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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Supplementary Methods

Cell culture and reagents

MV4-11 and OCI-AML2 cells were cultured in Iscove's modified Dulbecco's medium (IMDM). MOLM13, THP1 and NB4 cells were cultured in RPMI. OCI-AML3 cells were cultured in alpha-MEM medium. All media were supplemented with 10% fetal bovine serum and appropriate antibiotics. All cell lines were maintained in humidified incubators at 37 °C supplemented with 5% CO2. To enrich the plasma membrane of cell lines with cholesterol, cells were incubated overnight with 60 ug/ml water-soluble cholesterol (Sigma-Aldrich, C4951).

Animals

Immunodeficient NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were obtained from the University Health Network. For the in vivo experiments, the mice were grouped prior to treatment. The grouping and treatment of the mice were performed by an individual who was not involved in the analysis of the data from the experiment. Mice were randomly assigned to each experimental group. During all experiments, the weights of the mice were approximately 18–30 g with no animals losing greater than 10% body weight. All animals were housed in microisolator cages with temperature-controlled conditions under a 12-h light/dark cycle with free access to drinking water, and food. Primary AML cells (1x10^6) were injected into the right femur of pre-conditioned NSG mice (6–10 weeks) that were sub-lethally irradiated (2 Gy) 24h prior to injection. Eight weeks after cell injection, intravenous Ara-C treatment (60 mg/kg/day) was administered for 5 consecutive days. Mice were sacrificed on day 8 post-chemotherapy, and bone marrow samples were collected and processed using standard methods. Leukemic engraftment was determined by flow cytometry gating on the human CD45$^+$CD34$^+$ population. For the DNT killing assay, human CD45$^+$ cells were separated from pooled bone marrows from each treatment group using human magnetic CD45 MicroBeads (Miltenyi Biotec). For the in-vivo DNT killing experiments, MOLM13 cells (1x10^6) were injected into the right femur of pre-conditioned NSG mice sub-lethally irradiated (2 Gy) 24h prior to injection. Three days after cell injection, intravenous Ara-C treatment (40 mg/kg/day) was administered

for 5 consecutive days. 20×10^6 DNTs were then injected i.v. on days 11 and 13 along with rIL2 (Proleukin, 104 IU/mouse). Mice were sacrificed on day14 and engraftment was measured by flow cytometry gating on human $CD3^-CD45^+$ cells. All animal studies were performed in accordance with the University Health Network Animal Use Protocol.

Fatty acid uptake

Fatty acid uptake was assessed using BODIPY-FL-C_{16} (Invitrogen, #D3821). Cells were incubated in media with 1uM BODIPY-C16 for 30 minutes. Fatty acid uptake was measured using flow cytometry measuring the mean fluorescence intensity (MFI) of BODIPY-FL-C_{16}.

Viability

Cell viability following chemotherapy treatment was assessed by Alamar Blue viability kit (Invitrogen) as per the manufacturer's instructions. Viability of cells cultured at 31^0C was assessed using the Sulforhodamine B (SRB) assay following a previously described protocol[51].

Daunorubicin uptake

MOLM13 cells were treated with increasing concentrations of Daunorubicin for 3 hours. Daunorubicin uptake was assessed via the PE channel in flow cytometry.

Flow Cytometry

Flow cytometry analysis was performed on Cytoflex (Beckman Coulter Life Sciences) or LSR Fortessa (BD Biosciences). Data was analyzed post-acquisition with FlowJO Software Version 10.1. Patient samples were stained with anti-CD117 (Biolegend, #313230), anti-CD34 (BD, #560710), anti-CD45 (BD, #564585) antibodies and 7-AAD.

Cell cycle and proliferation analysis

Cells were incubated with EdU (10 uM) for 2 or 72 hours. Cell cycle was assessed using EdU Staining Proliferation Kit (Abcam, #219801) and DAPI, as per the manufacturer's instructions, and analyzed by flow cytometry. For the dye dilution assay, cells were stained

with CellTrace Violet (ThermoFisher, # C34557) according to manufacturer protocol. Dye dilution was determined through flow cytometry on days 1 and 5.

Lipidomics

Lipids were extracted from frozen cell pellets using a methyl tert-butyl ether (MTBE)-based extraction method[52,53]. Dried lipid extracts were reconstituted and analyzed using a Horizon Vanquish UHPLC system coupled to an Orbitrap IQ-X Tribrid Mass Spectrometer (Thermo Fisher Scientific). Raw spectral data were processed and analyzed using MS-DIAL (version 4.9.221218) and the MS-DIAL LipidBlast spectral library (version 68)[54]. To detect drug-/temperature-dependent changes in FA carbon chain lengths among lipids of the same lipid class, distributions of LC-MS signal (area), aggregated by FA carbon chain length and lipid class were compared. First, areas were summed for lipids of the same FA carbon chain length and class. These areas were then normalized to proportion values, which represent proportional contributions by lipids of a particular FA carbon chain length to the total area detected for a lipid class. To do so, each area was divided by the sum of all area values within its respective lipid class. To produce difference plots, difference values were calculated between mean proportion values of lipids with the same FA carbon chain length and class.

DNT killing assay

DNT expansion was performed as previously described[55]. Briefly, healthy donor–derived PBMCs depleted from $CD4^+$ and $CD8^+$ cells were cultured on anti-CD3 antibody-coated plates (OKT3; BioLegend) for 3 days in AIM-V (Thermo Fisher Scientific) with 250 IU/mL of interleukin-2 (IL-2) (Proleukin; Novartis Pharmaceuticals); soluble anti-CD3 antibody, IL-2 and fresh AIM-V were added to the cultures every 2 to 4 days. DNTs cells purity was evaluated by staining cells with fluorochrome-conjugated anti-human CD3, -CD4, -CD8, and -CD56 antibodies followed by flow cytometry analysis. For the killing assay, DNTs were used between days 10 and 20 of culture and co-cultured with target cells for 2 hours at a ratio of 1:2 target cells to DNT cells. Subsequently, cells were stained with anti-human CD3 (HIT3a), CD33 (WM53), CD45 (HI30), and CD34 (561) antibodies and Annexin V

(all from BioLegend) and analyzed by using flow cytometry. Specific killing was calculated by $\frac{\%AnnexinV^{+}_{with\ DNT} - \%AnnexinV^{+}_{without\ DNT}}{100 - \%AnnexinV^{+}_{without\ DNT}}$ x 100, as previously described[56].

Statistical analysis

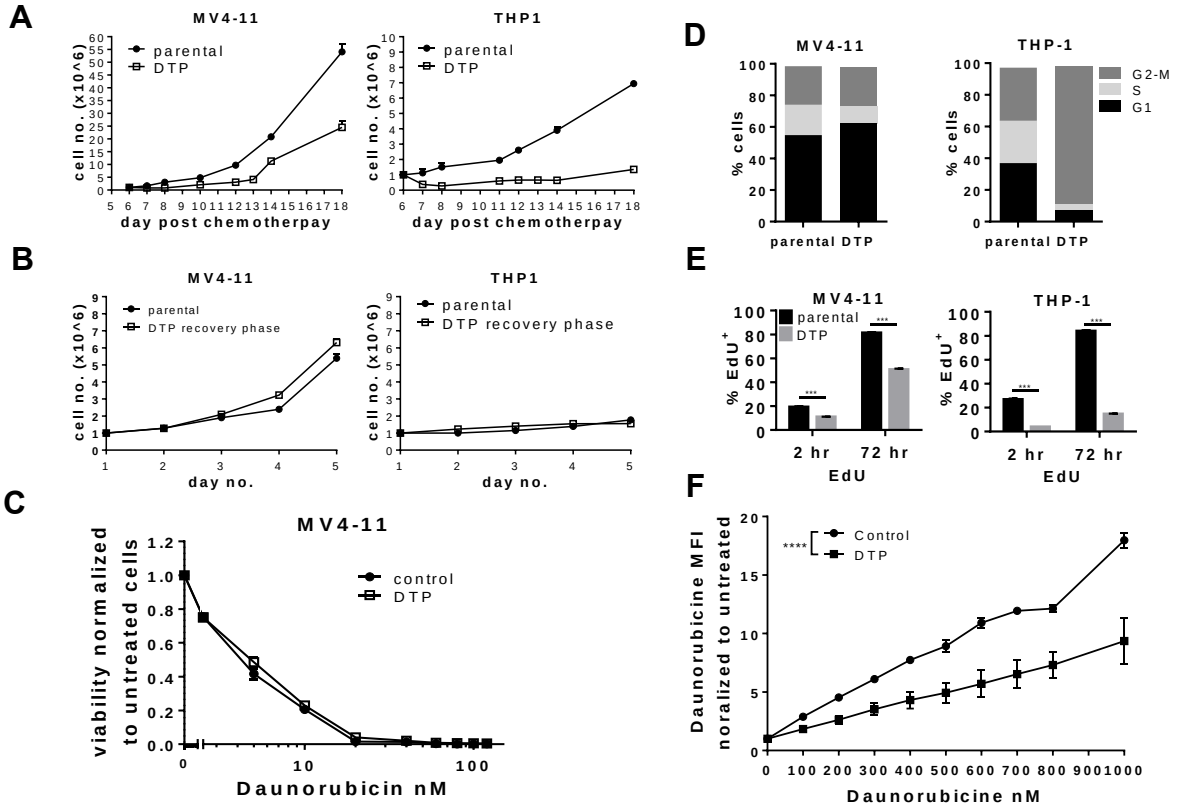
Statistical tests included unpaired Student's t-test or 2-way ANOVA. Statistical significance was established at $p < 0.05$. Comparable variability between groups was observed in both animal and in vitro studies. Sample sizes were determined based on experimental feasibility and previous experiences managing replicates.

Table S1. Clinical data of primary AML samples

Patient no.	Age	Gender	Diagnosis	Cytogenetics	Molecular
1401	59	M	AML	46,XY	NPM1 undetectable, FLT3-ITD undetectable
0856	52	M	AML	complex	NPM1 undetectable, FLT3-ITD undetectable
1211	57	M	AML post MDS	unsuccessful	NPM1 undetectable, FLT3-ITD undetectable
166315	72	F	AML	47,XX,+8[17]	NPM1 undetectable, FLT3-ITD undetectable

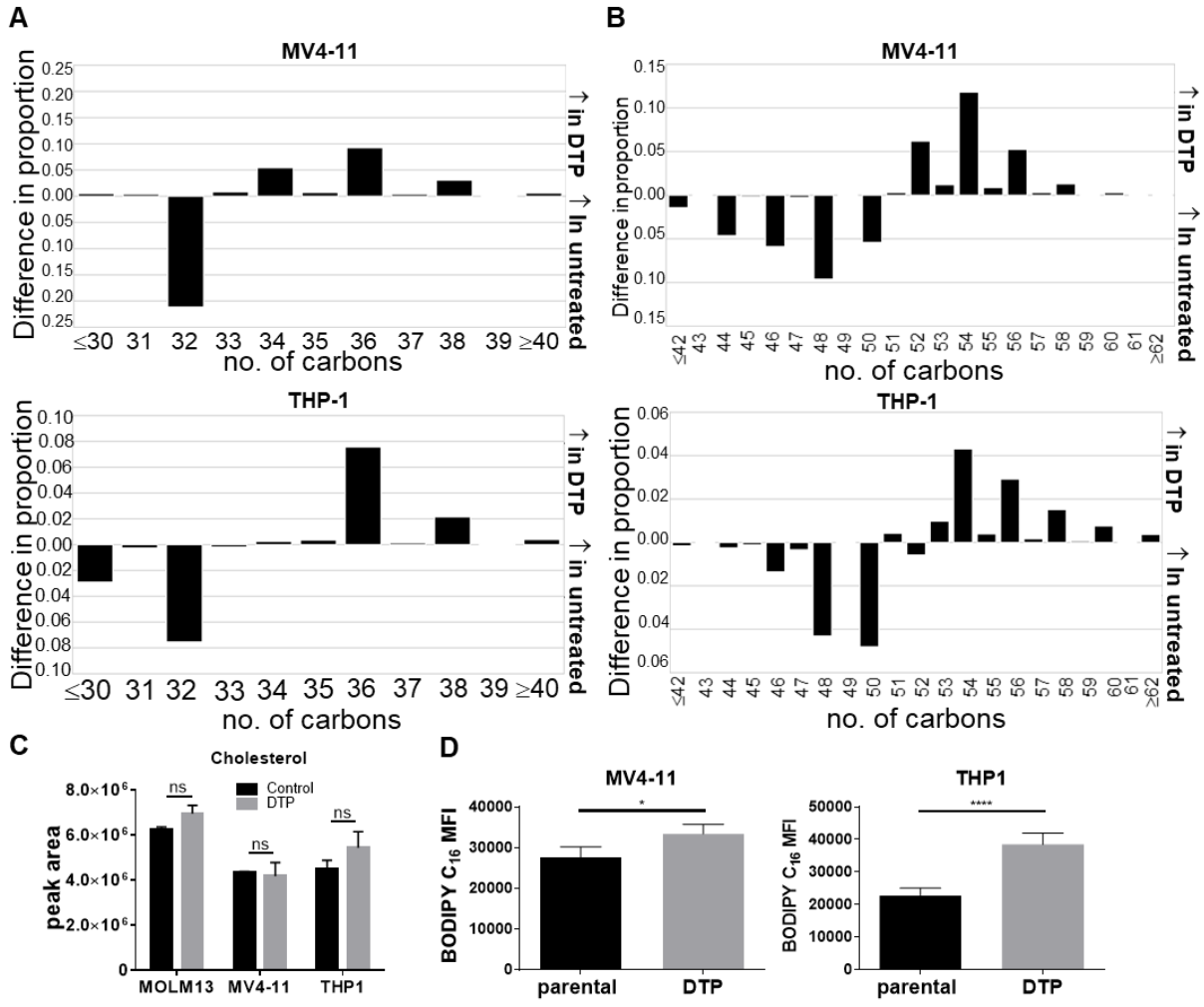
Supplementary Figures

Supplemental Figure 1



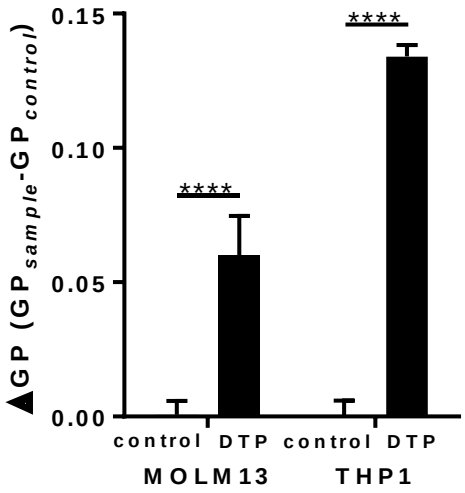
Supplemental Figure 1. AML DTP cells in-vitro model; A. MV4-11 and THP1 drug tolerant persistent (DTP) cells were treated with the combination of Daunorubicin (20 and 90 nM, respectively) and Ara-C (70, and 900 nM respectively) to achieve an IC90. Residual viable cells were collected on day 6 after Daunorubicin 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 +SD from 3 independent experiments. B. Mean + SD growth and viability of persisting MV4-11 and THP1 over time starting 20 days post Daunorubicin and Ara-C treatment as measured by trypan blue exclusion staining. Data represent the mean +SD from 3 independent experiments. C. Persisting MV4-11 cells were collected on day 6 after Daunorubicin (20nM) and Ara- C (70nM) 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 mean+SD growth and viability from a representative experiment (n=2). D. Cell cycle analysis as measured by PI staining and flow cytometry in persisting MV4-11, and THP1 cells collected on day 6 post Daunorubicin and Ara-C treatment. Data represents mean + SD from a representative experiment (n=2). E. Wild type and persisting MV411, and THP1 cells were labelled with EdU. EdU uptake was measured by flow cytometry 2 and 72 hours post treatment. Data represents mean + SD from a representative experiment (n=2). F. Control and DTP MOLM13 cells incubated with Daunorubicin at increasing concentrations. Daunorubicin uptake was assessed by flow cytometry. Data represents the mean + SD of 3 independent experiments. ****P < .0001 by 2-way ANOVA test.

Supplemental Figure 2



Supplemental Figure 2. Lipid composition in DTP cells; **A.** Difference in proportion of PC (phosphatidylcholine) species between DTP and control samples in MV4-11 and THP1 cell lines. Species above the x-axis are enriched in control samples, and species below are enriched in DTP samples. **B.** Difference in proportion of TG (Triglycerides) species between DTP and control samples in MV4-11 and THP1 cell lines. **C.** Cholesterol levels of MOLM13, MV4-11 and THP-1 DTP cells collected on day 6 after Daunorubicin and Ara-C treatment. Data represents the mean ± SD (n=3). **D.** MV41-11 and THP1 DTP cells were collected on day 6 after Daunorubicin and Ara-C treatment and incubated with BODIPY-FL-C16. BODIPY-FL-C16 MFI values measured by flow cytometry. Data represents the mean ± SD from 3 independent experiments.

Supplemental Figure 3



Supplemental Figure 3. Leukemic DTP cells exhibit decreased cell membrane fluidity. Quantiative analysis of di-4-ANEPPDHQ generalized polarization (GP) in MOLM13 and THP1 cells as measured by flow cytometry. Data represents the mean+SD of 3 independent experiments. **** $P < .0001$ by student t-test.