Acute myeloid leukemia drug-tolerant persister cells survive chemotherapy by transiently increasing plasma membrane rigidity, that also increases their sensitivity to immune cell killing

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Abstract

Resistance to chemotherapy remains a major hurdle to the cure of patients with acute myeloid leukemia (AML). Recent studies indicate that a minority of malignant cells, termed drug-tolerant persisters (DTP), stochastically upregulate stress pathways to evade cell death upon acute exposure to chemotherapy without acquiring new genetic mutations. This chemoresistant state is transient and the cells return to the baseline state after removal of chemotherapy. Nevertheless, the mechanisms employed by DTP to resist chemotherapy are not well understood and it is largely unknown whether these mechanisms are also seen in patients receiving chemotherapy. Here, we used leukemia cell lines, primary AML patients' samples and samples from patients with AML receiving systemic chemotherapy to study the DTP state. We demonstrated that a subset of AML cells transiently increases membrane rigidity to resist killing due to acute exposure to daunorubicin and Ara-C. Upon removal of the chemotherapy, membrane rigidity returned to baseline and the cells regained chemosensitivity. Although resistant to chemotherapy, the increased membrane rigidity rendered AML cells more susceptible to T-cell-mediated killing. Thus, we identified a novel mechanism by which DTP leukemic cells evade chemotherapy and a strategy to eradicate these persistent cells.

Introduction

Acute myeloid leukemia (AML) is a clonal disorder affecting early hematopoietic cells. It is an aggressive hematologic malignancy with a generally unfavorable prognosis. While several new therapeutic approaches for AML have been approved in recent years, conventional treatment still involves induction chemotherapy with a combination of chemotherapeutic agents, Ara-C and daunorubicin. Despite high initial response rates to induction chemotherapy, failure to achieve remission after one or two cycles of treatment is not unusual and continues to present a therapeutic challenge. Patients whose disease is refractory to first-line induction therapy have a dismal prognosis with a 5-year survival of only 17%.[1] Predictors of non-response to Ara-C and daunorubicin induction chemotherapy include poor-risk cytogenetics, high-risk molecular aberrations such as p53 mutations,[2] a cell hierarchy composition enriched for primitive, non-mature leukemic cells[3] and residual leukemic cells in the marrow at early times after chemotherapy (i.e., 5-14 days after chemotherapy).[4,5]

Classically, resistance to treatment in AML is attributed to leukemic stem cells (LSC). These cells are present at diagnosis and are inherently resistant to chemotherapy.[6-8] However, recent studies in AML, and other malignancies, report an additional mechanism of resistance, mediated by a non-LSC drug-tolerant persister (DTP) cell population. DTP cells alter their cellular state following treatment,[9] leading

to the development of an acute and transient resistance to chemotherapy,[10-13] When the stress of chemotherapy is removed, the cellular state returns to baseline and the cells regain sensitivity to chemotherapy.

DTP cells have been reported in several types of cancer, including breast,[14,15] colorectal,[16] and lung[17,18] cancers, in which they confer chemoresistance and contribute to disease relapse. DTP cells transiently upregulate stress mitigation pathways to facilitate their survival during therapy, without the acquisition of new genetic mutations. For example, enrichment in a diapause gene signature was detected in colon cancer and breast cancer DTP cells, reminiscent of an evolutionarily conserved embryonic strategy employed to survive unfavorable conditions.[14,16] DTP cells arise stochastically[19] during treatment and express a unique phenotype consisting of a slow proliferation rate[20] and a metabolic shift to increased autophagy[20] and fatty acid oxidation.[11,19,21,22] Notably, persistent cells that succeed in avoiding the effect of anti-cancer treatment, regain drug sensitivity upon drug removal, underscoring the plasticity of DTP cells and suggesting a timed window of opportunities for targeting this population. However, whether chemotherapy-resistant DTP cells are relevant in AML is unknown.

Here, we identified DTP cells in AML with alterations in their lipids and elongation of fatty acids. This shift manifested as changes in the physical characteristics of the cell membrane, enhancing membrane rigidity. Notably, cell membrane rigidity boosted the susceptibility of DTP cells to T-cell-mediated killing. Our findings indicate a novel mechanism for chemoresistance in the DTP state and highlight T-cell-based immunotherapeutic approaches as a strategy to eliminate these resistant cells.

Methods

Primary leukemic and normal hematopoietic cells

Primary human AML samples were obtained from peripheral blood or bone marrow of both male and female patients with AML, following informed consent, and then cryopreserved. Approval for the collection and use of the primary samples was obtained from the UHN Research Ethics Board and the Rambam Health Care Campus. AML cells were isolated using Ficoll-Paque differential density centrifugation and subsequently frozen in a solution comprising 50% fetal calf serum, 40% αMEM, and 10% dimethylsulfoxide. Information about the patients who were the source of the cells is provided in *Online Supplementary Table S1*.

Drug-tolerant persister model

MV4-11, MOLM13, THP-1, NB4, OCI-AML2 and OCI-AML3 cells were treated with daunorubicin and Ara-C at 90% inhibitory concentrations (IC_{90}) for 5 days. The concentrations of daunorubicin and Ara-C were 20 nM and 70 nM, respectively, for MV4-11 cells, 40 nM and 200 nM, respectively, for MOLM13 cells; 90 nM and 900 nM for THP-1 cells, 30 nM and 150 nm for NB4 cells, 60 nM and 600 nM for OCI-AML2 cells, and 90 nM and 900 nM for OCI-AML3 cells.

Dead cells were removed on day 6 after treatment via magnetic column depletion using the cell death removal kit (Miltenyi Biotec) according to the company's instructions.

Membrane fluidity

Membrane fluidity was assessed using pyrenedecanoic acid (PDA; Membrane Fluidity Kit; Abcam). Cells were incubated at room temperature with the labeling solution containing 5 μM of fluorescent lipid reagent and 0.08% Pluronic F127, for 1 hour. Cells were washed and then plated in a 96-well plate at a final volume of 100 μL. Fluorescence intensities were measured using a plate reader with excitation at 350 nm and emissions at 400 nm (monomer) and 470 nm (excimer). The membrane fluidity of patients' samples was measured by using flow cytometry, gating on the $CD45^{dim}CD34^{+}CD117^{+}$ population to obtain median fluorescence intensities (MFI) at 400 nm (monomer) and 470 nm (excimer). Membrane fluidity was assessed by calculating the ratio of 470 (excimer)/440 (monomer) fluorescence.

Membrane fluidity was also measured by staining cells with Di-4-ANEPPDHQ (Di4) (ThermoFisher, D36802) as previously described.[23] Cells were incubated with 1 μM Di4 for 20 minutes at room temperature. Di4 was excited at 488 nm and the fluorescence emission intensity of Di4 was recorded across the fluorescein thiocyanate and peridinin-chlorophyll protein channels, representing ordered and disordered wavelength, respectively. The generalized polarization (GP) value, reflecting relative membrane rigidity, was calculated as previously described:[23]

$$GP = \frac{Intensity\ Ordered - Intensity\ Disordered}{Intensity\ Ordered + Intensity\ Disordered}$$

Additional methods are provided in the *Online Supplementary Appendix*.

Results

Leukemic drug-tolerant persister cells transiently resist cell death from chemotherapy

Standard induction chemotherapy for patients with AML involves a combination of daunorubicin and Ara-C. To investigate DTP cells that survive acute exposure to chemotherapy, we treated MOLM13, MV4-11, and THP-1 leukemia cells with a combination of daunorubicin and Ara-C at an IC_{90} for 5 days to mimic standard therapy. Viable cells that persisted following chemotherapy were collected at 6 days after treatment and replated into fresh media. The DTP cells exhibited a lag phase in proliferation for 6-14 days after chemotherapy (Figure 1A, *Online Supplementary Figure S1A*), but subsequently regained normal proliferation (Figure 1B, *Online Supplementary Figure S1B*). We then re-

treated the recovered leukemic cells with chemotherapy and these cells retained a sensitivity equivalent to that of the wild-type leukemic cells (Figure 1C, *Online Supplementary Figure S1C*), underscoring the plasticity of these cells and the DTP state.

Quiescence and cell cycle delay are characteristic of the DTP state. We therefore measured cell cycle and 5-ethynyl-2 deoxyuridine (EdU) uptake in our DTP leukemic cells. We observed cell cycle delay in the DTP leukemic cells, as evidenced by a decrease in the percentage of cells in the S-phase compared to that of parental cells and accumulation in the G1 or G2-M phase (Figure 1D, *Online Supplementary Figure S1D*). Of note, THP-1 DTP cells arrested at the G1 phase while the other cell lines arrested at the G2-M phase.

Despite the delay in cell cycling, the DTP cells continued to proliferate albeit more slowly than the wild-type cells (Figure 1E, *Online Supplementary Figure S1E*). In addition,

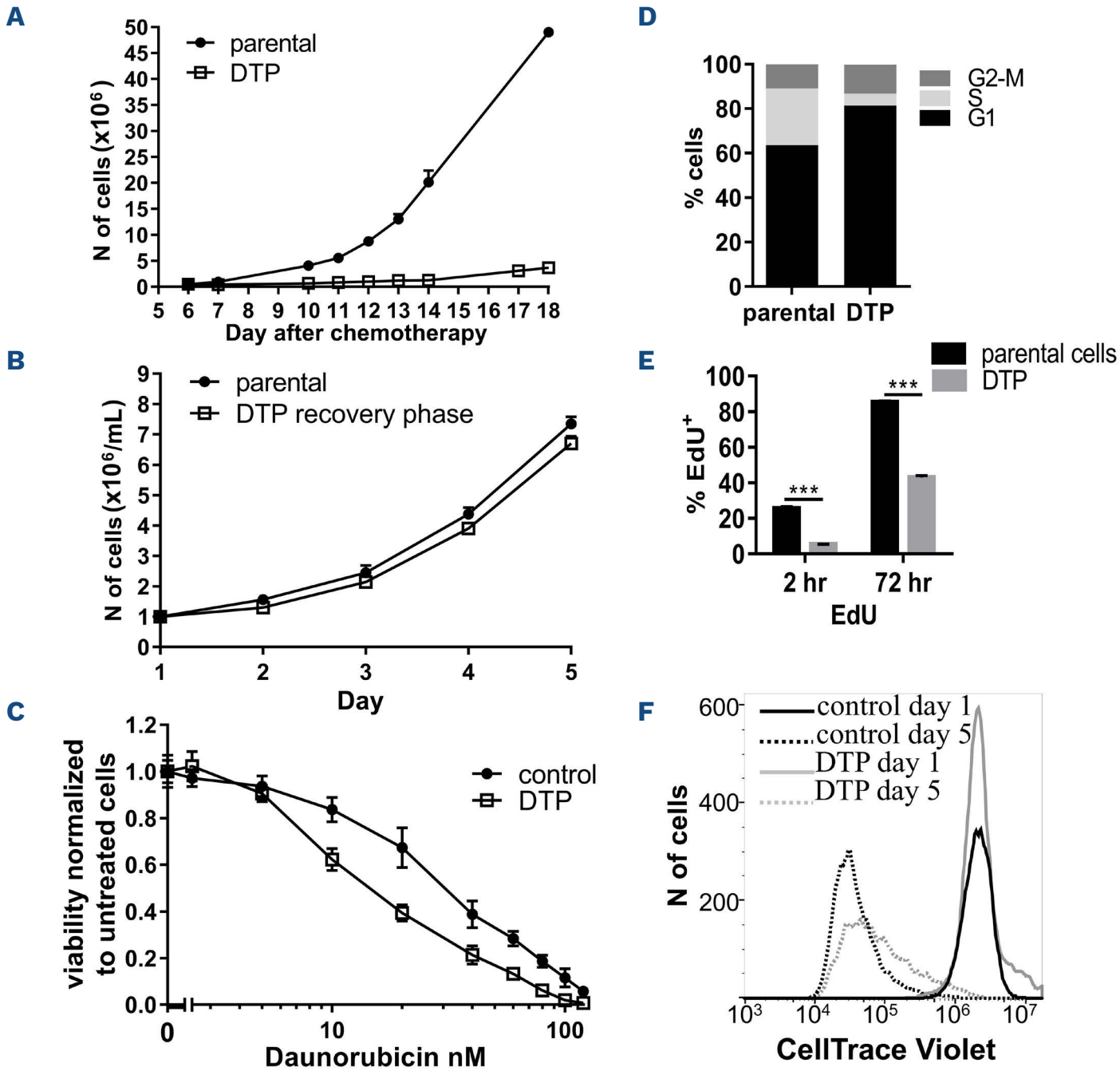

Figure 1. An *in-vitro* model for acute myeloid leukemia drug-tolerant persister cells. (A) MOLM13 drug-tolerant persistent (DTP) cells were treated with a combination of daunorubicin (40 nM) and Ara-C (200 nM) to achieve a 90% inhibitory concentration (IC_{90}). Residual viable cells were collected on day 6 after the daunorubicin and Ara-C treatment and plated in fresh medium. Growth and viability of the cells were measured over time by trypan blue exclusion staining. Data represent the mean ± standard deviation (SD) of three independent experiments. (B) Growth and viability of persisting MOLM13 over time starting 20 days after daunorubicin and Ara-C treatment as measured by trypan blue exclusion staining. Data represent the mean ± SD from three independent experiments. (C) Persisting MOLM13 cells were collected on day 6 after daunorubicin (40 nM) and Ara-C (200 nM) treatment and plated in fresh medium to recover until day 20. On day 20 cells were retreated with increasing concentrations of daunorubicin for 72 hours and growth and viability were measured using Alamar Blue staining. Data represent the mean ± SD growth and viability from a representative experiment (N=2). (D) Cell cycle analysis as measured by propidium iodide staining and flow cytometry in persisting MOLM13 cells collected on day 6 after daunorubicin and Ara-C treatment. Data represent mean ± SD from a representative experiment (N=2). (E) Wild-type and persisting MOLM13 cells were labeled with 5-ethynyl-2′-deoxyuridine (EdU). EdU uptake was measured by flow cytometry 2 and 72 hours after treatment. Data represent the mean ± SD from a representative experiment (N=2). (F) MOLM13 control and DTP cells were labeled with CellTrace Violet stain. Staining intensity and dilution over time were measured at days 1 and 5 after staining using flow cytometry.

we measured dilution of CellTrace Violet as an additional marker of proliferation. The population of DTP cells demonstrated decreased CellTrace Violet staining on day 5 than on day 1, although the reduction was less pronounced than in the control cells (Figure 1F), indicating slow proliferation of the DTP cells. Thus, our model recapitulates key features of the DTP state.

Finally, we compared the intracellular levels of daunorubicin in DTP and control cells. DTP and control cells were treated with increasing concentrations of daunorubicin for 3 hours. After incubation, intracellular levels of daunorubicin were measured by flow cytometry. Compared to control cells, DTP cells accumulated less daunorubucin intracellularly (*Online Supplementary Figure S1F*).

Leukemic drug-tolerant persister cells alter lipidome composition

To understand mechanisms by which DTP leukemic cells resist chemotherapy, we conducted an unbiased analysis of the cellular lipidome 6 days after chemotherapy. The lipidome heterogeneity in the different leukemic cells resulted in cell line-specific lipidome rearrangement. However, we focused our analysis on lipidomic changes common to the three tested cell lines.

Compared to control cells, MOLM13, MV4-11 and THP-1 DTP cells that resisted acute exposure to chemotherapy had increased elongated phosphatidylcholines with fatty acids chain lengths of more than 35 carbon atoms (Figure 2A, *Online Supplementary Figure S2A*), elongated triglycerides with a chain length of more than 53 carbon atoms (Figure 2B, *Online Supplementary Figure S2B*) and elongated phosphatidylethanolamine (Figure 2C). Additionally, an increase in the abundance of sphingomyelins of different fatty acid lengths was detected in the DTP cells (Figure 2D). In contrast, levels of cholesterol in the DTP cells were unchanged compared to those in the untreated cells (*Online Supplementary Figure S2C*).

To understand potential mechanisms for the increased elongated fatty acids, we measured fatty acid uptake in control and DTP leukemia cells. Compared to untreated controls, DTP leukemia cells increased uptake of fatty acids from the media, despite being in a slow cycle state (Figure 2E, *Online Supplementary Figure S2D*).

Leukemic drug-tolerant persister cells have increased membrane rigidity

Alterations in fatty acids influence the biophysical properties and fluidity of the cell membrane.[24] Specifically, elongation of fatty acids in membrane lipids and increased abundance of sphingomyelins increase membrane rigidity.[25-27] Previously, we and others demonstrated that increased plasma membrane rigidity decreases the intracellular accumulation of chemotherapy and renders cells chemoresistant.[28-31] Therefore, given the increased elongated fatty acids and sphingomyelins in DTP leukemia cells, we measured membrane fluidity by staining DTP and control cells with the fluorescent dye, lipophilic pyrenedecanoic acid (PDA). PDA is a fluorescent probe that integrates into the cell membrane, emitting two distinct spectra based on the membrane's fluidity. In MOLM13, MV4-11, THP1, NB4, OCI-AML2 and OCI-AML3 cells, membrane fluidity decreased (i.e., membrane rigidity increased) in the DTP cell population (Figure 3A). We confirmed increased membrane rigidity in the DTP cells by staining cells with di-4-ANEPPDHQ, a dye whose fluorescence reflects lipid-packing order in biomembranes[23] (*Online Supplementary Figure S3*). Importantly, when the DTP cells regained normal proliferation and chemosensitivity, their membrane fluidity returned to basal levels similar to that of control cells (Figure 3A).

To extend our investigation of membrane fluidity and the DTP state *in vivo* to primary AML cells, we engrafted primary AML cells into NSG mice. Eight weeks after cell injection, mice were treated with Ara-C (60 mg/kg/day) for 5 consecutive days and then sacrificed on day 8 (Figure 3B). Human $CD45^{+}CD33^{+}$ leukemic cells in the mouse marrow were quantified by flow cytometry. Treatment with Ara-C decreased the leukemic burden in the mice more than 3-fold compared to the reduction in vehicle-treated mice (Figure 3C). We isolated the residual leukemic cells flushed from the mouse marrow by magnetic separation and measured membrane fluidity in the leukemic cells. In alignment with our *in-vitro* DTP model, primary leukemic cells that persisted after Ara-C treatment had increased cell membrane rigidity (Figure 3D).

Next, we examined primary AML cells obtained from patients receiving systemic chemotherapy. Bone marrow samples were obtained before and on day 5 of induction chemotherapy with daunorubucin and Ara-C and the leukemic cells were identified by flow cytometry analysis and gating on $CD45^{dim}CD34^{+}CD117^{+}$ cells, representing patients' blasts. Compared to pre-treatment, membrane fluidity in the AML cells was decreased (i.e., rigidity increased) in the patients' leukemic cells that persisted on day 5 of chemotherapy (Figure 3E).

Non-pharmacological models that transiently increase membrane rigidity confer chemoresistance

As a non-pharmacological approach to examine the relationship between membrane rigidity and the DTP state, we incubated AML cells at 31°C. Temperature is a known factor influencing cell membrane fluidity[32-34] and, indeed, we observed increased cell membrane rigidity after culturing AML cells at 31°C (Figure 4C). Reminiscent of the DTP cell phenotype, cells cultured at 31°C remained viable and continued to proliferate, albeit more slowly (Figure 4A). Cells cultured at 31°C were more resistant chemotherapy compared to cells cultured at 37°C (Figure 4B). Importantly, the phenotypes of increased membrane rigidity, decreased proliferation, and chemoresistance, were all reversible upon returning the cells to 37°C (Figure 4A-C), in accordance

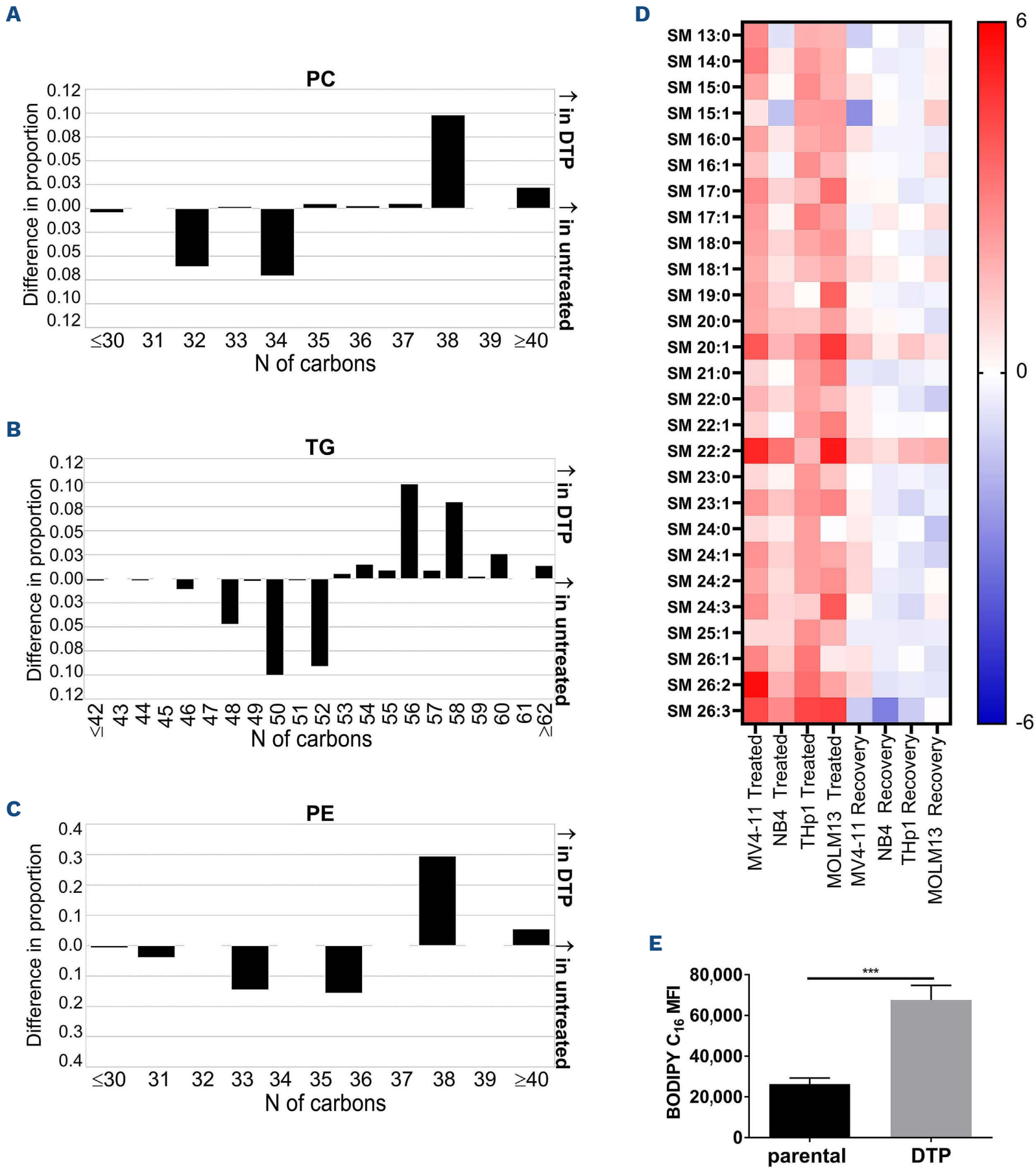

Figure 2. Lipid composition of leukemic drug-tolerant persister cells. (A) MOLM13 cells were treated with a combination of daunorubicin (40 nM) and Ara-C (200 nM) to achieve a 90% inhibitory concentration (IC_{90}). Residual viable cells were collected on day 6 after daunorubicin and Ara-C treatment for lipidomic analysis. Difference in proportion of phosphatidylcholine species between drug-tolerant persister (DTP) and control samples. Species above the x-axis are enriched in DTP samples, and species below are enriched in untreated samples. (B) Difference in proportion of triglyceride species between DTP and control samples. Species above the x-axis are enriched in DTP samples, and species below are enriched in untreated samples. (C) Difference in proportion of phosphatidylethanolamine species between DTP and control samples. Species above the x-axis are enriched in DTP samples, and species below are enriched in untreated samples. (D) Heatmap showing abundance of sphingomyelin species in MOLM13, MV4-11, THP-1 and NB4 cells in the DTP phase (day 6 after chemotherapy) and in the recovery phase (day 21 after chemotherapy). Relative fold-change levels are indicated on the color scale, with numbers indicating the $\log_2$ fold change normalized to untreated cells. (E) MOLM13 DTP cells were collected on day 6 after daunorubicin and Ara-C treatment and incubated with BODIPY-FL-C_{16}. The graph shows BODIPY-FL-C_{16} mean fluorescence intensities measured by flow cytometry. Data represent the mean ± standard deviation from three independent experiments. PC: phosphatidylcholines; TG: triglycerides; PE: phosphatidylethanolamines; SM: sphingomyelins; MFI: mean fluorescent intensity.

with the recovery phase observed in DTP cells after drug removal.

To further investigate the relationship between membrane rigidity and chemoresistance, we cultured MOLM13 cells with soluble cholesterol to increase the cholesterol of the cell membrane. Consistent with prior reports,[35] supplementation with soluble cholesterol decreased membrane fluidity (Figure 4D). These cells were then treated with increasing concentrations of daunorubicin for 3 hours, and daunorubicin uptake was measured using flow cytometry. Cholesterol-treated AML cells with more rigid membranes showed decreased daunorubicin uptake (Figure 4E), reinforcing the link between membrane rigidity and chemoresistance.

Drug-tolerant persister cells have increased sensitivity to T-cell-mediated killing

Recently, in a murine model of melanoma, increasing cell membrane stiffness by cholesterol depletion sensitized melanoma cells to T-cell-mediated killing.[36] The observed increase in cell membrane rigidity in AML DTP cells prompted us to investigate the sensitivity of these cells to T-cell-me-

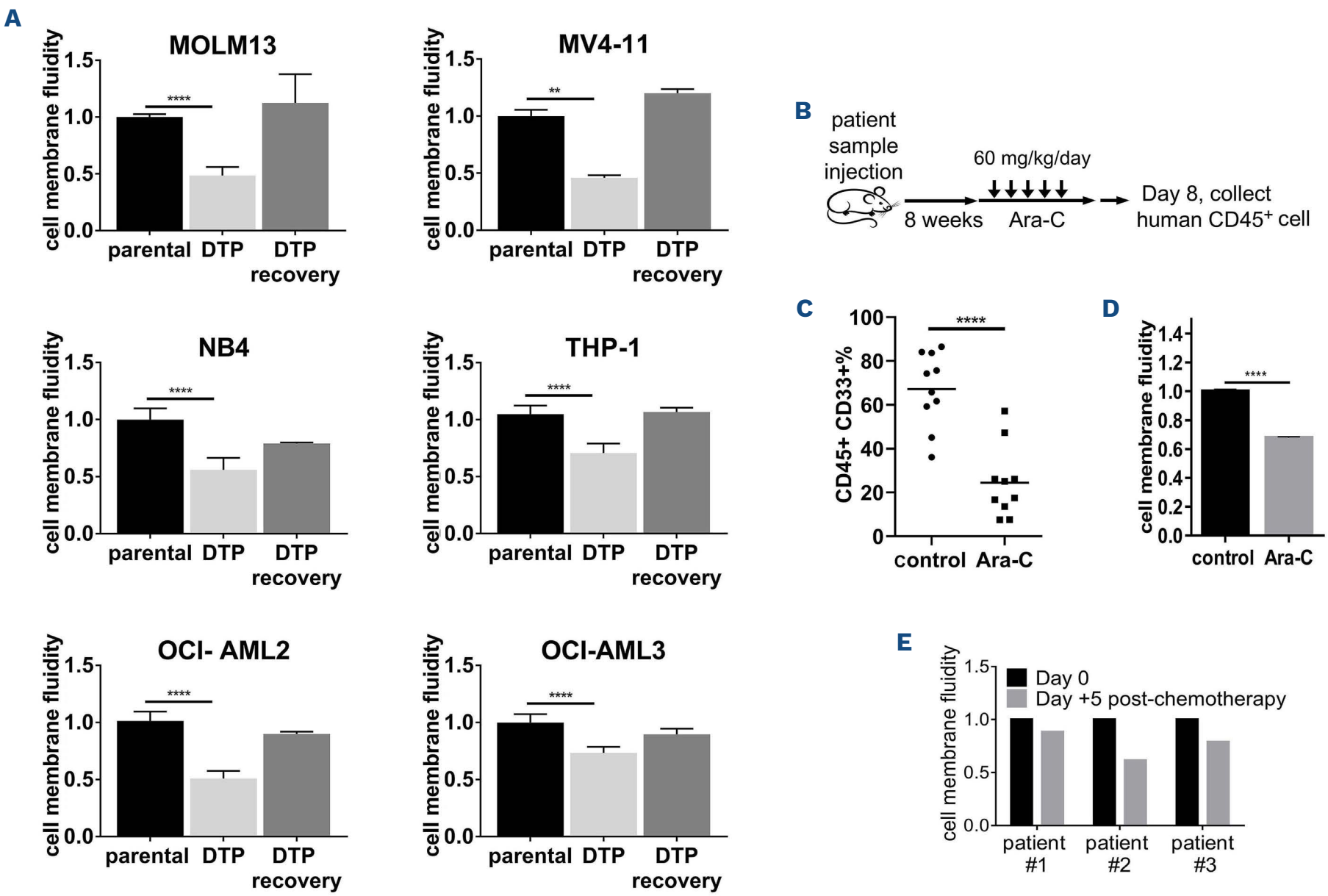

Figure 3. Leukemic drug-tolerant persister cells exhibit decreased cell membrane fluidity. (A) MOLM13, NB4, OCI-AML2, MV4-11, THP-1 and OCI-AML3 cells were treated with daunorubicin + AraC to achieve a 90% inhibitory concentration (IC_{90}). Residual viable cells were collected on day 6 after treatment and stained using the fluorescent PDA probe to assess membrane fluidity. Drug-tolerant persister (DTP) recovery represents DTP cells collected on day 6 after treatment, plated in fresh medium to recover until day 20. The emission fluorescence was quantified using a plate reader in triplicate. Data represent the mean ± standard deviation (SD) of three independent experiments. ***P*<0.01; *****P*<0.0001 by the Student *t* test. (B) Schematic illustration showing a patient-derived xenograft model. Primary acute myeloid leukemia (AML) cells were injected into the right femur of sub-lethally irradiated NSG mice. Eight weeks after cell injection, mice were treated with Ara-C 60 mg/kg/day or vehicle for 5 days. DTP cells were collected on day 8 after Ara-C treatment. (C) Primary AML cells were engrafted into NSG mice and treated with Ara-C as in Figure 3B. The percent of AML cells ($CD45^+CD33^+$) engrafted into the mouse femur was measured by flow cytometry. *****P*<0.0001 by the Student *t* test. (D) Pooled human $CD45^+$ patient-derived DTP cells were collected from mice on day 8 after Ara-C treatment. Cells were stained using the fluorescent PDA probe to assess membrane fluidity. The emission fluorescence was quantified using a plate reader in triplicate. Data represent the mean ± SD. *****P*<0.0001 by the Student *t* test. (E) Membrane fluidity of blast cells in paired patients' bone marrow samples collected on day 0 and day +5 after chemotherapy. Cells were stained using the fluorescent PDA probe and membrane fluidity was assessed using flow cytometry and analysis of the mean fluorescence intensity of $CD45^{dim}CD34^+CD117^+$ cells. MFI: mean fluorescence intensity.

diated killing using double-negative T (DNT) cells as a model. $CD3^+CD4^-CD8^-$ DNT cells are a rare subpopulation of mature T cells found in healthy individuals and can be expanded *ex-vivo* over 1,000-fold. DNT cells have anti-leukemic activity through perforin-dependent and -independent mechanisms but are not toxic to normal hematopoietic cells.[37-39] Phase I clinical trials demonstrated the safety and potential efficacy of DNT cells in treating AML patients who relapsed after allogeneic stem cell transplantation.[40]

To test how membrane rigidity influences sensitivity to T-cell-mediated killing, we first used our 31°C cell culture model. AML cell lines cultured at 31°C displayed heightened sensitivity to DNT-cell-mediated killing compared to cells cultured at 3°C (Figure 5A).

To further evaluate the sensitivity of DTP leukemic cells to killing by DNT cells, MOLM13 cells were injected into the femora of NSG mice. Mice were treated with Ara-C daily for 5 days. DNT cells were injected intravenously 4 and 6 days after completion of Ara-C chemotherapy, to target the chemoresistant persistent cells. Treatment of mice with Ara-C and DNT cells decreased the leukemic burden compared to treatment with either Ara-C or DNT cells alone (Figure 5B).

Next, we evaluated the sensitivity of persistent cells derived from primary AML patients to T-cell-killing using DNT cells. Primary AML samples were engrafted into immune-deficient mice and treated daily with Ara-C for 5 days. On day 8, the residual AML cells were collected and co-cultured *ex-vivo* with DNT cells. The persistent primary leukemic cells had increased susceptibility to T-cell-mediated cytotoxicity compared to the AML cells isolated from mice not treated with chemotherapy (Figure 5C).

Finally, we tested sensitivity to T-cell-mediated killing in residual leukemia cells isolated from a patient receiving

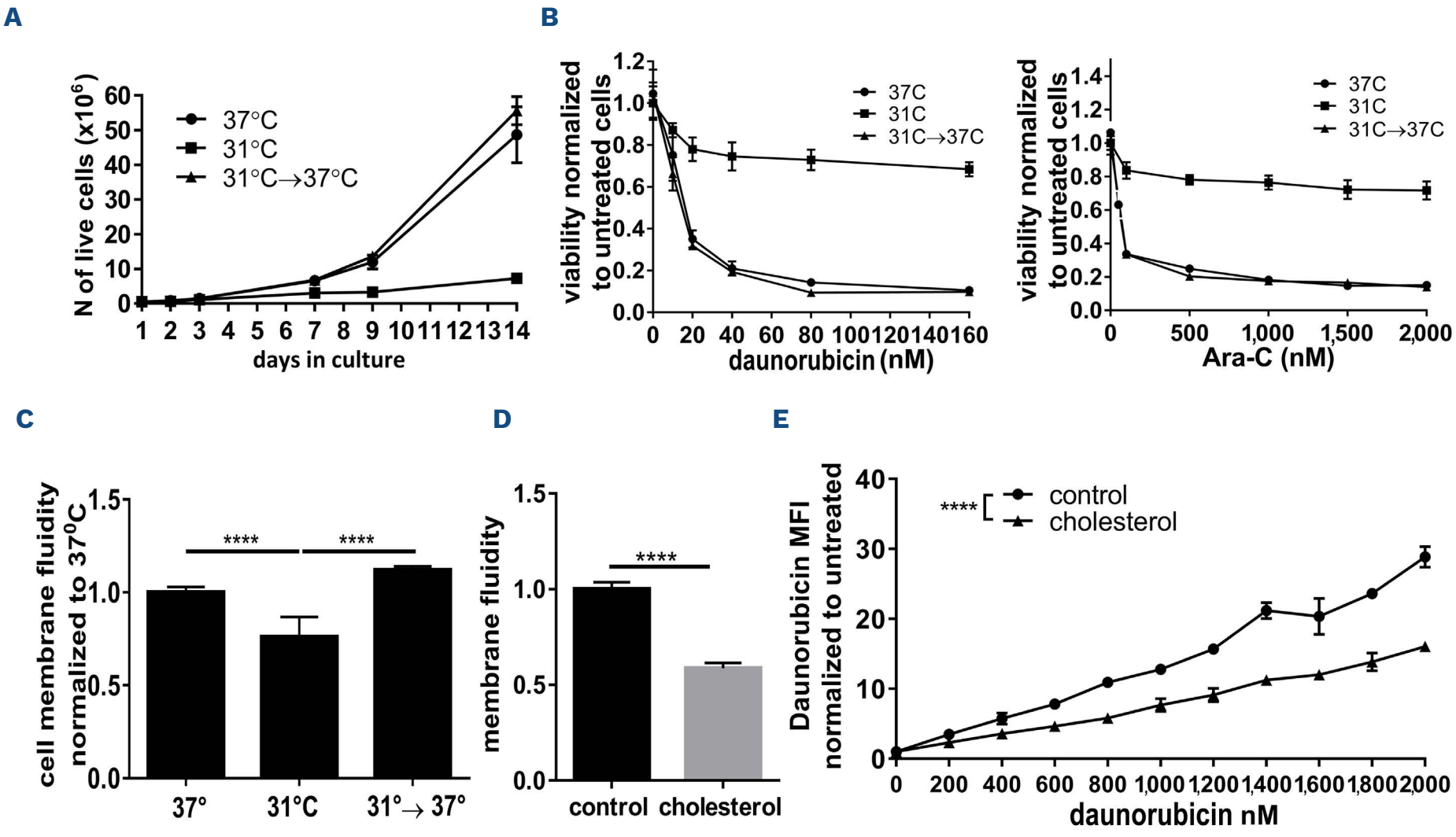

Figure 4. Non-pharmacological models that transiently increase membrane rigidity confer chemoresistance in acute myeloid leukemia. (A) MOLM13 cells were cultured at 31°C for 24 hours prior to analysis, at 37°C for 24 hours, or at 31°C for 24 hours and then transferred back to 37°C (31°C→37°C). Growth and viability of the cells were measured by trypan blue exclusion staining. Data represent the mean ± standard deviation (SD) of three independent experiments. (B) MOLM13 cells from Figure 4A were retreated with increasing concentrations of daunorubicin or Ara-C for 72 hours and growth and viability were measured using a sulforhodamine B (SRB) assay. Data represent the mean ± SD growth from a representative experiment (N=2). (C) MOLM13 cells cultured at 31°C, 37°C or 31°C→37°C were stained using the fluorescent PDA probe to assess membrane fluidity. Emission fluorescence was quantified using a plate reader in triplicate. Data represent the mean ± SD of three independent experiments. ****P<0.0001 by the Student t test. (D) MOLM13 cells treated with cholesterol (60 μg/mL) were stained using the fluorescent PDA probe to assess membrane fluidity. Data represent the mean ± SD of three independent experiments. ****P<0.0001 by the Student t test. (E) Control and cholesterol-treated MOLM13 cells incubated with daunorubicin at increasing concentrations. Daunorubicin uptake was assessed by flow cytometry. Data represent the mean ± SD of three independent experiments. ****P<0.0001 by two-way analysis of variance test. MFI: mean fluorescence intensity.

systemic induction chemotherapy with daunorubicin and Ara-C. Bone marrow samples were collected prior to treatment and on day 5 of chemotherapy. Cells persisting on day 5 had increased membrane rigidity compared to pre-treatment cells (Figure 3E). Leukemic cells before treatment and on day 5 of treatment were enriched from the bone marrow sample by magnetic separation and co-cultured with DNT cells for 2 hours. Compared to pre-treatment AML cells, those remaining on day 5 of chemotherapy had increased sensitivity to T-cell-mediated killing (Figure 5D). Thus, although AML cells transiently resist chemotherapy by increasing cell membrane rigidity, this increased membrane rigidity confered sensitivity to T-cell-mediated killing, highlighting a strategy to eradicate these persisting cells.

Discussion

Despite advancements in treatment options, chemoresistance and refractory disease remain a leading cause of death in AML patients.[41] The presence of LSC has long been implicated as a significant contributor to drug resistance. However, LSC-independent mechanisms of drug resistance also exist. Among those mechanisms, there is emerging interest in the transient DTP state. However, the mechanisms by which leukemic DTP cells transiently resist chemotherapy and how to target these residual cells remains unclear. Here, we demonstrate that leukemic DTP cells have increased plasma membrane rigidity, which contributes to chemoresistance but also increases sensitivity to immune-mediated killing. To our knowledge, we are the first to describe that AML DTP cells transiently increase plasma membrane rigidity to resist acute exposure to chemotherapy. To confirm that transient alterations in membrane rigidity influence chemosensitivity, we used cholesterol treatment and a non-pharmacological model in which cells were cultured under colder temperatures to increase membrane rigidity transiently. We demonstrated that these cells were transiently resistant to chemotherapy. Our findings are consistent with those of prior studies that demonstrated increased membrane rigidity is

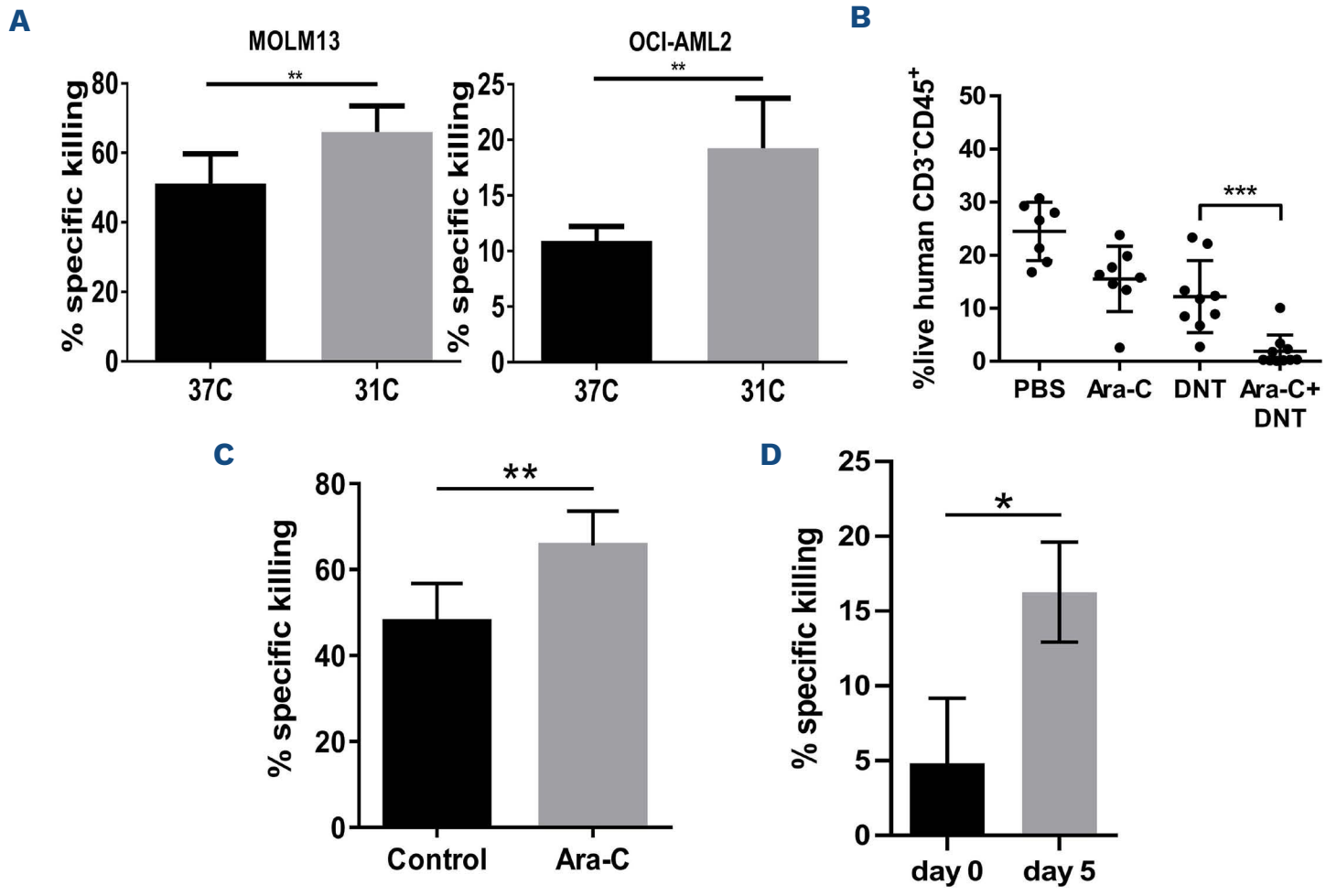

Figure 5. Increasing cell membrane rigidity sensitizes acute myeloid leukemia cells to T-cell-mediated cytotoxicity. (A) MOLM13 and OCI-AML2 cells cultured at 37°C or 31°C (24 hours) were incubated with double-negative T (DNT) cells for 2 hours. Percent specific killing of acute myeloid leukemia (AML) cells by DNT cells was determined by annexin V staining and flow cytometry. Data represent the mean ± standard deviation (SD) from a representative experiment (N=3). ***P*<0.01 by the Student *t* test. (B) MOLM13 cells engrafted and collected from mice after injection with Ara-C ± DNT cells. Percent engraftment was determined by flow cytometry gating on live human $CD3^{-}CD45^{+}$ cells. Data represent the mean ± SD (N=7-10). ***P*<0.001 by the Student *t* test. (C) A patient's sample (AML 166315) engrafted and collected from mice at day +8 after Ara-C treatment. Pooled human $CD45^{+}$ cells were incubated with DNT cells for 2 hours. Percent specific killing of AML cells by DNT cells was determined by annexin V staining in flow cytometry. Data represent the mean ± SD (N=3). ****P*<0.01 by the Student *t* test. (D) $CD34^{+}$ blast cells were separated from paired bone marrow samples (patient AML#3) collected on day 0 and day +5 after chemotherapy. Cells were incubated with DNT cells for 2 hours. Percent specific killing of $CD34^{+}CD117^{+}$ blast cells by DNT cells was determined by annexin V staining in flow cytometry. Data represent the mean ± SD (N=3). **P*<0.05 by the Student *t* test. PBS: phosphate-buffered saline.

associated with chemoresistance.[29] For example, increasing membrane rigidity in AML by applying biomechanical pressure decreases cellular uptake of daunorubicin and renders cells resistant to the drug.[28] Likewise, membrane rigidity was increased in lung and colorectal cells resistant to cisplatin and oxaliplatin, respectively.[42-45] While we demonstrated that DTP cells have increased plasma membrane rigidity, we concede that DTP cells also transiently upregulate other mechanisms that confer chemoresistance. As such, alterations in membrane rigidity are only one mechanism of chemoresistance in these cells.

Leukemic DTP cells increased uptake of fatty acids and had increased levels of elongated fatty acids, triglycerides and sphingomyelins. Prior studies demonstrated that alterations in the length of fatty acids and triglycerides and the amount of sphingomyelins[27] influence membrane fluidity. For example, in rat insulinoma β-cells, exposure to elongated fatty acids elevates plasma membrane rigidity.[46] Triglycerides are localized to lipid droplets, acting as reservoirs of cellular fatty acids, which ultimately will be incorporated in the plasma membrane.[47] The abundance of triglycerides contributes to membrane rigidity in lipopolysaccharide-stimulated human neutrophils.[48] However, we also recognize that altering metabolism and fatty acid composition can affect metabolism and cellular processes beyond the plasma membrane and these changes may also influence sensitivity to chemotherapy.

Recent studies have shown that remodeling the cytoskeleton of cancer cells decreases membrane rigidity and reduces T-cell-mediated cytotoxicity.[49] Conversely, increasing cell stiffness through modifications of the cancer cell membrane or cytoskeleton enhances T-cell-mediated killing.[36,50-52] We demonstrated that leukemic DTP cells have increased sensitivity to T-cell-mediated killing. For our model of T-cell-mediated killing, we employed DNT cells. DNT cells are a rare population of T cells that are cytotoxic to AML cells while sparing normal hematopoietic cells.[37-39] DNT cells can be collected from healthy donors, expanded *ex-vivo* and utilized as an "off-the-shelf" allogeneic T-cell therapy. Their efficacy was highlighted in a recent phase I clinical trial in which DNT-cell treatment led to a 50% progression-free survival rate at 1 year in a subgroup of poor prognosis, relapsed AML patients.[40] Thus, our findings suggest that cell membrane modifications during the DTP phase present an opportunity for selectively targeting the persistent cell population with a T-cell-based immunotherapy approach.

In conclusion, we delineate a transient non-genetic adaptation in AML DTP cells, marked by the acquisition of an abnormal cellular lipid profile, resulting in heightened membrane rigidity and subsequent treatment resistance. Although conferring resistance to chemotherapy, this transient alteration in the membrane also heightened the cells' susceptibility to T-cell-mediated elimination. Thus, our findings suggest that T-cell-based immunotherapy could be used immediately following induction chemotherapy to eradicate surviving leukemic DTP cells, which are important in mediating disease relapse.

Disclosures

ADS has received research funding from Takeda Pharmaceuticals, BMS and Medivir AB; has received consulting fees/honoraria from Takeda, Astra-Zeneca, BMS, and Novartis, and Pharmaceuticals; is on the medical and scientific board of the Leukemia and Lymphoma Society of Canada; and is a co-inventor of several DNT cell technology-related patents and intellectual properties for the treatment of AML. LZ has financial interests (e.g., holdings/shares) in WYZE Biotech Co Ltd has previously received research funding and consulting fees/honoraria from WYZE Biotech Co Ltd; and is a co-inventor of several DNT cell technology-related patents and intellectual properties for the treatment of AML. JBL is a co-inventor of several DNT cell technology-related patents and intellectual properties for the treatment of AML.

Contributions

YM designed and performed the experiments, analyzed data and wrote the article. JBL, YN, BYL, NSL, WDG, RH, LM, DL, MG, ATQ, and JRM-B performed the experiments and analyzed data. AA, AF, TZ, YO, and MDM provided AML patients' samples for the study. LZ and CO'B provided feedback and edited the manuscript. ADS conceived and designed the study.

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Data-sharing statement

Data files will be made available upon reasonable request to the corresponding authors.

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