to ALECSAT and assessed the antitumor effects of ALECSAT therapy in PDX mouse models generated from autologous patient tumor tissue.

Study treatment was continued until any of the following occurred: unacceptable toxicity, disease progression, or investigator/patient decision. Patients who discontinued chemotherapy due to toxicity or other reasons were allowed to continue ALECSAT monotherapy treatment at the investigator's discretion.

Response assessment according to RECIST V.1.1 was performed every 9 weeks during the first year, and thereafter with 12 weeks interval. Safety was monitored according to National Cancer Institute Common Terminology Criteria for Adverse Events V.5.0.

AUTOLOGOUS LYMPHOID EFFECTOR CELLS SPECIFIC AGAINST TUMOR

Second generation ALECSAT (hereafter referred to as ALECSAT) was manufactured from autologous peripheral blood at Cytovac A/S, Hørsholm, Denmark. For the initial preparation, 300 mL of peripheral blood was collected; subsequent donations comprised 200 mL. At each collection, an additional 20 mL of blood was drawn for serum preparation, which was used for cryopreservation. The procedure comprised sequential steps including dendritic cell (DC) differentiation, co-culture of DCs with lymphocytes in the presence of interleukin-2 (IL-2), exposure of activated lymphocytes to the demethylating agent 5-aza-2'-deoxycytidine (5-aza-CdR), and a final co-culture with fresh DCs and additional lymphocytes (figure 1B).

For DC generation, peripheral blood mononuclear cells (PBMCs) were seeded in AIM-V medium after preconditioning of culture flasks with RPMI-1640 containing 5% human AB serum. Following adherence of monocytes, non-adherent lymphocytes were collected and cryopreserved, while adherent cells were cultured further in AIM-V. Granulocyte-macrophage colony-stimulating factor (GM-CSF) (100 ng/mL) and IL-4 (25 ng/mL) were added on days 1 and 3. On day 4, IL-1β (10 ng/mL), IL-6 (1000 IU/mL), tumor necrosis factor-α (10 ng/mL), and prostaglandin E2 (PGE_2) (0.2 µg/mL) were introduced. On day 6, non-adherent cells were harvested, counted, and either used directly or cryopreserved in aliquots.

To prepare plasma-derived serum used for cultivation of lymphocytes, the lymphoprep centrifugation was done in 50 mL tubes first at low speed (200 g, 20 min) followed by collecting about 15 mL of the upper layer of plasma. After this, lymphoprep centrifugation was continued at 460 g 20 min. The collected plasma was placed in T225 TC flasks, and clotting was induced by addition of 1 mM of $CaCl_2$. After overnight incubation in CO2-incubator, serum was separated from the clot and placed at −20°C for at least 24 hours. After thawing, the tubes with serum were centrifuged at 2,000 g for 15 min, and the supernatant was transferred to a new tube.

Thawed lymphocytes were subsequently co-cultured with DCs at a 10:1 ratio in AIM-V supplemented with 1% autologous plasma-derived serum. IL-2 (25 IU/mL) was added on day 7 and replenished with medium on days 10 and 12. On day 13, cells were collected, washed, and resuspended in fresh AIM-V supplemented with IL-2 (150 IU/mL) and 5-aza-CdR (10 µM) for 2 days. On day 15, the 5-aza-CdR-treated cells were combined with thawed lymphocytes and DCs at a 10:10:1 ratio in AIM-V with 2% autologous serum. IL-2 (25 IU/mL) was added on day 17 and refreshed together with medium on days 20, 22, and 24. On day 26, part of the culture was used for flow cytometric analysis, while the remaining product was formulated for patient administration.

Each ALECSAT preparation contained 1×10^7 to 1×10^9 cells suspended in Plasma-Lyte (Baxter) with 5% autologous serum at a final volume of 20 mL. Patients received three ALECSAT doses during a loading phase (weeks 5–13) at 4-week intervals (±3 days), followed by a maintenance phase with administration every 6 weeks (±1 week) until discontinuation (figure 1A). An aliquot of each product was analyzed

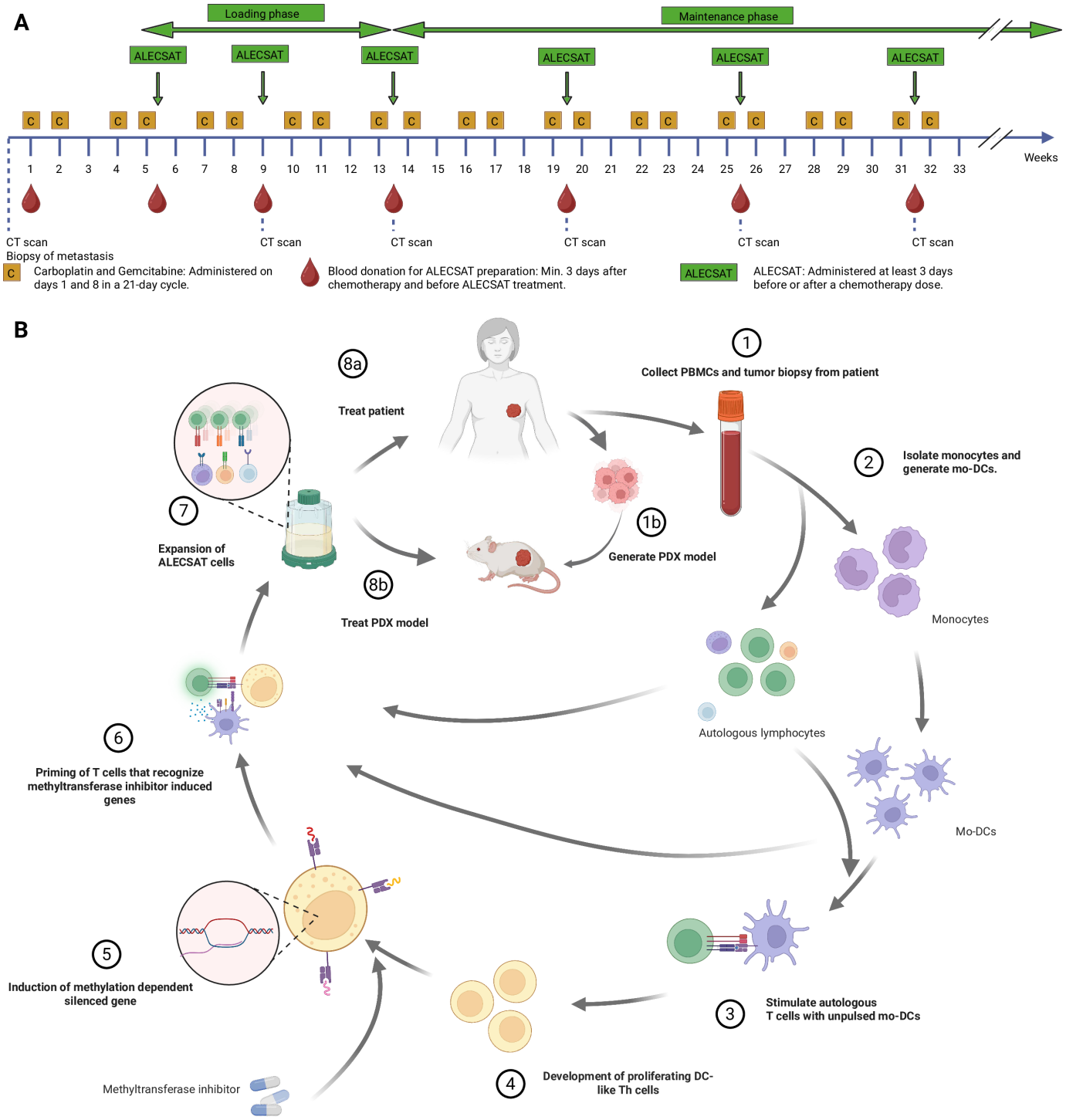

Figure 1 Study design. (A) Treatment schedule given in weeks. Carboplatin/gemcitabine was given as six consecutive cycles starting from week 0. During the loading phase, patients received three ALECSAT doses, administered every 28 days (±3 days). During the maintenance phase, ALECSAT doses were administered at 6-week intervals (±1 week). (B) Schematic representation of the individual steps in the ALECSAT production from individual patients and the generation of PDX models. Figure created with BioRender.com. ALECSAT, Autologous Lymphoid Effector Cells Specific Against Tumor; DC, dendritic cell; PDX, patient-derived xenograft.

by flow cytometry using antibodies against CD3, CD4, CD8, and CD56.

Chemotherapy

Carboplatin (area under the curve of longitudinal change 2 (AUC2)) and gemcitabine (1,000 mg/m^2) were administered on days 1 and 8 of a 21-cycle (every 3 weeks), following local guidelines and practices.

Patients

Female patients aged ≥18 years with mTNBC were enrolled at the Department of Oncology, Odense

University Hospital, Odense, Denmark. Key inclusion criteria included: histologically confirmed, inoperable, locally advanced, or mTNBC, not amenable to curative treatment. Eligibility for chemotherapy with carboplatin and gemcitabine. Eastern Cooperative Oncology Group (ECOG) performance status of 0–1 and an estimated life expectancy of ≥3 months at enrollment. Collection and submission of a fresh tumor biopsy from a local recurrence or distant metastasis site, along with blood samples for biomarker testing. At least one measurable target lesion per RECIST V.1.1. as determined by local radiology review. TNBC confirmation: TNBC was defined by absence of HER2 and estrogen receptor expression in the primary tumor as assessed by local pathologist. Patients initially diagnosed with hormone receptor-positive and/or HER2-positive breast cancer required a repeat biopsy from a local recurrence or distant metastasis site to confirm TNBC status. Whether patients had acceptable organ function (assessed within 10 days prior to treatment initiation) was determined based on the following criteria: hematological: absolute neutrophil count ≥1,500/µL, lymphocyte count >0.3×10^9/L, platelets ≥100×10^9/L; renal: creatinine ≤1.5 × upper limit of normal (ULN) or measured creatinine clearance (glomerular filtration rate (GFR) can also be used in place of creatinine or creatinine clearance (CrCl)) ≥30 mL/min for patient with creatinine levels >1.5 × institutional ULN); hepatic: total bilirubin ≤1.5 × ULN, aspartate aminotransferase and alanine transaminase ≤2.5 × ULN (≤5 × ULN for patients with liver metastases), albumin ≥3.0 g/dL. Women of childbearing potential were required to have a negative pregnancy test at screening and agreed to use acceptable methods of contraception during the study. The immune status (% TILs) in patients' metastatic biopsy samples was assessed on H&E stained tissue sections by an experienced breast cancer pathologist. PD-L1 status was assessed using the Ventana PD-L1 SP142 assay.

Generation of patient-derived xenograft models

Female NOG (NOD.Cg-Prkdc^{SCID}Il2rg^{tm1Sug}/JicTac, Taconic) mice were anesthetized, and the fourth mammary fat pad was surgically exposed. A total of 50 µL of extracellular matrix (ECM) gel (Merck, E1270-5) containing 5×10^6 ALECSAT cells was injected into the fat pad. Subsequently, the fat pad was opened, and a patient tumor fragment (approximately 8 mm^3) was implanted into the ECM gel. The mammary fat pad and the skin were then closed using internal and external stitches, respectively. On termination of the animals, tumors were surgically excised, and their mass was measured using a digital scale.

Animal experiments

All animal experiments were conducted at the animal core facility, University of Southern Denmark, in accordance with institutional and national ethical guidelines for animal research. Mice were housed under pathogen-free conditions with ad libitum access to food and water. Housing conditions were maintained at 21±1°C with 40–60% relative humidity, and the light/dark cycle was set to 12 hours (lights on from 06:00 to 18:00). Sample sizes were determined based on prior experiments and preliminary data. No animals were excluded from the analysis. Mice were acclimatized for 2 weeks prior to the start of experiments. Animals were sacrificed when tumors reached a diameter of 1.2 cm, or when mice showed signs of graft-versus-host disease, whichever occurred first.

Co-culture studies

For co-culture experiments, cancer cells (5×10^3) were plated in white 96-well plates using AIM V medium (Gibco, 12055–083) supplemented with 2% human serum and allowed to adhere for 2 hours. Immune effector cells were then added and co-cultured for 24 hours at 37°C. Cell viability was subsequently determined by adding D-luciferin (3 mg/mL in phosphate-buffered saline) and measuring luminescence immediately on a Victor3 Multilabel Plate Reader. Cancer-cell viability was calculated as (sample – background) / (cancer cells only – background) × 100%.

Degranulation assays

For degranulation assays, cancer cells (1×10^5) were seeded in AIM V medium containing 5% human serum and co-incubated with effector cells (3×10^5). Cultures were supplemented with 2 µg/mL GolgiStop (BD, 554724) either alone or together with anti-CD107a. After 5 hours, cells were collected, stained, and evaluated by flow cytometry.

Real-time monitoring of cell viability

Real-time cytotoxicity assays were carried out using the iCELLigence system (ACEA Biosciences, San Diego, California, USA) to monitor lymphocyte-mediated killing. Cancer cells (3×10^4 per well) were seeded in 400 µL RPMI 1640 supplemented with 10% fetal calf serum (FCS) and incubated for 20–24 hours. Subsequently, three different doses of lymphocytes (0.05×10^6, 0.1×10^6, and 0.2×10^6) lymphocytes were added in 200 µL AIM-V medium. Tumor cell killing was detected as a reduction in impedance (Cell Index), which was recorded every 15 min for 20–25 hours and expressed as Normalized Cell Index (NCI). Cytotoxic activity (percentage) was calculated as $(NCI_{Control} - NCI_{experiment}) / NCI_{Control} \times 100$.

Statistics

The statistical output was generated using GraphPad Prism. Patient characteristics, safety, and efficacy results were summarized descriptively. All patients who received at least one dose of ALECSAT were included in the safety and efficacy analysis. Point estimates and exact two-sided Clopper-Pearson 95% CI were provided for ORR and DCR. The median follow-up time was calculated using the Kaplan-Meier method and asymmetrical exact two-sided 95% CI were provided for PFS and OS. Spearman's rank correlation test was used to assess the correlation between cell types in the administered ALECSAT product and time to progression, with Spearman's rho

(r) and corresponding p values reported. A linear regression model was fitted to assess the relationship between variables, with the best-fit regression line, R^2 value, and equation reported. The Mann-Whitney U test was used to compare the distribution of different cell types between response groups, given the non-normal distribution of the data.

RESULTS

Study population

Between May 15, 2020, and August 25, 2023, a total of 15 patients with mTNBC were enrolled. One patient (patient 8) received one treatment with carboplatin and gemcitabine but was discontinued from the study due to rapid disease progression and clinical deterioration before receiving the first ALECSAT dose. The remaining 14 patients received between 1 and 10 ALECSAT doses (5 patients receiving 1–2 doses, 7 patients receiving 3–4 doses and 2 patients receiving 10 doses). All patients had discontinued treatment at the time of data collection. The baseline patient characteristics are listed in table 1.

The median age of patients was 49 years and all had ECOG performance status of 0 or 1. Nine patients had recurrent metastatic disease, with five experiencing a disease-free interval of ≥12 months before relapse. The remaining six patients had de novo metastatic disease. Most patients (80%) had received one to two prior lines of systemic therapy for mTNBC, while the remaining patients (20%) had received four prior lines of therapy. Before receiving ALECSAT treatment, patients had primarily received chemotherapy, while four patients also receiving prior atezolizumab (anti-PD-L1). None had received pembrolizumab (anti-PD-1).

Feasibility and safety

Overall, the combination of ALECSAT and chemotherapy treatment was well tolerated, with toxicities that were consistent with the expected profile of the chemotherapy. The most common adverse events (AEs) were fatigue, nausea and pain, as summarized in table 2. Grade ≥3 AEs were predominantly hematologic, including neutropenia and thrombocytopenia. The median duration of grade ≥3 neutropenia was 7 days (range: 6–10 days). A total of seven serious adverse events (SAEs) were reported, including back pain, hypokalemia, thrombocytopenia, bladder infection and pneumonitis. Two patients discontinued treatment due to AEs, while five patients required chemotherapy dose modification due to AEs. There were no grade 5 AEs.

Clinical efficacy

The antitumor activity in 14 patients with mTNBC treated with ALECSAT in combination with carboplatin and gemcitabine is shown in table 3, based on RECIST V.1.1. One patient (7%) had a complete response (CR) and four patients (29%) had partial responses (PR). The ORR per RECIST V.1.1 was 36% (5/14 patients; 95% CI 12.8%

Table 1 Patient characteristics

	Total, N=15 (%)
Age, median, y (IQR)	49 (37–64)
ECOG performance status	
0	9 (60)
1	6 (40)
≥2	0
Disease presentation	
De novo metastatic	6 (40)
Recurrent metastatic	9 (60)
Disease-free interval*	
<12 months	4 (44)
≥12 months	5 (56)
Prior systemic therapy for metastatic disease	39 (100)
Anthracycline	4 (10)
Taxane	8 (21)
Capecitabine	7 (18)
Vinorelbine	3 (8)
Eribulin	6 (15)
Cyclophosphamide	4 (10)
Anti-PD-L1 immunotherapy	4 (10)
Other†	3 (8)
Prior lines of therapy (advanced setting)	
0	0
1	7 (47)
2	5 (33)
3	0
4	3 (20)
Metastatic sites, number	
≤2	8 (53)
>2	7 (47)
Localization of metastases	
Opposite breast	2 (6)
Liver	3 (9)
Bone	4 (12)
Skin	2 (6)
Lung	6 (18)
Regional lymph nodes	8 (24)
Distant lymph nodes	5 (15)
Central nervous system	1 (3)
Other‡	3 (9)
% TILs	
<5% TILs	7 (47)
≥5% TILs	8 (53)
PD-L1 status	

Continued

Table 1 Continued

	Total, N=15 (%)
Negative (<1%)	4 (27)
Positive (≥1%)	9 (60)
Unknown	2 (13)
Germline BRCA mutational status	
BRCA1/2 mutation	2 (13)
No BRCA1/2 mutation	8 (53)
Unknown	5 (33)

*From time of primary tumor resection to recurrence of disease. Percentage of recurrent.
†Other is endocrine treatment, CDK4/6 inhibitor, and unknown if patients had received blinded treatment within clinical trial.
‡Other is peritoneal carcinomatosis and ovaries.
§
BRCA, breast cancer gene; ECOG, Eastern Cooperative Onocology Group; PD-L1, programmed death-ligand 1; TIL, tumor infiltrating lymphocytes.

to 64.9%), and the DCR was 71% (10/14 patients; 95% CI 41.9% to 91.6%). Percentage change in tumor size over time from baseline is shown in a spider plot (figure 2A), while largest percent change in tumor size is depicted in the waterfall plot (figure 2B). The median PFS was 4.3 months (95% CI 1.6 to 7.0) (figure 2C and table 3). The two patients who received 10 doses of ALECSAT had PFS of 17.7 and 15.2 months, respectively. The median OS for the population was 8.7 months (95% CI 5.1 to 12.4) (figure 2D and table 3) and for the two patients receiving 10 doses of ALECSAT, the OS was 23.7 and 16.9 months, respectively. Two patients were still alive at the time of data retrieval. These two patients received two and five doses of ALECSAT, respectively. The immune status of patients' metastatic biopsies, as determined by the % of TILs, was evaluated. TIL levels ranged from 0% to 90%, with eight patients exhibiting ≥5% TILs and seven patients <5%. No significant correlation was observed between TIL levels and PFS or OS. Tumors from nine patient were PD-L1 positive, four were PD-L1 negative, and two had unknown PD-L1 status. No significant correlation between PD-L1 status and PFS or OS was observed.

Evaluation of cancer-reactivity and product characteristics

The tumor reactivity of ALECSAT has previously been demonstrated in multiple preclinical settings, including cytotoxicity assays against solid tumor cell lines as well as tumor inhibition in autologous, HLA-matched PDX models.[24 25] Unlike TIL or CAR-T approaches that rely on selecting or engineering antigen-specific clones, ALECSAT is designed to generate a polyclonal T-cell and natural killer (NK)-cell response against a spectrum of tumor-associated antigens. This breadth is considered a key strength of the platform, as it may reduce the risk of immune escape compared with therapies targeting single antigens. To further characterize the ALECSAT products used in the ImmunoBreast trial, we analyzed their cellular composition by flow cytometry. Across all clinical batches (n=61 doses from 14 patients), ALECSAT comprised $CD4^+$ and $CD8^+$ T cells together with NK cells, with interpatient variability. Median frequencies and ranges, as well as data for each patient product, are provided in online supplemental table S1. To assess functional tumor reactivity, we evaluated cytotoxicity of each ALECSAT batch. Initially, products generated from healthy donors tested against MDA-MB-231 (n=17) and MDA-MB-468 (n=14), exhibited reproducible killing of breast cancer cells, although with inter-individual variability (figure 3A,B). In addition, CD107a degranulation assays performed for two clinical preparations demonstrated degranulation of $CD3^+$ T cells and $CD3^-CD56^+$ NK cells in response to tumor cell exposure, providing direct evidence of effector activation (figure 3C,D). All clinical ALECSAT products tested before patient use (n=49) exhibited tumor-reactive cytotoxicity in vitro. While the magnitude of reactivity varied between donors, all batches responded as expected with a clear dose-dependent increase in target cell killing (figure 3E,F). Together, these data demonstrate that ALECSAT products exhibit consistent cellular composition and harbor tumor-reactive effector cells when generated from both healthy donor and patient-derived preparations.

PDX models suggest patient-specific anticancer activity of ALECSAT therapy

Multiple studies have demonstrated the utility of PDX models in predicting treatment responses in patients with cancer.[27–29] Emerging studies suggest that combining PDX models with autologous immune cell transfer may provide valuable insights into immunotherapy responses, particularly in the context of ACT.[30] To further explore this, we established PDX models from metastatic core needle biopsies obtained from four patients enrolled in this trial. Each TNBC PDX model was subsequently treated with autologous ALECSAT cells derived from the corresponding patient or received no treatment (controls). Among these four patients, one achieved a CR, one had a PR, and two patients experienced SD based on RECIST V.1.1 criteria. Notably, PDX models treated with autologous ALECSAT from the CR and PR patients exhibited significant tumor growth inhibition, whereas PDX models derived from stable disease (SD) patients did not show tumor growth reduction on ALECSAT treatment (figure 3G). As the mice did not receive chemotherapy, the observed effects are attributable to ALECSAT itself. In this context, although in a very limited sample, it is noteworthy that tumor regression in PDX models coincided with objective clinical responses, suggesting that product characteristics may influence clinical activity.

Characteristics of the administered ALECSAT products and correlation with clinical outcomes

We investigated whether clinical outcomes correlated with the total number of administered ALECSAT cells or

Table 2 Safety

			ALECSAT+carboplatin/gemcitabine		
			Total, N=14		
			Any grade		Grade ≥3
Patients with any AE			14 (100)		10 (71)
Patients with SAE			5 (36)		4 (29)
Patients with treatment-related AE			14 (100)		7 (50)
Patients with AEs leading to treatment discontinuation			2 (14)		1 (7)
Patients with AEs leading to dose reduction			5 (36)		5 (36)
AEs in >10% patients by preferred term	**CTC grade**				
	1	**2**	**3**	**4**	**Total**
Laboratory					
Anemia	0	3	2	0	5
Thrombocytopenia	0	2	0	3	5
Neutropenia	0	4	1	3	8
Hypokalemia	0	1	1	0	2
Clinical					
Constitutional symptoms					
Fatigue	6	4	0	0	10
Dyspnea	4	1	0	0	5
Cough	2	2	0	0	4
Nausea	8	2	0	0	10
Fever	7	0	0	0	7
Hot flashes	2	0	0	0	2
Alopecia	2	0	0	0	2
Pain	5	7	1	0	13
Blurred vision	2	1	0	0	3
Edema	4	0	0	0	4
Tinnitus	1	1	0	0	2
Pneumonitis	0	2	0	0	2
Mucositis	4	1	0	0	5
Anorexia	4	3	0	0	7
Constipation	4	2	0	0	6
Diarrhea	5	0	0	0	5
Abdominal pain	4	1	0	0	5
Vomiting	5	0	0	0	5
Dizziness	2	2	0	0	4
Headache	4	1	0	0	5
Peripheral neuropathy	2	1	0	0	3

For grade 1–3 AEs, only those reported in at least 10% of the patients are listed in the table. All grade 4 AEs are listed.
AE, adverse event; ALECSAT, Autologous Lymphoid Effector Cells Specific Against Tumor; CTC, common terminology criteria; SAE, serious AE.

the cellular composition of ALECSAT products. A positive correlation was observed between the total administered cell number per dose and time to progression (figure 4A, r=0.57, p<0.05). Patients who achieved a PR tended to receive higher total cell doses compared with those with SD or progressive disease (PD), although this difference was not statistically significant (figure 4B). The ALECSAT products contain a heterogeneous mixture of immune cell populations, including NK cells and various T-cell subsets. Due to the small sample size, we compared the cellular composition between patients with PR and those with SD or PD. The number of administered NK cells

Table 3 Efficacy

ALECSAT+carboplatin/gemcitabine, N=14	N (%)
CR	1 (7)
PR	4 (29)
SD	5 (36)
PD	4 (29)
ORR (CR+PR) (95% CI)	5 (36) (0.13 to 0.65)
DCR (CR+PR+SD) (95% CI)	10 (71) (0.4 to 0.9)
Median PFS (95% CI), months	4.3 (1.6, 7.0)
Median OS (95% CI), months	8.7 (5.1 to 12.4)

ALECSAT, Autologous Lymphoid Effector Cells Specific Against Tumor; CR, complete response; DCR, disease control rate; OCR, objective response rate ; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease.

was not significantly correlated with time to progression. However, ALECSAT products administered to patients with PR contained significantly fewer NK cells compared with those given to patients with SD or PD (figure 4,D, p<0.05). In contrast, a significant positive correlation was observed between the total number of T cells per dose and time to progression (figure 4E, r=0.59, p<0.05). Additionally, ALECSAT products administered to patients who obtained a PR contained significantly higher total T-cell numbers (figure 4F, p<0.01). Further stratification of T-cell subsets revealed a significant positive correlation between CD8+T cell numbers per dose and time to progression (figure 4G, r=0.65, p=0.01). ALECSAT products administered to patients with PR contained significantly higher numbers of CD8+T cells compared with those with SD or PD (figure 4H, p<0.0001). No correlation was observed between the numbers of CD4+T cell per dose and time to progression. CD4+T cell numbers were similar across response groups (figure 4I,J). However, a positive correlation was detected between the number of injected double-negative (DN) T cells and time to progression (figure 4K, r=0.61, p<0.05). Despite this correlation, DN T-cell counts were similar across response groups (figure 4K,L). Taken together, these findings indicate that higher T-cell numbers per ALECSAT dose, particularly $CD8^+$ T cells, are linked to longer time to progression. In combination with the PDX data, where tumor control occurred in the absence of chemotherapy, this supports the notion that autologous ALECSAT products themselves can mediate cancer-reactive activity.

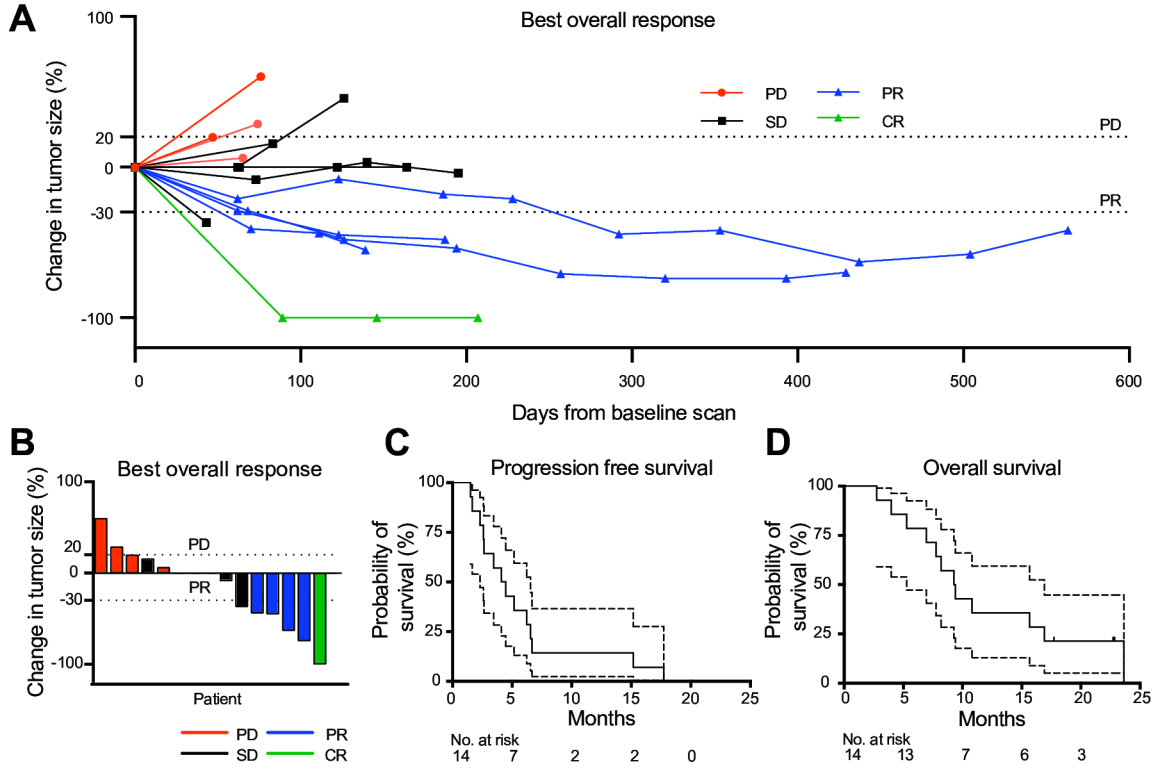

Figure 2 Antitumor activity and response duration on therapy among patients treated with ALECSAT in combination with carboplatin/gemcitabine (n=14). In (A) spider plot depicting percentage change in tumor size per RECIST V.1.1 over time from baseline scan (at start of protocol treatment). In (B) waterfall plot depicting largest percentage change in target lesion size from baseline per RECIST V.1.1. In (C) Kaplan-Meier estimates of PFS in the study population receiving ≥1 dose of ALECSAT. In (D) Kaplan-Meier estimates of OS in the study population receiving ≥1 dose of ALECSAT. Two patients were still alive at the time of data retrieval and censored. ALECSAT, Autologous Lymphoid Effector Cells Specific Against Tumor; CR, complete response; PD, progressive disease; PFS, progression-free survival; PR, partial response; RECIST, Response Evaluation Criteria In Solid Tumors; OS, overall survival; SD, stable disease.

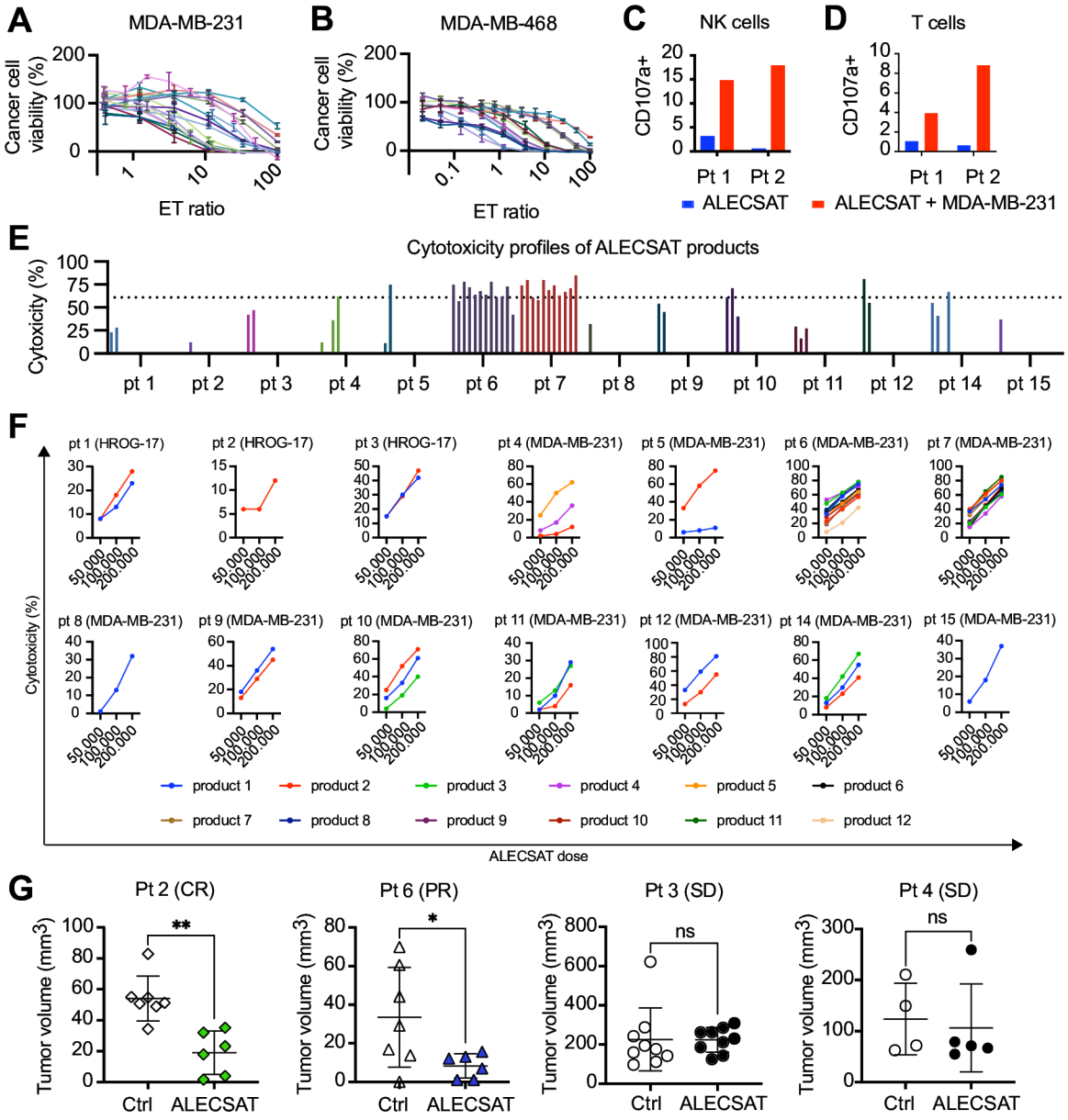

Figure 3 Cytotoxic activity of ALECSAT products in vitro and in PDX models. (A–B) ALECSAT products generated from healthy donors exhibited consistent, dose-dependent cytotoxic activity against breast cancer cell lines (MDA-MB-231 and MDA-MB-468), measured by luminescence from labeled target cells across a range of E:T ratios. (C–D) Degranulation assays with patient-derived ALECSAT showed induction of CD107a expression in NK cells and T cells following co-culture with MDA-MB-231 target cells. (E) Cytotoxicity profiles of clinical ALECSAT batches tested against tumor cell lines (patients 1–3 against HROG17; all other patients against MDA-MB-231), assessed by real-time impedance measurements (iCELLigence), revealed variability between patients; the dotted line indicates median cytotoxicity across all products. (F) Dose-response analyses of patient-derived ALECSAT batches, also measured by impedance, demonstrated increasing cytotoxicity with escalating effector cell doses. (G) PDX models established from trial patients were treated with autologous ALECSAT products in the absence of chemotherapy. Tumor regression was observed in models derived from patients with CR or PR, whereas models from patients with SD showed no significant growth inhibition. Mice bearing tumors from patients 2, 3, 4, and 6 were sacrificed on days 38, 130, 28, and 28, respectively. Diamonds represent patients with CR, triangles represent patients with PR, and circles represent patients with SD. Open symbols indicate untreated mice, while closed symbols represent mice treated with ALECSAT. Statistical differences were determined using unpaired t-tests (*p<0.05, **p<0.01; ns, not significant). ALECSAT, Autologous Lymphoid Effector Cells Specific Against Tumor; CR, complete response; E:T, effector-to-target; NK, natural killer; PD, progressive disease; PDX, patient-derived xenograft; PR, partial response; SD, stable disease.

DISCUSSION

ACT therapy with cancer-directed immune cells has emerged as a promising therapeutic strategy. In contrast to TIL-cell and CAR T-cell therapies, which are constrained by antigen selection or genetic engineering, ALECSAT generates NK-cell and a broad, polyclonal T-cell response. This feature may reduce the risk of tumor immune escape and potentially extend ACT applicability

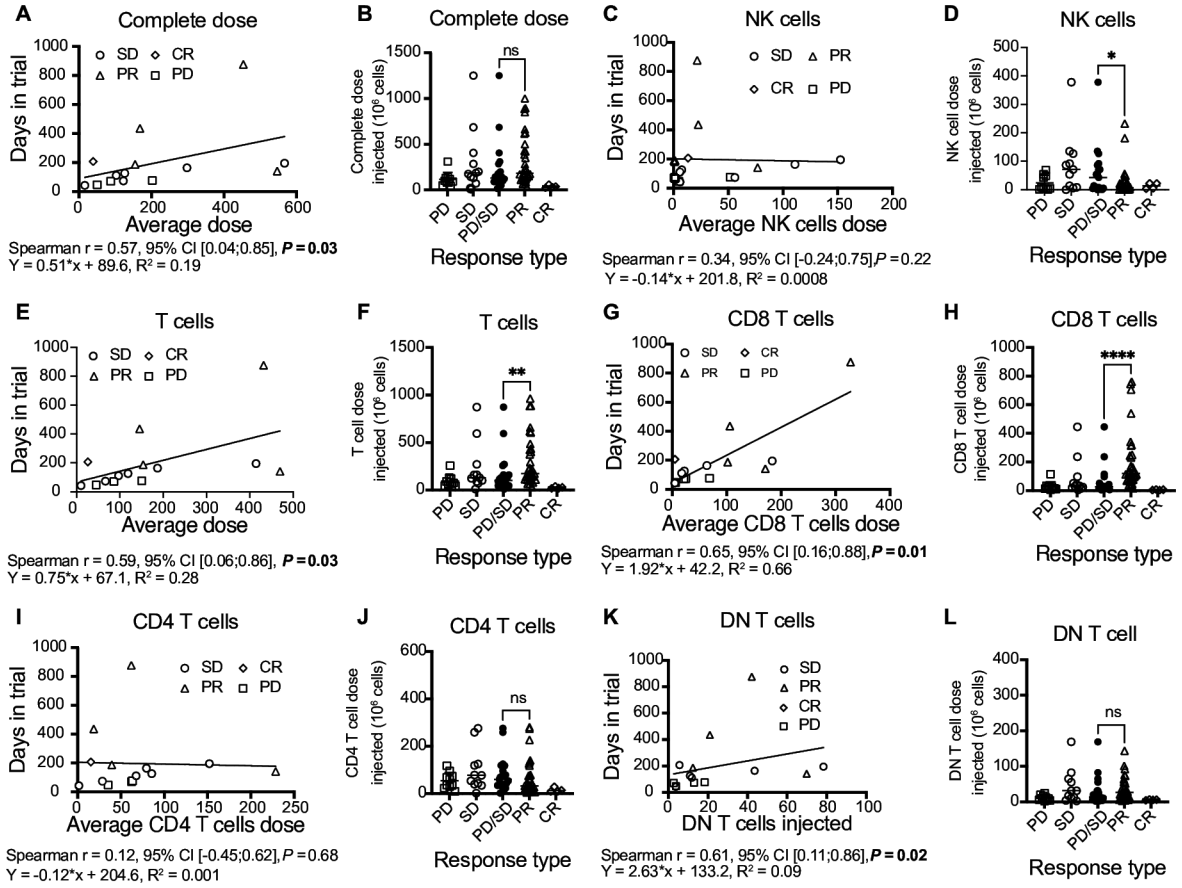

Figure 4 Patient outcomes correlate with higher T cell and CD8+T cell amounts in the administered ALECSAT preparations. Total administered cell numbers and the cellular composition of ALECSAT products were analyzed in relation to patient outcomes. (A) A significant positive correlation was observed between the total administered cell number per dose and time to progression (Spearman r=0.57, p<0.05). (B) Patients achieving a PR tended to receive higher total cell doses than patients with SD or PD, although differences were not statistically significant. (C–D) The number of NK cells per dose did not correlate with time to progression; however, ALECSAT products administered to PR patients contained significantly fewer NK cells compared with those given to patients exhibiting SD/PD. (E–F) In contrast, the total number of T cells per dose showed a positive correlation with time to progression (r=0.59, p<0.05), and products given to patients exhibiting PR contained significantly higher numbers of T cells than those with SD/PD. (G–H) Stratification of T-cell subsets revealed that $CD8^+$ T-cell numbers per dose were strongly correlated with time to progression (r=0.65, p=0.01) and were significantly higher in PR patients exhibiting PR compared to those with SD/PD. (I–J) $CD4^+$ T-cell numbers did not correlate with time to progression and were similar across response groups. (K–L) Numbers of DN T cells correlated positively with time to progression (r=0.61, p<0.05), but absolute counts did not differ significantly between response groups. Overall, higher numbers of administered T cells, particularly $CD8^+$ T cells, were associated with prolonged time to progression, supporting a central role of the T-cell compartment in ALECSAT activity. Statistical comparisons were performed using unpaired t-tests; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant. ALECSAT, Autologous Lymphoid Effector Cells Specific Against Tumor; CR, complete response; DN, double-negative; NK, natural killer; PD, progressive disease; PR, partial response; SD, stable disease.

to patients where TIL expansion is challenging. Within the landscape of adoptive immunotherapy, ALECSAT therefore represents a complementary strategy, with early signs of efficacy supporting further clinical evaluation. This phase 1b trial evaluated the safety, tolerability, and signs of efficacy of ALECSAT in combination with chemotherapy in patients with mTNBC, demonstrating encouraging results. These findings contribute to the growing clinical interest in immune cell-based adoptive cell therapies, highlighting their potential role in the oncology treatment landscape.

The combination of ALECSAT and chemotherapy demonstrated a manageable safety profile, with primarily hematologic toxicities, in this heavily pretreated mTNBC population. Common AEs, such as fatigue, nausea, and hematological abnormalities, were consistent with the expected toxicity profiles of carboplatin and gemcitabine. A total number of seven SAEs was reported, including back pain, hypokalemia, and pneumonitis. While two patients discontinued treatment due to toxicity, the absence of life-threatening complications or treatment-related deaths is encouraging. These findings suggest that ALECSAT can be safely integrated into standard chemotherapy regimens. To minimize potential negative effects of chemotherapy on the administrated ALECSAT cells, chemotherapy was withheld for 3 days before and 3 days after ALECSAT administration.

The observed clinical responses, including an ORR of 36% and DCR of 71%, are notable in this pretreated mTNBC population. The median PFS of 4.3 months and OS of 8.7 months are comparable to or exceed historical benchmarks for this patient population. The efficacy of gemcitabine and carboplatin treatment in late-line mTNBC remains incompletely characterized. However, in first-line mTNBC, this regimen has demonstrated an ORR of 30.2%, a median PFS of 4.6 months, and a median OS of 12.6 months.[5] While direct comparisons between first-line and late-line settings are challenging, the observed survival outcomes in this study suggest that the addition of ALECSAT to chemotherapy warrants further investigations. The two patients who received 10 doses of ALECSAT achieved prolonged survival, with PFS of 17.7 and 15.2 months, and OS of 23.7 and 16.9 months, respectively.

In this study, a subset of patients exhibited longer-than-expected clinical responses. Similar observations were made in a phase I trial evaluating ALECSAT in advanced glioblastoma,[24] where some patients demonstrated prolonged survival beyond historical benchmarks. Whether ALECSAT directly contributes to these durable responses requires confirmation in a randomized controlled trial. Our exploratory analysis further suggests that clinical outcome was associated with product composition, in particular the presence of $CD8^+$ T cells. This is consistent with the central role of cytotoxic $CD8^+$ T cells as key effectors of antitumor immunity, and with our functional assays demonstrating tumor reactivity across patient-derived products. Although limited by size, these observations point to strategies that selectively enrich for CD8+T cells as a potential means to enhance ALECSAT therapy.

PDX models were successfully generated from metastatic biopsies and treated with autologous ALECSAT. To preserve tumor architecture, heterogeneity, and stromal interactions, intact tumor fragments were engrafted, which provides a more biologically realistic and resistant setting than cell suspensions. ALECSAT was administered at the time of implantation, a strategy that in our prior work proved most reliable for achieving consistent engraftment of both tumor and immune cells.[25] Although this differs from the conventional practice of treating established tumors, early intervention models are commonly used in preclinical research to evaluate therapeutic activity during tumor outgrowth. Tumors initially expanded in size in nearly all animals, indicating that the experiments represented an early therapeutic rather than a prophylactic setting. As spontaneous metastases rarely occur in PDX models, metastatic dissemination could not be assessed; instead, effects on metastasis were previously demonstrated in complementary xenograft models, where ALECSAT significantly inhibited both spontaneous and experimental metastases.[25] No chemotherapy was administered, underscoring that the observed anticancer effect was attributable to the administrated immune cells. The concordance between responses observed in the patient and their corresponding PDX models indicates that such models may provide insights into patient-specific responsiveness. This was exemplified by PDX derived from patients with CR or PR, which demonstrated growth inhibition when treated with autologous ALECSAT, in contrast to PDX from patients with SD, which showed little or no measurable response.

In preclinical models, including TNBC cell line xenografts and PDX models, we recently observed that combined anti-PD-L1 therapy with ALECSAT resulted in robust anticancer responses.[25] This combination led to a significant reduction in primary tumor expansion, inhibition of metastasis and prolonged survival, superior to treatment with ALECSAT alone. These findings highlight PD-L1 upregulation as one potential compensatory mechanism of immune evasion; however, it is likely that additional suppressive pathways and cell populations in the tissue microenvironment also contribute. Identifying such mechanisms will be important for guiding rational ALECSAT-based combination strategies going forward. Based on our current data and the observed predictive value of the PDX model, a trial evaluating the safety and sign of efficacy of ALECSAT in combination with anti-PD-L1 therapy is warranted.

CONCLUSION

This study provides preliminary evidence that ALECSAT, in combination with chemotherapy, is a feasible and potentially effective treatment approach for patients with mTNBC. While the small cohort size and exploratory design limit definitive conclusions, the favorable safety profile and observed clinical responses support further investigation in larger, controlled clinical trials. Our findings suggest that clinical outcomes in ALECSAT-treated patients are strongly correlated with the composition of the administered cell product, particularly the presence of CD8+T cells. This underscores the potential role of specific immune cell populations in mediating therapeutic effects and highlights the importance of optimizing manufacturing strategies to enhance treatment efficacy. These results lay a foundation for future research aimed at refining ALECSAT therapy and evaluating its broader application in advanced cancer treatment. Further investigation into the mechanistic basis of these findings will be essential for translating these insights into improved immunotherapeutic strategies.

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Competing interests AFK is a co-founder and was an employee of Cytovac A/S, a biotechnology company that commercializes the ALECSAT therapy, during the study. AFK is a co-inventor on a patent on ALECSAT and owns warrants and stock in Cytovac A/S. OLG, AFK and HJD are co-inventors of a patent application related to combined ALECSAT and anti-PDL1 therapy. The other authors declare no conflicts of interest.

Patient consent for publication Not applicable.

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