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Combination Adoptive Cell Transfer Immunotherapy and Chemotherapy in Advanced Triple-Negative Breast Cancer: Phase Ib Results from the ImmunoBreast Trial

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ABSTRACT

Adoptive cellular immunotherapy has emerged as a potential treatment modality for advanced malignancies, yet its broader clinical application is constrained by difficulties in producing large quantities of immune cells with diverse tumor-recognition capabilities. To address this limitation, we developed Autologous Lymphoid Effector Cells Specific against Tumor (ALECSAT), a personalized approach that generates functionally mature, polyclonal lymphocytes from peripheral blood with antitumor activity. This phase Ib, single-institution study explored the safety profile, feasibility, and early signals of clinical benefit associated with ALECSAT administered alongside carboplatin and gemcitabine in patients with locally advanced or metastatic triple-negative breast cancer (mTNBC). A total of fifteen patients diagnosed with mTNBC were enrolled. ALECSAT was infused at 4-week intervals for the first three administrations, followed by maintenance dosing every six weeks. Standard chemotherapy with carboplatin and gemcitabine was delivered on days 1 and 8 of repeated 21-day cycles. Phenotypic characterization of infused cellular products was performed using multiparameter flow cytometry. In parallel, tumor biopsies were used to establish patient-derived xenograft (PDX) models, which were subsequently treated with ALECSAT to evaluate concordance with clinical outcomes. Fourteen patients who had previously undergone one to four systemic treatment regimens received between one and ten ALECSAT infusions. The combined treatment regimen demonstrated acceptable tolerability, with adverse events largely attributable to chemotherapy rather than cell therapy. Frequently reported toxicities included asthenia, gastrointestinal symptoms, and hematologic suppression. Severe adverse events (grade ≥3) were primarily hematologic, consisting mainly of transient neutropenia and thrombocytopenia. Antitumor activity was observed, with one patient achieving complete tumor regression, four exhibiting partial responses, five maintaining disease stabilization, and four experiencing disease progression. The resulting objective response rate was 36% (95% CI 12.8–64.9%). Median progression-free survival was 4.3 months (95% CI 1.6–7.0), while median overall survival reached 8.7 months (95% CI 5.1–12.4). Improved clinical outcomes were associated with higher cumulative numbers of infused ALECSAT cells, particularly cytotoxic $CD8^+$ T lymphocytes. Notably, therapeutic responses in patients were reflected by treatment effects observed in matched PDX models. The administration of ALECSAT in combination with platinum-based chemotherapy was feasible and exhibited an acceptable safety profile in patients with mTNBC, with preliminary evidence of antitumor efficacy. These findings provide a rationale for further clinical development of ALECSAT in larger trials.

Keywords: Cell transfer, Immunotherapy, Chemotherapy, Breast cancer

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Introduction

Triple-negative breast cancer (TNBC) lacks hormone receptors and human epidermal growth factor receptor 2 (HER2), making it a highly aggressive type that represents 10–15% of breast cancer diagnoses. Those with inoperable advanced or metastatic TNBC (mTNBC) typically face limited survival, averaging 12 to 18 months [1, 2]. Systemic treatment has long relied on chemotherapy, with agents like taxanes or anthracyclines as initial options [3]. Yet, these often yield only temporary benefits, and prior exposure in early-stage care can reduce their

value in later stages. Gemcitabine combined with carboplatin stands as a guideline-recommended frontline choice for mTNBC, per the European Society for Medical Oncology [3, 4]. Trials show this duo achieves a 30.2% objective response rate (ORR), 4.6-month median progression-free survival (PFS), and 12.6-month median overall survival (OS) [5].

Many TNBC cases show immune-related traits, like elevated tumor mutational load and abundant tumor-infiltrating lymphocytes (TILs), but rapid advancement still leads to unfavorable results [6–8]. This aligns with frequent high levels of programmed death-ligand 1 (PD-L1) in TNBC, signaling a suppressed immune environment. Blocking PD-1 or PD-L1 checkpoints has offered some hope but modest results in mTNBC, with response rates under 20% overall. In PD-L1-positive cases (combined positive score ≥10), adding pembrolizumab to chemotherapy markedly boosts PFS and OS (23.0 versus 16.1 months; HR 0.73; 95% CI 0.55–0.95; p=0.0185) [9, 10], earning FDA and EMA approvals. Atezolizumab with nab-paclitaxel also gained initial FDA and EMA nods for PD-L1-positive mTNBC, but no clear OS gain in the full population led to FDA withdrawal after confirmatory data fell short [11]. Based on KEYNOTE-522's success, pembrolizumab plus chemotherapy is now standard for neoadjuvant use in stage II–III early TNBC, with adjuvant pembrolizumab following.

Recent approvals include targeted agents like the anti-Trop2 antibody-drug conjugate (ADC) sacituzumab govitecan (linked to SN-38, irinotecan's active form) and the HER2 ADC trastuzumab deruxtecan, backed by ASCENT and DESTINY-Breast04 phase III data [12, 13]. PARP inhibitors are also available for the ~20% of TNBC patients with germline BRCA1/2 mutations [14].

Adoptive cell transfer (ACT) therapy taps into the patient's immune defenses to combat cancer by transferring activated cells [15, 16]. Cells like cytotoxic T lymphocytes (CTLs) detect tumor antigens, prompting cell destruction or cytokine release. Despite their presence in many cancers, CTLs often face suppression in the tumor setting [17]. Activating and growing these cells outside the body before reinfusion shows strong potential. ACT includes TILs and engineered T cells with custom receptors or chimeric antigen receptors (CARs) [18–21]. TIL therapy has proven highly effective in trials [22], but reliable tumor-specific TILs are mainly from melanoma [23]. This highlights the need to refine cellular therapies for wider use, especially in hard-to-treat cancers like mTNBC.

ALECSAT, or Autologous Lymphoid Effector Cells Specific against Tumor, is a tailored ACT using the patient's lymphocytes. Unlike methods needing specific T-cell selection or engineering, ALECSAT uses epigenetically altered antigen-presenting cells to grow varied T-cell groups targeting tumors. Lab studies confirm its action against breast cancer lines and interferon-γ responses to antigens like MAGE-A, GAGE, and CTAG1 [24, 25].

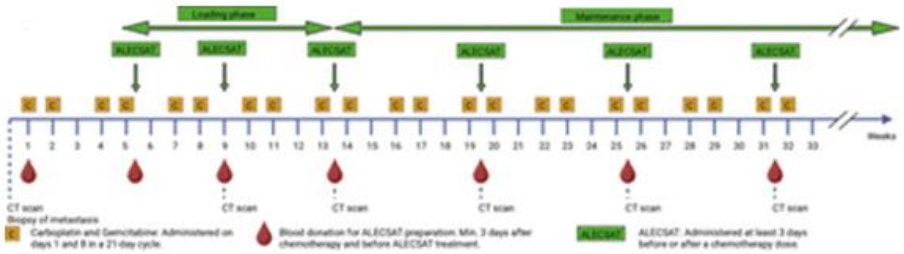
This phase Ib trial tested ALECSAT alongside gemcitabine-carboplatin in mTNBC patients. Key goals were evaluating safety and practicality. Other measures tracked early efficacy signs: ORR, duration of response (DOR), disease control rate (DCR), PFS, and OS. We also examined the ALECSAT product for links between cell types and outcomes. Patient-derived xenograft (PDX) models from biopsies helped predict responses.

Materials and Methods

Trial design

Conducted as a phase Ib, single-site, open-label, single-arm, investigator-led study at Odense University Hospital's Oncology Department in Denmark from May 15, 2020, to December 29, 2023 (ClinicalTrials.gov: NCT00891345).

Test safety and tolerability of ALECSAT added to standard gemcitabine-carboplatin in women with confirmed inoperable advanced or mTNBC, limited to ≤4 prior metastatic treatments. Treatment timeline shown in **Figure 1a**. Secondary aims: Assess efficacy trends via ORR, DOR, DCR, PFS, and OS per RECIST v1.1 [26]. Exploratory: Compare ORR, DOR, PFS using immune-modified RECIST 1.1 alongside standard RECIST v1.1, via local radiology. Also, identify response predictors for ALECSAT and test its effects in PDX models from patients' tumors.



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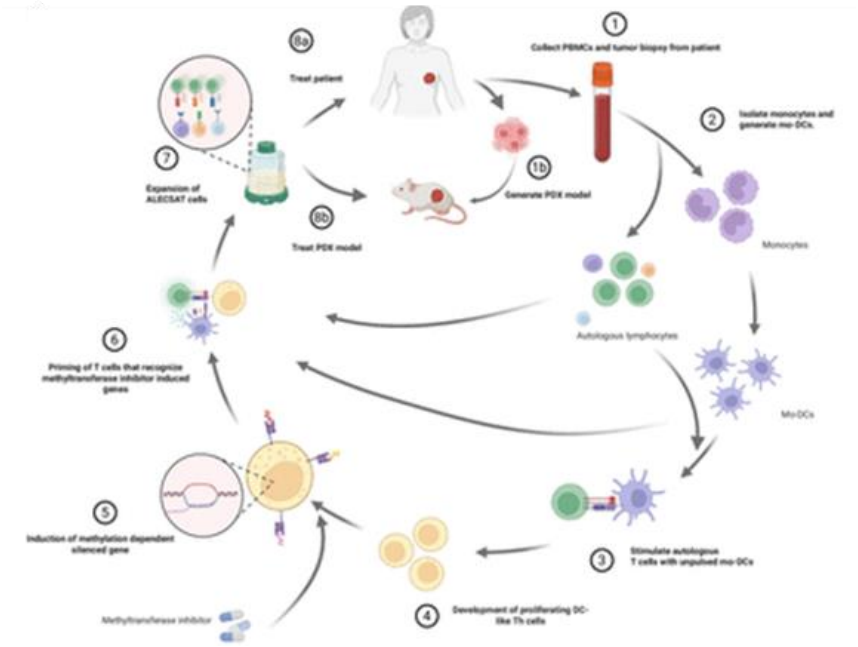


b)

Figure 1. Study overview and experimental workflow.
(a) Timeline of therapeutic interventions shown in weeks. Patients received six sequential cycles of carboplatin and gemcitabine beginning at study initiation (week 0). ALECSAT administration consisted of an initial induction phase with three infusions delivered at 28-day intervals (±3 days), followed by a maintenance phase in which ALECSAT was administered every six weeks (±1 week).
(b) Diagram outlining the stepwise manufacturing process of ALECSAT from patient-derived peripheral blood samples, together with the establishment of corresponding patient-derived xenograft (PDX) models. Illustrations were generated using BioRender.com. PDX, patient-derived xenograft; DC, dendritic cell; ALECSAT, Autologous Lymphoid Effector Cells Specific against Tumor.

Study therapy was maintained until treatment discontinuation criteria were met, including the occurrence of unacceptable adverse effects, radiographic or clinical disease progression, or a decision by the patient or investigator to stop treatment. Patients who discontinued chemotherapy because of toxicity or other considerations were permitted to continue receiving ALECSAT alone at the discretion of the treating investigator.
Tumor response was evaluated in accordance with Response Evaluation Criteria in Solid Tumors version 1.1 at 9-week intervals during the first year of treatment, followed by assessments every 12 weeks thereafter. Adverse events were documented and graded using the National Cancer Institute Common Terminology Criteria for Adverse Events version 5.0.

Autologous lymphoid effector cells specific against tumor
Second-generation Autologous Lymphoid Effector Cells Specific against Tumor (ALECSAT) were produced from patients' own peripheral blood at Cytovac A/S (Hørsholm, Denmark). The initial manufacturing process used 300 mL of blood, while subsequent collections consisted of 200 mL. At each blood draw, an additional 20 mL sample was obtained to generate autologous serum for cryopreservation purposes. ALECSAT production involved a multistep protocol that included differentiation of dendritic cells (DCs), stimulation of lymphocytes through DC

co-culture in the presence of interleukin-2 (IL-2), epigenetic modulation using the demethylating agent 5-aza-2′-deoxycytidine (5-aza-CdR), and a final expansion phase with newly prepared DCs and lymphocytes **(Figure 1b).** For DC generation, peripheral blood mononuclear cells (PBMCs) were cultured in AIM-V medium following preconditioning of culture flasks with RPMI-1640 supplemented with 5% human AB serum. After monocyte adherence, non-adherent lymphocytes were collected and cryopreserved, while adherent monocytes were further differentiated. 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, maturation was induced by supplementation with IL-1β (10 ng/mL), IL-6 (1000 IU/mL), tumor necrosis factor-α (10 ng/mL), and prostaglandin E_2 (0.2 µg/mL). Mature DCs were harvested on day 6, enumerated, and either used immediately or cryopreserved.

Autologous serum was prepared from plasma obtained during lymphoprep centrifugation. Plasma was collected after an initial low-speed centrifugation (200 g, 20 min), followed by a second centrifugation step (460 g, 20 min). Clotting was induced by adding $CaCl_2$ (1 mM), and samples were incubated overnight. Serum was separated, frozen for at least 24 hours, thawed, centrifuged at 2,000 g for 15 min, and the clarified supernatant was collected for use in cell culture.

Previously cryopreserved lymphocytes were thawed and co-cultured with DCs at a 10:1 ratio in AIM-V medium supplemented with 1% autologous serum. IL-2 (25 IU/mL) was introduced on day 7 and replenished during medium changes on days 10 and 12. On day 13, cells were harvested and exposed to IL-2 (150 IU/mL) together with 5-aza-CdR (10 µM) for 48 hours. Following epigenetic treatment, cells were combined with thawed lymphocytes and DCs at a 10:10:1 ratio and expanded further in AIM-V containing 2% autologous serum. IL-2 supplementation resumed on day 17, with additional medium exchanges on days 20, 22, and 24. On day 26, a portion of the culture was allocated for immunophenotyping, while the remaining cells were formulated for clinical use.

Each ALECSAT infusion consisted of 1×10^7 to 1×10^9 viable cells suspended in 20 mL Plasma-Lyte containing 5% autologous serum. Patients received three infusions during an induction phase between weeks 5 and 13 at four-week intervals, followed by maintenance dosing every six weeks until treatment discontinuation **(Figure 1a).** Cell composition was assessed by flow cytometry using antibodies targeting CD3, CD4, CD8, and CD56.

Chemotherapy

Patients received carboplatin (AUC 2) and gemcitabine (1,000 mg/m²) on days 1 and 8 of each 21-day treatment cycle, in accordance with institutional standards.

Patients

Eligible participants were women aged 18 years or older with metastatic TNBC treated at Odense University Hospital, Denmark. Inclusion criteria required histologically verified, unresectable locally advanced or metastatic TNBC not suitable for curative therapy, eligibility for carboplatin and gemcitabine chemotherapy, ECOG performance status of 0–1, and a projected life expectancy of at least three months. Patients were required to provide a fresh tumor biopsy from a recurrent or metastatic site and blood samples for biomarker analyses. At least one measurable lesion per RECIST V.1.1 was mandatory.

TNBC status was defined by absence of estrogen receptor and HER2 expression in tumor tissue. Patients with prior hormone receptor-positive or HER2-positive disease were required to undergo re-biopsy to confirm TNBC at recurrence. Adequate organ function was assessed within 10 days before treatment initiation, including hematologic, renal, and hepatic parameters. Women of reproductive potential were required to have a negative pregnancy test and to use effective contraception during the study period. Tumor immune infiltration was evaluated on H&E-stained sections by an experienced breast pathologist, and PD-L1 expression was determined using the Ventana SP142 assay.

Generation of patient-derived xenograft models

Female NOG mice were anesthetized, and tumor implantation was performed into the fourth mammary fat pad. An extracellular matrix gel containing 5×10^6 ALECSAT cells was injected, followed by implantation of a patient-derived tumor fragment (~8 mm³). Surgical closure was performed using internal and external sutures. Tumors were excised at study termination and weighed using a digital balance.

Animal experiments

All in vivo experiments were conducted under approved ethical guidelines at the University of Southern Denmark animal facility. Animals were housed in pathogen-free conditions with controlled temperature, humidity, and light cycles, and provided food and water ad libitum. Mice were euthanized when tumor diameter reached 1.2 cm or upon signs of graft-versus-host disease.

Co-culture studies

Tumor cells were plated in 96-well plates and allowed to adhere prior to addition of effector cells. After 24 hours of co-culture, cell viability was determined by luminescence following D-luciferin addition. Tumor cell survival was calculated relative to untreated controls.

Degranulation assays

Effector and tumor cells were co-incubated in the presence of GolgiStop with or without anti-CD107a antibody. After five hours, cells were harvested, stained, and analyzed by flow cytometry.

Real-time cytotoxicity assays

Tumor cell killing was continuously monitored using the iCELLigence platform. Cancer cells were seeded and allowed to adhere before addition of graded doses of lymphocytes. Changes in impedance were recorded and expressed as normalized cell index values, from which cytotoxic activity was calculated.

Statistics

All statistical analyses were performed using GraphPad Prism. Safety and efficacy outcomes were summarized descriptively. ORR and DCR were reported with exact two-sided 95% confidence intervals. Time-to-event endpoints were estimated using Kaplan–Meier methodology. Correlations between ALECSAT cell populations and clinical outcomes were evaluated using Spearman's rank correlation, while group comparisons employed the Mann–Whitney U test.

Results and Discussion

Study population

From May 15, 2020, to August 25, 2023, fifteen patients with metastatic TNBC were enrolled. One patient discontinued prior to receiving ALECSAT due to rapid clinical deterioration. The remaining fourteen patients received between one and ten ALECSAT infusions. At the time of analysis, all participants had completed or discontinued treatment. Baseline demographic and clinical characteristics are summarized in **Table 1.**

Table 1. Patient characteristics

Characteristic	Value
Median age, years (IQR)	49 (37–64)
Total patients	N=15 (100%)
Disease presentation	
Recurrent metastatic	9 (60%)
De novo metastatic	6 (40%)
ECOG performance status	
0	9 (60%)
1	6 (40%)
≥2	0 (0%)
Disease-free interval* (among recurrent cases, n=9)	
≥12 months	5 (56%)
<12 months	4 (44%)
Prior systemic therapies in metastatic setting	(Percentages may exceed 100% due to multiple therapies per patient)
Taxane	8 (21%)
Anthracycline	4 (10%)
Vinorelbine	3 (8%)

Capecitabine	7 (18%)
Eribulin	6 (15%)
Anti-PD-L1 immunotherapy	4 (10%)
Cyclophosphamide	4 (10%)
Other†	3 (8%)
Number of metastatic sites	
≤2	8 (53%)
>2	7 (47%)
Sites of metastases	(Percentages exceed 100% due to multiple sites per patient)
Opposite breast	2 (6%)
Skin	2 (6%)
Lung	6 (18%)
Distant lymph nodes	5 (15%)
Central nervous system	1 (3%)
Liver	3 (9%)
Bone	4 (12%)
Regional lymph nodes	8 (24%)
Other‡	3 (9%)
Number of prior lines of therapy (advanced setting)	
1	7 (47%)
2	5 (33%)
4	3 (20%)
Tumor-infiltrating lymphocytes (% TILs)	
≥5%	8 (53%)
<5%	7 (47%)
Germline BRCA1/2 mutational status	
No mutation	8 (53%)
Mutation present	2 (13%)
Unknown	5 (33%)
PD-L1 status	
Positive (≥1%)	9 (60%)
Negative (<1%)	4 (27%)
Unknown	2 (13%)

*Defined as the interval from the date of primary tumor resection to the date of disease recurrence. Percentages are calculated among patients with recurrent metastatic disease.

† Other prior therapies include endocrine treatment, CDK4/6 inhibitors, and cases where it is unknown whether patients received blinded treatment as part of a clinical trial.

‡ Other metastatic sites include ovaries and peritoneal carcinomatosis.

§

ECOG, Eastern Cooperative Onocology Group; BRCA, breast cancer gene; TIL, tumor infiltrating lymphocytes; PD-L1, programmed death-ligand 1.

The median patient age was 49 years, and all enrolled individuals had an Eastern Cooperative Oncology Group (ECOG) performance status of either 0 or 1 at baseline. Metastatic disease at study entry was recurrent in nine patients, of whom five experienced a disease-free period of at least 12 months following initial treatment before relapse. Six patients presented with metastatic disease at first diagnosis. Prior to enrollment, the majority of patients (80%) had received one or two previous systemic treatment regimens for mTNBC, whereas a smaller subset (20%) had undergone four prior lines of therapy. Treatment histories predominantly consisted of chemotherapy; four patients had also been exposed to the anti–PD-L1 antibody atezolizumab. No patients had previously received anti–PD-1 therapy with pembrolizumab.

Feasibility and safety

The combined administration of ALECSAT with carboplatin and gemcitabine was generally well tolerated. The observed toxicity profile aligned with the known adverse effects associated with the chemotherapy backbone. As outlined in **Table 2**, the most frequently reported adverse events included fatigue, nausea, and pain. Severe (grade ≥3) toxicities were mainly hematological in nature, with neutropenia and thrombocytopenia occurring most often.

The median duration of grade ≥3 neutropenia was 7 days, with a range of 6 to 10 days. In total, seven serious adverse events were documented, comprising back pain, hypokalemia, thrombocytopenia, urinary tract infection, and pneumonitis. Adverse events led to permanent treatment discontinuation in two patients, while dose adjustments of chemotherapy were required in five patients. No treatment-related deaths or grade 5 adverse events were reported.

Table 2. Safety

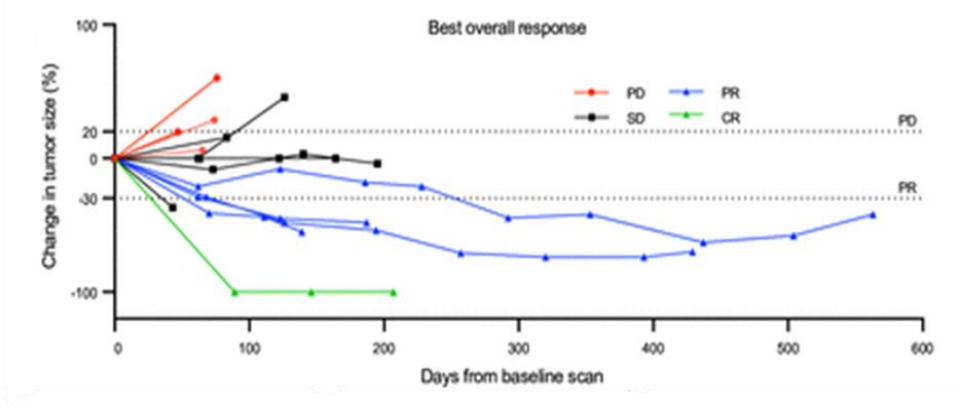
			ALECSAT+carboplatin/gemcitabine		
			Total, N=14		
			Any grade		Grade ≥3
Patients with SAE			5 (36)		4 (29)
Patients with any AE			14 (100)		10 (71)
Patients with AEs leading to treatment discontinuation			2 (14)		1 (7)
Patients with treatment-related AE			14 (100)		7 (50)
Patients with AEs leading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

Only adverse events of grades 1–3 occurring in ≥10% of patients are included in the table, whereas all grade 4 events are reported regardless of frequency. SAE, serious adverse event; CTC, Common Terminology Criteria; ALECSAT, Autologous Lymphoid Effector Cells Specific against Tumor; AE, adverse event.

Clinical activity

Treatment responses in the 14 patients with metastatic TNBC receiving ALECSAT together with carboplatin and gemcitabine are summarized in **Table 3** and were evaluated using RECIST V.1.1 criteria. Objective tumor regression was observed in five patients: one patient (7%) achieved a complete radiologic remission, and four patients (29%) demonstrated partial tumor shrinkage. This resulted in an overall response rate of 36% (5/14; 95% CI 12.8%–64.9%). When patients with stable disease were included, the disease control rate reached 71% (10/14; 95% CI 41.9%–91.6%). Individual tumor dynamics over the course of treatment are presented **in Figure**

2a, showing percentage change from baseline over time, while the greatest reduction in tumor size per patient is illustrated in the waterfall plot **(Figure 2b).** Across the study population, the median progression-free survival was 4.3 months (95% CI 1.6–7.0) **(Figure 2c and Table 3).** Markedly longer disease control was observed in the two patients who received the maximum number of ALECSAT infusions (10 doses), with progression-free survival times of 17.7 and 15.2 months, respectively. Median overall survival for all treated patients was 8.7 months (95% CI 5.1–12.4) (**Figure 2d and Table 3).** In contrast, the two patients receiving prolonged ALECSAT treatment exhibited overall survival durations of 23.7 and 16.9 months. At the time of analysis, two patients remained alive; these individuals had received two and five ALECSAT doses, respectively. Baseline immune characteristics were assessed using metastatic tumor biopsies. The proportion of tumor-infiltrating lymphocytes varied widely, ranging from complete absence to 90%. Eight patients displayed TIL levels ≥5%, whereas seven had low infiltration (<5%). Statistical analyses did not reveal any association between TIL abundance and either progression-free or overall survival. With respect to PD-L1 expression, nine tumors were classified as PD-L1 positive, four as PD-L1 negative, and PD-L1 status was unavailable in two cases. No meaningful relationship was identified between PD-L1 expression and clinical outcomes, including PFS and OS.



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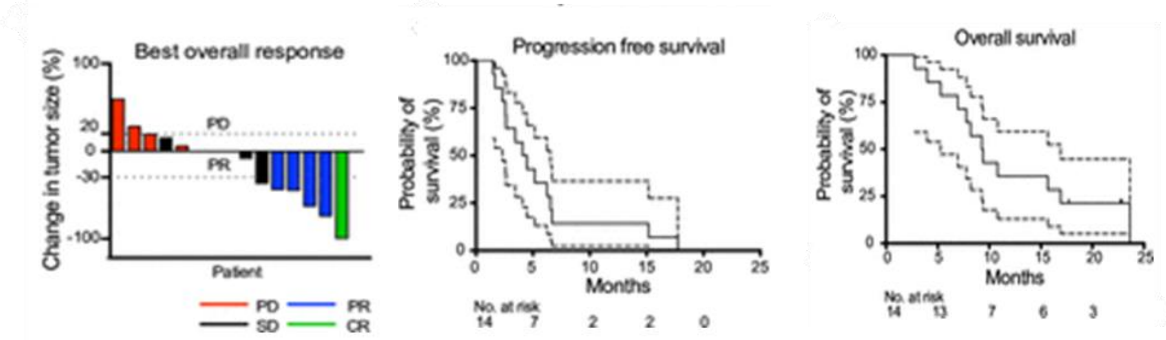


b) c) d)

Figure 2. Tumor response patterns and durability of clinical benefit in patients treated with ALECSAT combined with carboplatin and gemcitabine (n=14). Panel (a) shows a spider plot illustrating longitudinal percentage changes in tumor burden from baseline, assessed according to RECIST V.1.1 at the start of study treatment. Panel (b) presents a waterfall plot summarizing the maximum relative change in target lesion size for each patient based on RECIST V.1.1 criteria. Panel (c) displays Kaplan–Meier curves for progression-free survival among patients who received at least one ALECSAT infusion. Panel (d) depicts Kaplan–Meier estimates of overall survival in the same population. At the time of analysis, two patients remained alive and were censored in the survival analyses. OS, overall survival; PFS, progression-free survival; RECIST, Response Evaluation Criteria in Solid Tumors; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ALECSAT, Autologous Lymphoid Effector Cells Specific Against Tumor.

Table 3. Efficacy

Response Category	**N=14**
Partial Response (PR)	4 (29%)

Stable Disease (SD)	5 (36%)
Complete Response (CR)	1 (7%)
Progressive Disease (PD)	4 (29%)
Disease Control Rate (DCR; CR + PR + SD) (95% CI)	10 (71%) (0.42 to 0.90)
Objective Response Rate (ORR; CR + PR) (95% CI)	5 (36%) (0.13 to 0.65)
Median Overall Survival (OS) (95% CI), months	8.7 (5.1 to 12.4)
Median Progression-Free Survival (PFS) (95% CI), months	4.3 (1.6 to 7.0)

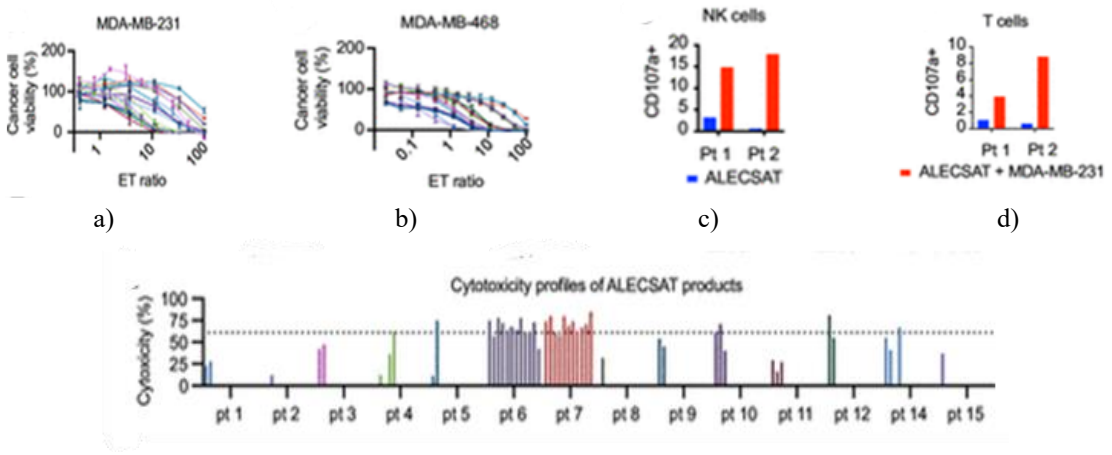
CR, complete response; ALECSAT, Autologous Lymphoid Effector Cells Specific Against Tumor; OCR, objective response rate ; DCR, disease control rate; PD, progressive disease; OS, overall survival; PR, partial response; SD, stable disease; PFS, progression-free survival.

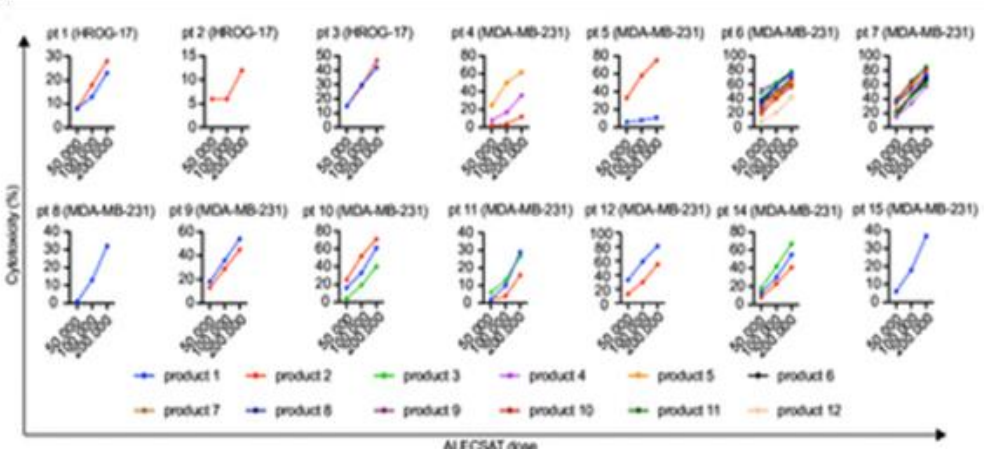
Assessment of tumor reactivity and product features

Antitumor activity of ALECSAT has been established previously in a range of preclinical models, including in vitro cytotoxicity assays against solid tumor cell lines and suppression of tumor growth in autologous, HLA-matched patient-derived xenograft systems [24, 25]. In contrast to TIL- or CAR-T–based strategies, which depend on the isolation or genetic modification of antigen-specific lymphocyte clones, ALECSAT is intended to induce a broad, polyclonal immune response involving both T cells and natural killer (NK) cells directed against multiple tumor-associated antigens. This multi-antigen targeting strategy is viewed as a major advantage, as it may limit tumor immune escape mechanisms commonly associated with single-antigen therapies.

To further define the characteristics of the ALECSAT products administered in the ImmunoBreast trial, we performed immunophenotypic profiling using flow cytometry. Analysis of all manufactured clinical products (61 infusions derived from 14 patients) revealed that ALECSAT consisted predominantly of $CD4^+$ and $CD8^+$ T lymphocytes, along with a variable proportion of NK cells. Considerable interpatient heterogeneity was observed. Functional tumor-directed activity was assessed by measuring cytotoxic capacity of individual ALECSAT preparations. Products generated from healthy donors demonstrated consistent cytolytic activity against the breast cancer cell lines MDA-MB-231 (n=17) and MDA-MB-468 (n=14), although the intensity of responses varied between donors **(Figures 3a and 3b).** Furthermore, CD107a degranulation assays conducted on two clinical ALECSAT preparations showed activation of both $CD3^+$ T cells and $CD3^-CD56^+$ NK cells following co-culture with tumor cells, confirming functional engagement of effector populations **(Figures 3c and 3d).**

All ALECSAT batches tested prior to clinical administration (n=49) displayed measurable in vitro tumor cell killing. Although the degree of cytotoxicity differed among individual products, each preparation demonstrated a clear dose–response relationship, with increasing effector cell numbers resulting in enhanced target cell lysis **(Figures 3e and 3f).** Collectively, these findings indicate that ALECSAT products generated from both healthy donors and patients possess reproducible cellular composition and retain robust tumor-reactive effector function.





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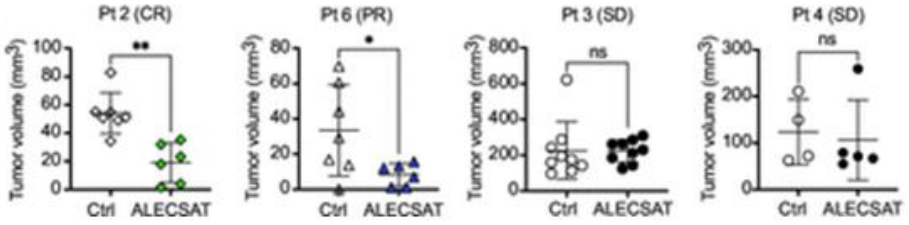

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Figure 3. Functional evaluation of ALECSAT products in vitro and in vivo. Panels (a) and (b) show that ALECSAT preparations derived from healthy donors mediated effective killing of breast cancer cell lines MDA-MB-231 and MDA-MB-468. Cytotoxicity increased with rising effector-to-target ratios and was quantified by luminescence-based readouts, demonstrating reproducibility across donors despite inter-individual variation. Panels (c) and (d) illustrate functional activation of patient-derived ALECSAT following exposure to MDA-MB-231 cells, as evidenced by CD107a mobilization on both $CD3^+$ T lymphocytes and $CD3^-CD56^+$ natural killer cells. In panel (E), real-time impedance monitoring (iCELLigence) was used to assess cytolytic activity of clinical ALECSAT batches against tumor targets (HROG17 for patients 1–3 and MDA-MB-231 for all other patients), revealing marked heterogeneity between individual products; the dotted line represents the cohort median response. Panel (f) confirms a dose-dependent increase in cytotoxicity with escalating effector cell numbers. Panel (g) depicts tumor growth kinetics in patient-derived xenograft models treated exclusively with autologous ALECSAT, without concomitant chemotherapy. Suppression of tumor growth was observed in models corresponding to patients achieving complete or partial clinical responses, whereas tumors derived from patients with stable disease were largely unaffected. Animals bearing tumors from patients 2, 3, 4, and 6 were euthanized on days 38, 130, 28, and 28, respectively. Symbol coding indicates clinical outcome (diamonds: complete response; triangles: partial response; circles: stable disease), with open symbols denoting untreated controls and filled symbols indicating ALECSAT-treated mice. Group comparisons were performed using unpaired t-tests (*$p<0.05$, **$p<0.01$; ns, not significant). PDX, patient-derived xenograft; NK, natural killer; E:T, effector-to-target; CR, complete response; SD, stable disease; ALECSAT, Autologous Lymphoid Effector Cells Specific Against Tumor; PR, partial response; PD, progressive disease.

Patient-specific antitumor effects observed in PDX models

Patient-derived xenografts have been widely used to model therapeutic responses in cancer and increasingly explored as platforms to evaluate immunotherapeutic strategies [27–29]. Recent work suggests that pairing PDX models with autologous immune cell transfer may be particularly informative for adoptive cell therapies [30]. To investigate this concept, metastatic biopsy samples from four trial participants were used to establish TNBC PDX models. Each model received treatment with ALECSAT generated from the same individual patient, while matched controls remained untreated.

Among the corresponding patients, clinical outcomes included one complete response, one partial response, and two cases of stable disease according to RECIST V.1.1. In alignment with these outcomes, ALECSAT

administration led to pronounced tumor growth inhibition in PDX models derived from patients with objective responses. In contrast, ALECSAT treatment failed to restrict tumor expansion in models established from patients whose disease remained stable **(Figure 3g).** As no cytotoxic chemotherapy was administered to the animals, these findings indicate that tumor control in responsive models was driven by ALECSAT alone. Although limited by the small number of cases, the concordance between in vivo tumor behavior and patient responses supports a link between intrinsic properties of individual ALECSAT products and clinical efficacy.

Relationship between ALECSAT composition and therapeutic outcome

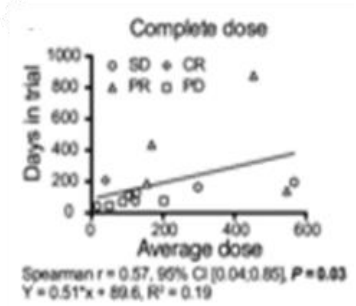
To explore potential determinants of response, we examined whether clinical benefit was associated with either the quantity or immunologic composition of administered ALECSAT products. Analysis revealed a significant positive association between the total number of infused cells per dose and time to disease progression **(Figure 4a)**; ($r=0.57$, $p<0.05$). Patients achieving partial responses generally received higher cell doses than those with stable or progressive disease, although this trend did not reach statistical significance **(Figure 4b).**

Given the heterogeneous immune makeup of ALECSAT, further analyses focused on specific cell subsets. The number of infused natural killer cells did not correlate with progression-free intervals. Interestingly, products administered to patients with partial responses contained lower NK-cell numbers compared with those given to patients with stable or progressive disease **(Figure 4d)**; ($p<0.05$).

In contrast, T-cell content demonstrated a clear association with outcome. The total T-cell dose per infusion correlated positively with time to progression **(Figure 4e)**; $r=0.59$, $p<0.05$), and patients achieving partial responses received significantly higher T-cell numbers overall **(Figure 4f)**; ($p<0.01$). Subset analysis identified $CD8^+$ T cells as a key contributor: higher $CD8^+$ T-cell counts per dose were strongly associated with prolonged disease control **(Figure 4g)**; $r=0.65$, $p=0.01$), and ALECSAT products given to responding patients were markedly enriched for $CD8^+$ T cells compared with those administered to non-responders **(Figure 4h)**; ($p<0.0001$).

No association was observed between $CD4^+$ T-cell numbers and time to progression, and $CD4^+$ T-cell content was comparable across response categories **(Figures 4i and 4j).** A modest correlation was detected between the number of infused double-negative ($CD4^-CD8^-$) T cells and time to progression **(Figure 4k)**; ($r=0.61$, $p<0.05$), although absolute counts of these cells did not differ significantly between clinical outcome groups **(Figure 4l).**

Taken together, these analyses indicate that higher overall T-cell dosing—particularly increased $CD8^+$ T-cell content—is associated with improved disease control following ALECSAT therapy. When considered alongside the PDX findings demonstrating tumor inhibition in the absence of chemotherapy, these results support the conclusion that ALECSAT products can exert direct, patient-specific antitumor effects.



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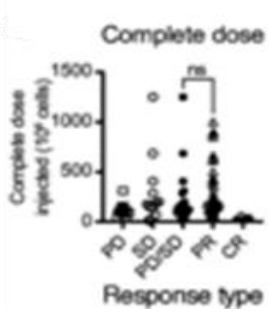

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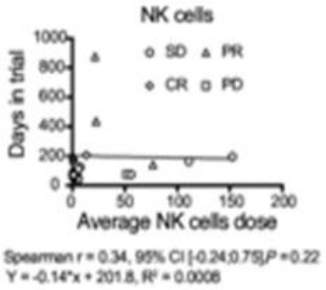

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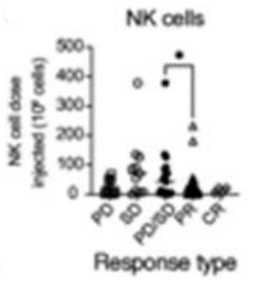

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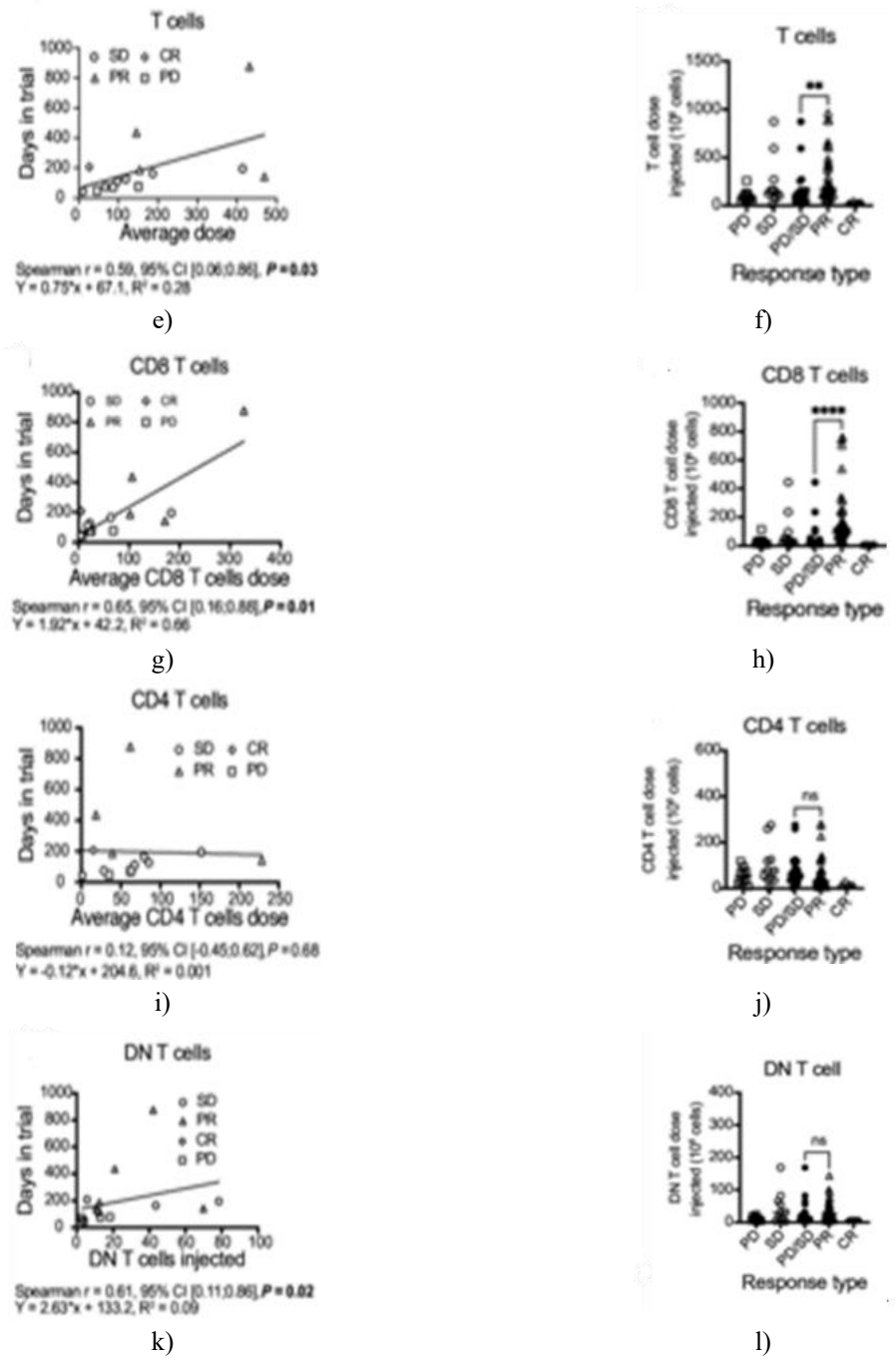

Figure 4. Association between ALECSAT cellular content and clinical outcomes. Clinical responses were evaluated in relation to both the total number of infused cells and the immune cell composition of ALECSAT products. (a) Increasing total cell numbers per infusion were significantly associated with longer time to disease progression (Spearman r = 0.57, $p < 0.05$). (b) Although not reaching statistical significance, patients achieving partial responses generally received higher total cell doses than those with stable or progressive disease. (c–d) No relationship was detected between infused natural killer (NK) cell counts and time to progression; however, ALECSAT preparations administered to patients with partial responses contained significantly lower NK-cell numbers compared with those given to patients with stable or progressive disease. (e–f) In contrast, total T-cell dose per infusion correlated positively with time to progression (r = 0.59, $p < 0.05$), and products administered to patients with partial responses were enriched for T cells relative to those used in patients with stable or progressive disease. (g–h) Analysis of T-cell subsets demonstrated a strong positive association between $CD8^+$ T-cell numbers per dose and time to progression (r = 0.65, p = 0.01); $CD8^+$ T-cell counts were also significantly higher in ALECSAT products given to patients with partial responses than in those administered to patients with stable or progressive disease. (i–j) $CD4^+$ T-cell content

showed no association with progression timing and did not differ between response categories. (k–l) Although the number of infused double-negative (DN) T cells correlated positively with time to progression ($r = 0.61$, $p < 0.05$), DN T-cell quantities were comparable across clinical response groups. Collectively, these findings indicate that higher T-cell dosing—most notably increased $CD8^+$ T-cell content—is linked to prolonged disease control, underscoring the importance of the T-cell compartment in mediating ALECSAT therapeutic activity. Statistical analyses were conducted using unpaired t-tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; ns, not significant). Autologous Lymphoid Effector Cells Specific against Tumor (ALECSAT); double-negative (DN); natural killer (NK); partial response (PR); stable disease (SD); progressive disease (PD); complete response (CR).

Adoptive cell therapy (ACT) has gained increasing attention as a cancer treatment modality due to its ability to harness immune effector cells with antitumor activity. Unlike tumor-infiltrating lymphocyte (TIL) or chimeric antigen receptor (CAR) T-cell approaches, which depend on predefined antigen targets or genetic modification, ALECSAT is designed to induce a diverse immune response composed of natural killer (NK) cells and polyclonal T cells. This broader immune repertoire may lower the likelihood of tumor immune evasion and expand the applicability of ACT to patient populations in whom TIL expansion is not feasible. Within the evolving field of cellular immunotherapy, ALECSAT therefore represents a distinct and complementary strategy, and the results of this phase 1b study provide early clinical support for its continued development.

In this trial, ALECSAT administered alongside chemotherapy demonstrated an acceptable and manageable safety profile in patients with heavily pretreated metastatic triple-negative breast cancer (mTNBC). The adverse event pattern was dominated by hematologic toxicities and constitutional symptoms, aligning closely with the known safety profiles of carboplatin and gemcitabine. The seven reported serious adverse events included conditions such as hypokalemia, back pain, and pneumonitis. Although treatment discontinuation due to toxicity occurred in two patients, no fatal adverse events or unexpected safety signals were observed. Importantly, chemotherapy administration was intentionally paused for three days before and after ALECSAT infusion to reduce potential detrimental effects on the infused immune cells, supporting the feasibility of integrating ALECSAT into standard chemotherapy schedules.

Clinical activity observed in this study is notable given the advanced disease stage and prior treatment exposure of the enrolled population. The objective response rate of 36% and disease control rate of 71% compare favorably with historical outcomes in late-line mTNBC. Median progression-free survival (4.3 months) and overall survival (8.7 months) were within or above expected ranges for this setting. While the activity of carboplatin and gemcitabine in heavily pretreated mTNBC remains insufficiently defined, first-line studies have reported an objective response rate of 30.2%, median progression-free survival of 4.6 months, and median overall survival of 12.6 months [5]. Although cross-line comparisons should be interpreted cautiously, the survival outcomes observed here suggest that the addition of ALECSAT may contribute meaningful clinical benefit. This is further supported by the two patients who received ten ALECSAT doses and experienced prolonged progression-free and overall survival.

A subset of patients demonstrated durable clinical benefit exceeding expectations for this disease context. Similar patterns were previously reported in a phase I study of ALECSAT in advanced glioblastoma, where extended survival beyond historical norms was observed in some individuals [24]. While causal attribution cannot be established in the absence of randomized controls, these findings raise the possibility that ALECSAT contributes to sustained disease control in selected patients. Exploratory analyses in the present study suggest that treatment outcomes were associated with the cellular composition of the infused product, particularly the abundance of $CD8^+$ T cells. This observation is consistent with the established role of cytotoxic $CD8^+$ T cells in antitumor immunity and aligns with in vitro data demonstrating tumor-reactive effector function across patient-derived ALECSAT products. Although limited by sample size, these findings indicate that enrichment strategies favoring $CD8^+$ T cells may enhance therapeutic efficacy.

Patient-derived xenograft (PDX) models were successfully established from metastatic tumor biopsies and treated with autologous ALECSAT products. To preserve tumor heterogeneity and stromal context, intact tumor fragments rather than dissociated cells were engrafted, creating a biologically stringent experimental system. ALECSAT was administered at the time of tumor implantation, an approach previously shown to optimize co-engraftment of immune and tumor components [25]. Although this differs from conventional treatment of established tumors, initial tumor growth was observed in most animals, supporting the interpretation that

ALECSAT exerted therapeutic rather than prophylactic effects. As spontaneous metastasis is uncommon in PDX systems, metastatic spread could not be evaluated; however, prior xenograft studies demonstrated that ALECSAT effectively reduced metastatic burden [25]. The absence of chemotherapy in these experiments further confirms that tumor growth inhibition resulted directly from immune cell activity. Notably, tumor responses in PDX models mirrored clinical outcomes, with growth suppression observed in models derived from patients achieving complete or partial responses but not in those from patients with stable disease, highlighting the potential utility of PDX systems for predicting patient-specific responsiveness.

Recent preclinical studies have shown that combining ALECSAT with anti–PD-L1 therapy produces enhanced antitumor effects compared with ALECSAT alone, including greater suppression of primary tumor growth, reduced metastatic spread, and improved survival [25]. These results suggest that PD-L1 upregulation may represent a compensatory immune resistance mechanism, although additional inhibitory pathways within the tumor microenvironment are likely involved. Elucidating these mechanisms will be critical for optimizing combination regimens. Based on the present findings and the apparent predictive value of PDX modeling, clinical evaluation of ALECSAT in combination with immune checkpoint blockade is justified.

Conclusion

This phase 1b study provides early clinical evidence that ALECSAT administered alongside chemotherapy is both feasible and potentially beneficial for patients with metastatic triple-negative breast cancer. Although the small cohort size and exploratory nature of the trial limit definitive interpretation, the favorable tolerability profile and observed antitumor activity support continued investigation in larger, controlled studies. Importantly, treatment outcomes appeared to correlate with the immune composition of the infused product, particularly $CD8^+$ T-cell content, underscoring the relevance of product optimization in adoptive cell therapy. Collectively, these findings establish a rationale for further refinement of ALECSAT manufacturing strategies and for evaluating combination approaches aimed at enhancing immune-mediated tumor control. Continued mechanistic and clinical studies will be essential to fully define the therapeutic potential of ALECSAT in advanced malignancies.

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