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Marisa Mercadante, Armin Scheben, Jacob Estrada, Jan Savas-Carstens, William Sullivan, Nicholas Housel, Maria Sapor, Tatiana Volpari, Jax Hebner, Tomasz Rusielewicz, Frederick J. Monsma Jr, Stefan Semrau, Yinan Wang & Laura A. Martin

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Large scale quantification of natural killer cell-induced apoptosis in patient-derived organoids reveals intratumoral response heterogeneity

Marisa Mercadante^{1,3#}, Armin Scheben^{1,3#}, Jacob Estrada^{1#}, Jan Savas-Carstens¹, William Sullivan¹, Nicholas House², Maria Sapor^{1,3}, Tatiana Volpari^{1,4}, Jax Hebner¹, Tomasz Rusielewicz^{1,3}, Frederick J Monsma Jr.¹, Stefan Semrau^{1,3*}, Yinan Wang^{1,3*}, Laura A Martin^{1,3*}

¹ The New York Stem Cell Foundation Research Institute, New York, NY

² The Stevens Institute of Technology, Hoboken, NJ

³ Current affiliation: JAX-NYSCF Collaborative

⁴ Current address: San Raffaele Telethon Institute for Gene Therapy

#Contributed equally

*Correspondence: Laura A. Martin (Laura.AndresMartin@jax.org); Yinan Wang (Yinan.Wang@jax.org); Stefan Semrau (Stefan.Semrau@jax.org).

Abstract

Intratumoral heterogeneity drives immunotherapy resistance, hindering clinical efficacy. Functional assays that capture heterogeneous responses to immunotherapies remain limited. Here, we present a 3D live imaging assay using patient-derived ovarian cancer organoids to quantify natural killer (NK) cell-induced apoptosis across hundreds of individual organoids. Our assay reveals inter- and intratumor response heterogeneity and detects rare populations unresponsive to NK cytotoxicity, enabling the study of subclonal immune responses and underlying mechanisms.

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Main Text

Inter- and intratumor heterogeneity drive resistance to therapies and most cancer-related deaths¹. Immunotherapies are revolutionizing cancer treatment for certain patients, but heterogeneity in advanced-stage solid cancers hinders their efficacy. A major roadblock to developing effective immunotherapies for heterogeneous cancers is characterizing patient-specific tumor-immune cell interactions that are driving a diverse array of responses, including resistance.

Ovarian cancer is one of the most heterogeneous cancer types, diagnosed in the metastatic stage for most patients. Ovarian cancer tumors arise from divergent clonal trajectories and present heterogeneous immune cellularity and immune escape mechanisms^{2,3}. In contrast to the results of T cell-based therapies and immune checkpoint inhibitors⁴, natural killer (NK) cells have shown promise in clinical trials for patients presenting with refractory ovarian cancer⁵. Allogeneic NK cells are emerging as a promising strategy with a better safety profile than CAR-T cells and cytotoxic activity independent of surface target antigens, which might have advantages in heterogeneous tumors⁶. Among NK cell sources, induced pluripotent stem cells (iPSCs)-derived NK cells (iNKs) are amenable to genetic engineering, and can be produced at scale with uniform quality. Thus, clinical-grade iNKs are in development to exploit them as a potential off-the-shelf cell therapy product⁷.

Further optimization of NK cell – or other immunotherapies requires suitable preclinical models that recapitulate tumor heterogeneity. Patient-derived tumor organoids (PDOs) provide an invaluable model system for studying tumor specific immune-cancer cell interactions and for optimizing immunotherapy strategies^{8,9}. PDOs maintain the complex 3D tissue architecture of primary tumors and recapitulate individual tumor phenotypes, including inter- and intratumor drug response heterogeneity¹⁰ and patient responses to chemotherapies^{11,12}. In short-term patient-derived ovarian cancer 3D cultures NK cells showed an anti-tumoral molecular phenotype after immune checkpoint inhibition *ex vivo*¹³. This demonstrated the critical role of NK cells in the

response to immunotherapy in ovarian cancer. Breast cancer PDOs have been shown to capture heterogeneous responses to T cells, intriguingly even within the same PDO culture¹⁴. This shows that PDO-based functional immunoassays can be used to screen for sensitivity and resistance to T-cell immunotherapies across patients and within the same tumor. Similar strategies to evaluate the effectiveness and heterogeneous tumor responses to NK cells are lacking. All these findings encouraged us to explore whether variations in responsiveness to NK cells exist and could be captured in patient-derived ovarian cancer organoids.

Here, we report the development of an ovarian cancer PDO co-culture system with iNK cells in combination with 3D live-cell high-content imaging. We found that responses to iNK cytotoxic activity vary between individual organoids within the same culture. We show that this heterogeneity was different between PDOs established from different ovarian cancer patients. In addition, we identified a rare subpopulation of organoids that are unresponsive to NK cell cytotoxicity. Our results provide evidence that ovarian cancer PDOs can be used to reveal subclonal response heterogeneity to NK cell-based immunotherapies and discern differences between patients or tumor subtypes.

In this study, we adapted published protocols¹⁵ to derive PDOs from a high-grade serous ovarian carcinoma resected from the omentum (PDO-1) and an ovarian mucinous carcinoma resected from the left ovary (PDO-2) (Figure 1a; Figure S1). Histological analysis and whole exome sequencing (WES) of tumor/organoid pairs demonstrated that PDOs recapitulated the morphological and molecular features of the tumor they were established from (Text S1-S2; Figures S1-S3; Data S1-S3). We found 80% concordance of the gene copy number alterations and 100% concordance of the single-nucleotide variants across previously characterized genomic signatures of ovarian cancer^{2,16,17}. iNKs were generated from iPSCs using established differentiation protocols¹⁸ (Figure 1b). We consistently obtained >90% CD45+CD56+ cells that also expressed NK-specific inhibitory and activating receptors (Figure 1b-c) and displayed cytotoxic activity against the leukemia cell line K562 (Figure 1d). To reduce assay variability, we

generated a large batch of iNK cells that were then cryopreserved after phenotypic and functional validation. We also verified that cryopreserved iNKs maintain killing activity comparable to freshly differentiated iNKs in cytotoxic assays against K562 cells, confirming that they could be used in downstream killing assays (Figure 1e).

We next tested which media conditions enable simultaneous maintenance of ovarian cancer PDOs and iNKs in co-culture. First, we evaluated the viability of iNK cells exposed to organoid media (tOva) or a 1:1 (v/v) iNK media-organoid media mixture (iNK:tOva media). We also tested viability in organoid media without nicotinamide (tOva-B3), since a previous study showed that nicotinamide interferes with the functionality of CAR-engineered NK-92 cells in a patient-derived colorectal cancer organoid system⁸. When compared to iNK media, tOva and tOva-B3 conditions significantly decreased the amount of CD45+CD56+ cells after 24 h, although the remaining CD45+CD56+ cells remained viable. In contrast, iNK:tOva media conditions allowed the CD45+CD56+ population to remain stable and viable even after 24 hours (Figure 1f). To determine PDO viability in iNK:tOva and iNK media conditions, we performed cell viability assays using CellTiter-Glo® 3D reagent to quantify ATP levels. Intact organoids were collected, counted using optimized pipelines with the Keyence hybrid cell count analysis software (Figure S4), and plated in 384-well plates at 1,000 organoids per well. In comparison to the viability in standard tOva media conditions, exposure to iNK media resulted in a significant decrease in PDO-1 viability. In contrast, both PDO lines remained viable in iNK:tOva media for at least 20 h (Figure 1g). Based on these results, we chose iNK:tOva media conditions to develop our live cell imaging assay in iNK/ovarian PDO co-cultures.

For live-cell imaging, formed organoids were collected intact from the Cultrex® Basement Membrane Extract (BME) at day 6 after plating, a timepoint at which most organoids range from

30-70 μm in diameter (Figure 2a). The collected organoids were then labeled with CellTracker™ Orange CMRA, filtered using a 70 μm cell strainer, and counted as described above (Figure S4). In parallel, iNKs were collected from an overnight culture after thaw and labeled with CellTrace™ Calcein Blue. Next, labeled iNKs and organoids were mixed in iNK:tOva media containing 5% Cultrex® BME, and plated in a pre-coated 96-well plate at a 20:1 effector (iNKs) to target (organoids) (E:T) ratio per well^{8,19}. To detect apoptotic events, CellEvent™ Caspase-3/7 Green detection reagent was added at the time of plating. This reagent consists of a peptide conjugated to a nucleic acid binding dye that only binds to DNA and produces a fluorogenic signal after cleavage by activated caspase-3/7. As a control, labeled organoids were plated in the same conditions without iNKs in parallel wells (i.e., monoculture controls). Co-culture and monoculture controls were incubated for 30-45 minutes and then subjected to live cell imaging in an Opera Phenix™ High Content Screening system, which utilizes an advanced confocal spinning-disk technology providing high 3D imaging resolution with four fluorescence channels imaged simultaneously. Images were acquired every 15 minutes for up to 11 h (Figure 2b), capturing the tumor organoid-iNK cell interactions and caspase-3/7 activation dynamics across >500 individual organoids simultaneously per condition (Figure 2c; Figure S5e-f). We next created an image analysis pipeline, using the Opera Phenix™ Harmony software, to quantify the area of each individual CMRA-labeled organoid positive for activated caspase-3/7 signal at each acquisition time point as a measurement of each organoid's susceptibility to iNK cytotoxic activity (Figure 2d-f; Figure S5a-d; Figure S6). As evident from monoculture controls, our labeling and co-culture media conditions did not significantly induce apoptosis. In contrast, we observed a robust apoptotic signal in organoids co-cultured with iNKs that increased over time (Figure 2e-f; Figure S5a-d). We observed differences in response kinetics (Figure 2e) as well as overall response (Figure 2f) between the PDO lines studied (Figure S5a-d). Importantly, individual organoids within the same PDO culture displayed varying levels of apoptotic signal, suggesting that tumor immune heterogeneity is preserved in ovarian cancer PDOs (Figure 2f; Figure S5a-d). Interestingly, we

also observed that, in both PDO lines, a small fraction of organoids displayed no apoptotic events throughout the assay, which we hypothesize could correspond to resistant subpopulations (Figure 2g).

We next sought to further characterize the non-apoptotic and apoptotic organoid subpopulations and compare their frequency between the studied PDO lines. Thus, we applied a Bayesian Beta mixture model with two components to the distribution of organoid apoptotic area at the experimental endpoint (Figure 3a). With this model, we inferred that the fraction of apoptotic organoids was 0.93 (CI:0.92-0.94) and their average apoptotic area was 43.8% (CI:43.3-44.5%), whereas the potentially resistant non-apoptotic population had an average apoptotic area of 12.0% (CI:10.1-14.6%). Based on the modeling results, we found a threshold of 8.6% apoptotic area to optimally distinguish between non-apoptotic and apoptotic organoid subpopulations. By applying this threshold across all time points of the experiment, we found that the mean apoptotic area increased from 13.5-23.1% at the initial quantification time point to 32.0-49.1% during the experiment, but was stable for non-apoptotic organoids, resembling the control range (Figure 3b). Moreover, we found that the proportion of apoptotic organoids increased sharply at the beginning of the experiment, and thus, by 5 h, the majority of organoids (>75%) displayed apoptotic events in both PDO co-cultures (Figure 3c). Towards the end of the experiment, the proportion of apoptotic organoids plateaued in a sigmoid fashion, indicating a persisting population of resistant organoids. In contrast, most organoids (>90%) remained alive in monoculture controls throughout the duration of the assay (Figure 3c). We then tested logistic, Hill, and Gompertz models for fitting the kinetics of organoid apoptosis and found the Gompertz model to fit best to both datasets ($p < 0.001$ and $p < 0.004$, respectively) (Figure S9). From this model, we estimated, for both PDO lines combined, a non-apoptotic organoid fraction of 6.2% (CI:4.9-7.5%) and a difference of 1.4% (CI:0.2-2.8%) in non-apoptotic fraction between PDOs (Figure 3d). Intriguingly, we were able to visualize iNKs actively engaging with organoids with low or entirely absent apoptotic signals

throughout the duration of the assay (Figure 4; Videos S1-S2). To rule out that the non-apoptotic subpopulation was an artefact of incomplete detection of apoptosis, we conducted an agent-based simulation and fit the results to our data, which supported the presence of a non-apoptotic subpopulation (Text S6; Figures S7-S8; Data S4). We next evaluated whether individual organoid size could be a predictor of the observed differential response. When we looked at the relationship between organoid diameter and apoptotic area in the monoculture controls, it showed a weak negative relationship, indicative of smaller organoids exhibiting higher apoptotic area rates with Pearson's correlation (R) ranging from -0.084 to -0.25 across replicates (Figure 3e). Interestingly, in the co-culture conditions, we observed two subpopulations at the endpoint (31% are small organoids with a mean of 28 μm diameter and 69% are larger ones with a mean of 247 μm diameter) (Figure S10) with potentially resistant non-apoptotic organoids enriched in the small subpopulation (odds ratio=3.64, $p<2\text{e-}16$, Fisher's exact test), for both PDO lines (Figure 3f). Apoptotic organoids were found in both the small and large subpopulations. This suggests that small size is necessary but not sufficient for a resistant response. Intriguingly, PDO lines differed in the relationship between size and apoptotic area: While sensitive organoids in PDO-1 showed a negative relationship between organoid diameter and apoptotic area across the three replicates, this relationship was positive in PDO-2 ($R=0.43\text{-}0.59$) (Figure 3f). Taken together, these results demonstrate the usefulness of our platform to detect and characterize subclonal response heterogeneity and patient-specific responses to immunotherapies.

Genomic and transcriptomic characterization of advanced-stage cancers is not sufficient to identify immune-evading subclones²⁰, underscoring the need for new functional platforms to study immune responses in heterogeneous tumors. PDOs in co-culture with immune cells are emerging as powerful *in vitro* functional systems to assess and optimize immunotherapeutic strategies for treating solid tumors. A variety of platforms have been established, but for the most part, these capture organoid culture responses in bulk, precluding the observation of sub-clonal response

differences to immune cells that may be recapitulated in organoid culture systems. A pioneering study reported intratumoral heterogeneous responses to T cells in breast cancer organoid cultures¹⁴, further highlighting the value of PDO-based immunoassays to understand the complexity of the immune-cancer crosstalk. Here we present a PDO-based functional platform for ovarian cancer that can effectively probe NK cells' cytotoxic activity and capture inter- and intra-tumor ovarian cancer heterogeneity. Ovarian cancer is one of the most heterogeneous cancer types, leading to frequent relapses with tumors resistant to treatments. Emerging evidence strongly supports the development of NK cell-based immunotherapies to treat ovarian cancer²¹⁻²³, but the detailed mechanisms that drive responses to NKs in ovarian tumors remain largely unexplored²⁴. Our assay enables the quantification of iNK-mediated apoptosis across hundreds of individual organoids within the same tumor and across different PDOs (Figure 2; Figure S5). Our assay is robust, reproducible, and provides the means to correlate responses with tumor-iNK cell interactions, capturing heterogeneous response phenotypes within the same tumor, allowing assessment of the potential effectiveness of NK cell-based therapies in candidate patient populations. Intriguingly, our system captures individual organoid responses, including a rare subset that remains intact even after active interaction with iNK cells, suggesting that these organoids potentially represent an immune-evading sub-clonal population that may drive therapy resistance (Figures 4; Videos S1-S2). During the revision of our study, Dijkstra *et al* have just reported the characterization of sub-clonal immune evasion in non-small cell lung cancer organoids co-cultured with primary immune cells, using clonal organoid libraries²⁰. These studies^{14,20}, together with ours, demonstrate the usefulness of PDO-based co-culture platforms for studying immune evasion in complex solid tumors. Our platform is amenable to functional immunotherapy and/or compound testing, which, in combination with our live imaging assay, enables us to assess the effectiveness of candidate strategies with the potential to sensitize immune-evading subclones. The methodology presented here also allows us to enrich for immune-evading organoid subclones given the distinctive self-renewal capability of PDOs that

enables further expansion after exposure to therapeutic interventions. Once isolated, these organoid subclones can be used to identify the molecular underpinnings of their specific responses and devise strategies to overcome resistance. Overall, our technology offers a promising platform for functional testing that can be adapted to other solid tumor types and/or immune cells so that the effects of immune modulatory agents can be assessed and optimized to enhance immune cell killing activity in resistant cells.

Methods

Ethical regulations

The activities performed, as approved by the Biomedical Research Alliance of New York Institutional Review Board (IRB), under IRB 23-02-739-1209 protocol were conducted in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards (the “Study”).

Informed consent for material acquisition used in the Study was obtained from the participants (or their parent or legal guardian in the case of children under the age of 16) by the Cooperative Human Tissue Network (CHTN) and Memorial Sloan Kettering Cancer Center (MSKCC). Coded tumor material were obtained from participants diagnosed with ovarian cancer and provided by MSKCC (IRB 06-107 A (21)) and CHTN (2014H0130). CHTN is funded by the National Cancer Institute. Other investigators may have received specimens from the same subjects.

Cell lines

K-562 (CCL-243™) or K-562-GFP (CCL-243-GFP™) cells were obtained from The American Type Culture Collection (ATCC®) and cultured according to ATCC’s instructions, in Gibco™ RPMI (ThermoFisher Scientific, Cat. No. 61870036), 10% Gibco™ certified, heat-inactivated FBS (ThermoFisher Scientific, 10082147), 1 x Gibco™ MEM Non-essential amino acids (NEAA) (ThermoFisher Scientific, Cat. No. 11140050), 1 x Gibco™ GlutaMAX™ Supplement (ThermoFisher Scientific, 35050061), 1 x Gibco™ Penicillin/Streptomycin (50 U/mL)

(ThermoFisher Scientific, Cat. No. 15070063) 50 μ M Gibco™ 2-Mercaptoethanol (ThermoFisher Scientific, Cat. No. 21985-023), 10 mM Gibco™ HEPES (ThermoFisher Cat. No. 15-630-080).

Derivation and culture of patient-derived organoids from ovarian cancer tissues

Patient-derived organoids (PDOs) were established from fresh tumor resections as described previously¹⁵ with slight modifications. Briefly, tumor tissues were digested in Gibco™ Advanced DMEM/F12 media (ThermoFisher Scientific, Cat. No. 12634-028) containing 1 x Gibco™ GlutaMAX (ThermoFisher Scientific, Cat. No. 35050061), 1 x Penicillin Streptomycin (10,000 U/ml) (Life Technologies, Cat. No. 15140122), 10 mM HEPES (ThermoFisher Scientific, Cat. No. 15-630-080), 100 μ g/mL Primocin® (InvivoGen, Cat. No. ant-pm-1), 10 μ M Y-27632 ROCK inhibitor (AbMole, Cat. No. M1817) and 0.7 mg/ml Collagenase XI (Sigma, Cat. No. C9407) for 25 minutes at 37°C. The digested tissue was then filtered through a 100 μ m cell strainer and centrifuged at 300 x g for 5 minutes at 4°C. When needed, the resulting pellet was treated with 3 mL of Red Blood Cell Lysis buffer (Sigma, Cat. No. 11814389001) for 5-minutes at room temperature and then centrifuged at 300 x g, 4°C for 5 minutes. For plating, the pellet was resuspended in an ice-cold 100% Cultrex® Reduced Growth Factor BME, Type 2, PathClear™ (Cultrex® BME) (R&D Systems, Cat. No. 3533-010-02) and droplets containing 4000-10,000 cells/ μ l were plated on pre-warmed multiwell tissue culture plates (Greiner CELLSTAR®, Cat. No. 677102). After plating, plates were placed in the incubator at 37°C to allow the Cultrex® BME to solidify for 30-60 minutes at 37°C. Once solidified, the domes with embedded cells were topped with pre-warmed ovarian tumor organoid medium (tOva) composed of Gibco™ Advanced DMEM/F12 (ThermoFisher Scientific, Cat. No. 12634-028), 1 x Gibco™ GlutaMAX (ThermoFisher Scientific, Cat. No. 35050061), 1 x Penicillin Streptomycin (10,000 U/ml) (Life Technologies, Cat. No. 15140122), 10 mM HEPES (ThermoFisher Scientific, Cat. No. 15-630-080), 100 μ g/mL Primocin® (InvivoGen, Cat. No. ant-pm-1), 1 x Gibco™ B-27™ Supplement (50x), (Life Technologies, Cat. No. 17504-044), 1.25 mM N-Acetyl-L-cysteine (Sigma-Aldrich, Cat. No.

A9165), 10 mM Nicotinamide (Sigma-Aldrich, Cat. No. N0636), 0.5 μ M A83-01 (Tocris, Cat. No. 2939), 0.5 μ g/mL Hydrocortisone (Sigma-Aldrich, Cat. No. H0888), 10 μ M Forskolin (R&D Systems, Cat. No. 1099), 100 nM β -Estradiol (Sigma-Aldrich, Cat. No. E2758), 16.3 μ g/ml Bovine Pituitary Extract (BPE) ThermoFisher Scientific, Cat. No. 13028014), 10 ng/mL Recombinant Human FGF-10 (PeproTech, Cat. No. 100-26), 5 ng/mL Recombinant Human KGF (FGF-7) (PeproTech, Cat. No. 100-19), 37.5 ng/mL Recombinant Human Heregulin Beta-1 (PeproTech, Cat. No. 100-03), 5 ng/mL Recombinant Human EGF (PeproTech, Cat. No. AF-100-15), 100 ng/ml Recombinant Human R-Spondin-1 (PeproTech, Cat. No. 120-38), 1% Noggin-Fc Fusion Protein Conditioned Medium (Immunoprecise, Cat. No. N002), 0.5 nM WNT Surrogate-Fc fusion protein (ImmunoPrecise, Cat. No. N001) and containing 10 μ M Y-27632 ROCK inhibitor (AbMole, cat. No. M1817) and then incubated for organoid formation with media changes every 2–3 days and passaging every 1-2 weeks for expansion and biobanking. PDOs are routinely tested with our PDO culture-adapted PCR-based Mycoplasma contamination test (Mycoplasma PCR Detection Kit, Cat. No. G238). For biobanking, dense organoid cultures were cryopreserved as small cell clusters/single cells in Recovery™ Cell Culture Freezing Medium (ThermoFisher Scientific, Cat. No. 12648010) at a concentration of $0.8\text{--}1.2 \times 10^6$ cells per 500 μ l. For co-culture and live cell imaging experiments, ovarian cancer PDOs were retrieved from the NYSCF organoid biobank. Cryopreserved organoids were thawed, plated, and expanded for at least 7 days prior to use in co-culture experiments. Briefly, after thawing, cells were resuspended in 70-100% (depending on the specific PDO line requirements) cold Cultrex® Reduced Growth Factor BME, Type 2, PathClear™ (Cultrex® BME) (R&D Systems, Cat. No. 3533-010-02) and droplets were plated on pre-warmed multiwell tissue culture plates (Greiner CELLSTAR®, Cat. No. 677102). Droplets were allowed to solidify at 37°C inside an incubator for at least 30 minutes, then overlaid with tOva medium as described above, containing 10 μ M Y-27632 ROCK inhibitor (AbMole, Cat. No. M1817). The medium was changed every 2–3 days for 7–10 days until dense organoid cultures were formed with organoids ranging in size from 100 to 500 μ m. Organoids were then

passaged with Gibco™ TrypLE™ Select Enzyme (ThermoFisher Scientific, Cat. No. 12563011), containing 10 μ M Y-27632 ROCK inhibitor (AbMole, Cat. No. M1817) and 10 μ g/mL DNase I (Sigma-Aldrich, Cat. No. DN25), resuspended in 70-100% cold Cultrex® Reduced Growth Factor BME, Type 2, PathClear™ (Cultrex BME) (R&D Systems, Cat. No. 3533-010-02) plated in droplets at 800 cells/ μ L and cultured for further expansion or use in co-culture experiments.

iPSC generation and culture

To reduce line-to-line variability, we used only one iPSC line generated by reprogramming human hematopoietic stem/progenitor cells isolated from peripheral whole blood from a healthy donor. Peripheral whole blood was obtained from Comprehensive Cell Solutions, an operating division of the New York Blood Center, Inc. Following Ficoll separation, PBMCs were sorted to obtain hematopoietic stem/progenitor cells (EasySep™ Human Progenitor Cell Enrichment Kit, Stemcell Technologies, Cat. No. 17936,) and cultured for three days in media containing IL-3 (CellGenix, Cat. No. 1002-050), IL-6 (CellGenix, Cat. No. 1004-050), FLT-3 (CellGenix, Cat. No. 1015-050), TPO (CellGenix, Cat. No. 1017-050), and SCF (CellGenix, Cat. No. 1018-050) to enrich for CD34+ cell population, after which they were reprogrammed using CytoTune™-iPS 2.0 Sendai Reprogramming Kit (ThermoFisher, Cat. No. # A16517) according to manufacturer instructions. Individual clones were manually picked and passaged/expanded in Gibco™ Essential 8™ Medium (ThermoFisher Scientific, Cat. No. A1517001) on Recombinant human truncated Vitronectin-coated tissue culture-treated vessels (Gibco™ CTS™ VTN-N, ThermoFisher Scientific, Cat. No. CTS279S3) using Gibco™ CTS™ TrypLE™ Select Enzyme (ThermoFisher Scientific, Cat. No. A4738001) and cryopreserved in BiolifeSolutions® CryoStor® CS10 Cell Freezing Medium according to the manufacturer instructions (StemCell Technologies, Cat. No. 07930).

Natural killer cells generation from iPSCs and culture

iPSCs were retrieved from our biobank and expanded as described prior to their differentiation into Natural Killer cells (iNKs). For iNK generation, we used a spin embryoid body (EB) protocol

adapted from previously published procedures²⁵. For EB formation, iPSCs were treated with CTS™ TrypLE™ Select (ThermoFisher Scientific, Cat. No. A1285901) to yield a single cell iPSC suspension. To render one EB per well, iPSCs were seeded into an entire 96-well Clear Round Bottom Not-TC Treated Microplates (Falcon®, Corning, Cat. No. 351177) at 8,000 per well, in 150 µL of STEMdiff™ APEL™2 Medium (StemCell Technologies, Cat. No. 05270) containing 50 ng/mL recombinant human bone morphogenetic protein 4 BMP-4 (R&D Systems, Cat. No. 314-BP/CF), 20 ng/mL recombinant human FGF basic (bFGF) (146 aa) Protein (R&D Systems, Cat. No. 233-FB), 50 ng/mL recombinant human vascular endothelial growth factor (VEGF) 165 Protein, CF (R&D Systems, Cat. No. 293-VE), 50 ng/mL recombinant human stem cell factor SCF (Peprotech, Cat. No. 300-07) and 10 µM Y-27532 ROCK inhibitor (StemCell Technologies, Cat. No. 72302). After spinning the plates at 300 x g for 5 min at room temperature (RT), they were cultured at normoxic (20% O₂, 5% CO₂) condition in a 37 °C incubator for 6 days. After 6 days, 15 EBs were collected and plated into one well of a 6-well TC-treated culture vessels coated with 10 µg/mL laminin 521 (BioLamina, Cat. No. MX521). For coating, plates were incubated for 1 hour at 37 °C. EB were then cultured for 28-30 days (D28-D30) in 5 mL NK differentiation medium with half volume media changes every 2-4 days. Using this protocol, approximately 1-5 x 10⁶ iNK cells can be collected from one single well of a 6-well plate. During the first 7 days, the NK differentiation medium consisted in 85% 2:1 (v/v) mixture of Gibco™ DMEM (ThermoFisher Scientific, Cat. No. 10569010) and Gibco™ Ham's F-12 Nutrient Mix (ThermoFisher Scientific, Cat. No. 31765035), 15% human AB serum (GeminiBio, Cat. No. 100-612), 1 x Gibco™ MEM Non-essential amino acids (NEAA) (ThermoFisher Scientific, Cat. No. 11140050), 1 x Gibco™ GlutaMAX™ Supplement (ThermoFisher Scientific, Cat. No. 35050061), 1 x Gibco™ Penicillin/Streptomycin (50 U/mL) (ThermoFisher Scientific, Cat. No. 15070063) 25 µM Gibco™ 2-Mercaptoethanol (ThermoFisher Scientific, Cat. No. 21985-023), 5 ng/ml Sodium selenite (Sigma-Aldrich, Cat. No. S5261), 50 µM Ethanolamine (Sigma-Aldrich, Cat. No. E0135), 20 mg/L

L-Ascorbic acid (Millipore Sigma, Cat. No. A4544) and supplemented with 20 ng/ml recombinant human SCF (PeproTech, Cat. No. 300-07), 20 ng/ml recombinant human IL-7 (PeproTech, Cat. No. 200-07), 10 ng/ml recombinant human IL-15 (PeproTech, Cat. No. 200-15), 10 ng/ml recombinant human FMS-related tyrosine kinase 3 ligand (Flt3-Ligand) (PeproTech, Cat. No. 300-19) for the entire duration of NK differentiation, and 5 ng/ml recombinant human IL-3 (PeproTech, Cat. No. 200-03) for the first week of differentiation only. Every 2 to 4 days, a half-volume medium change was performed using NK differentiation medium with 2 x cytokine concentrations. At the end of the differentiation, on day 28-30, the supernatant containing iNKs was collected into 15 mL conical tubes. A fraction of the cells was kept on ice for flow cytometry as described below to check for purity. The remaining cells were spun down at 300 x g for 5 minutes at room temperature and cryopreserved in BiolifeSolutions® CryoStor® CS5- Cell Freezing Medium (StemCell Technologies, Cat. No. 07933) at 5×10^6 cells per mL per vial according to the manufacturer's instructions. For co-culture experiments, iNKs were thawed and cultured overnight in NK differentiation medium without IL-3, containing 20 ng/ml recombinant human SCF (PeproTech, Cat. No. 300-07), 20 ng/ml recombinant human IL-7 (PeproTech, Cat. No. 200-07), 10 ng/ml recombinant human IL-15 (PeproTech, Cat. No. 200-15), 10 ng/ml recombinant human FMS-related tyrosine kinase 3 ligand (Flt3-Ligand) (PeproTech, Cat. No. 300-19) at $0.3-0.5 \times 10^6$ cells per mL in Costar® 6-well Ultra-Low attachment plates (Corning, Cat. No. 3471). The next day, recovered iNKs were collected and counted on a NucleoCounter® NC-202™ Cell Counter (Chemometec) before being used for co-culture experiments.

Phenotypic characterization of iPSC-derived NK cells via flow cytometry

For every round of iPSC differentiation into NK cells, we first confirmed endothelial-hematopoietic differentiation of embryoid bodies (EBs) cultures via flow cytometry. At least 20 EBs were collected from the initial 96-well differentiation plate. To generate a single-cell suspension from EBs, EBs were enzymatically dissociated with TrypLE™ Express enzyme (Gibco, ThermoFisher Scientific Cat. No. 12605-010) for an initial 5-minute incubation at 37°C, followed by shearing of

EBs with vigorous pipetting through a P1000 tip, and then a subsequent 2-minute incubation at 37°C and a second round of vigorous pipetting. Next, the enzymatic dissociation was inactivated by adding 2 mL of Cell Staining Buffer (Biolegend, Cat. No. 420201). The cell suspension was then filtered through a CellTrics™ 50 µm filter (Sysmex, Cat. No. 04-004-2327) and stained with fluorescent antibodies to label cell surface markers. As previously described²⁶, EB-derived hemogenic endothelium (HE) was defined as CD34⁺/CD307^{high}/CD43^{low}/. FITC anti-human CD34 antibody (Biolegend, Cat. No. 343603), APC anti-human CD43 antibody (Biolegend, Cat. No. 343205), and PE anti-human CD309 (Biolegend, Cat. No. 359903) were used according to the manufacturer's instructions. We typically obtained 15-50% HE cells in a 6-day EB culture with the iPSC line used in this study as a source of iNKs. Note that the HE cells yield can vary depending on the iPSC line used. We have typically observed 15-50% HE cells yields across different iPSC lines. For an optimal differentiation into iNKs that renders good yields, EBs should contain at least 30% HE cells. At the end of the differentiation (Day 28-30), iNKs are collected from the supernatant and checked for purity via flow cytometry. iNKs were identified as CD45⁺CD56⁺ suspension cells using Pacific Blue™ anti-human CD45 (Biolegend, Cat. No. 368539) and APC anti-human CD56 (Biolegend, Cat. No. 392406). Additionally, cell surface expression of a panel of NK cell markers on CD45⁺CD56⁺ iNKs was evaluated via flow cytometry^{19,27}: CD2 (Biolegend, Cat. No. 300207), CD16 (Biolegend, Cat. No. 302007), CD94 (Biolegend, Cat. No. 305506), NKG2A (Miltenyi Biotec, Cat. No. 130-113-566), NKG2D (Biolegend, Cat. No. 320805), NKp30 (Biolegend, Cat. No. 325207), NKp44 (Biolegend, Cat. No. 325107), NKp46 (Biolegend, Cat. No. 331907). For flow cytometry, 0.1 - 0.5 x 10⁶ iNK cells were stained in 50 - 100 µL staining buffer containing an antibody cocktail for 20-30 minutes at room temperature in the dark. After staining, samples were washed with 1 mL of Cell Staining Buffer and then spun down for 5 minutes at room temperature. Cell pellets were resuspended in Cell Staining Buffer containing either SYTOX™ Blue (ThermoFisher Scientific, Cat. No. S34857), with the HE antibody cocktail, or SYTOX™ Green (ThermoFisher Scientific, Cat. No. S34860), with

the NK antibody panel, Dead Cell Stain at 1:1,000 for excluding dead cells in flow cytometry data analysis. To assess iNK phenotype and viability across different media conditions, iNKs were thawed and cultured overnight in NK differentiation medium without IL-3 at $0.3\text{--}0.5 \times 10^6$ cells per mL in Costar® 6-well Ultra-Low attachment plates (Corning, Cat. No. 3471). The next day, recovered iNKs were collected and counted on a NucleoCounter® NC-202™ Cell Counter (Chemometec). A fraction of the iNKs was subjected to Flow Cytometry to quantify the CD45⁺CD56⁺ population using Pacific Blue™ anti-human CD45 (Biolegend, Cat. No. 368539) and APC anti-human CD56 (Biolegend, Cat. No. 392406) and cell viability with SYTOX™ Green (ThermoFisher Scientific, Cat. No. S34860). The remaining iNKs were plated evenly and cultured in different media conditions for 12 h and 24 h, after which viability and the number of CD45⁺CD56⁺ cells were quantified via Flow cytometry as described above. Flow cytometry samples were analyzed on an Attune NxT Acoustic Focusing Cytometer (ThermoFisher Scientific). Flow cytometry data was analyzed with FlowJo™ Software (BD Biosciences).

Functional characterization of iPSC-derived NK cells

To determine iNK cells' killing capability, we performed a flow cytometry-based assay to quantify iNK cell cytotoxic activity against K-562 cells. To distinguish target tumor cells from iNKs, 10×10^6 K-562 cells K-562 or K-562-GFP were first labeled with 2 μM CellVue™ Maroon (Invitrogen™, Cat. No. 88-0870-16) according to the manufacturer's instructions. For the assay a two-fold dilution series of iNKs starting from 1×10^6 cells per mL to was plated onto Nunc™ 96-Well Polystyrene Conical Bottom MicroWell™ Plate (ThermoFisher Scientific, Cat. No. 249935) with each dilution plated in triplicate in iNK Assay Medium consisting in a 2:1 (v/v) mixture of DMEM (ThermoFisher Scientific, Cat. No. 10569010) and Ham's F-12 (Life Technologies, Cat. No. 31765035), 10% human AB serum (GeminiBio, Cat. No. 100-612), and 25 mM Gibco™ HEPES (ThermoFisher Cat. No. 15-630-080). Next, CellVue™ Maroon-labeled K-562 cells were added onto the assay plate at 0.1×10^6 cells per mL in K-562 culture medium. Wells containing K-562 or iNK alone in iNK Assay Medium were used as controls. The assay plate was then spun down

at 120 x g for 2 minutes at room temperature and placed in a 37 °C incubator for 4 hours. After 4 hours, the plate was spun down at 400 x g for 5 minutes at 4°C and the supernatant was removed. Co-cultures were then washed with 200 µL per well of Annexin V Binding Buffer (Biolegend, Cat. No. 422201) and then stained with 1.6 µg/mL Pacific Blue™ Annexin V (Biolegend, Cat. No. 640918) and 20 µg/mL Propidium Iodide (Invitrogen™, Cat. No. P3566) in 50 µL Annexin V Binding Buffer per well for 15 minutes at room temperature after which an additional 150 µL per well of Annexin V Binding Buffer was added. Co-cultures and monocultures controls were analyzed on an Attune NxT Acoustic Focusing Cytometer with a CytKick™ Autosampler (ThermoFisher Scientific). Flow cytometry data were analyzed with FlowJo™ Software (BD Biosciences). From each well, the percentage of CellVue™ Maroon-labelled cell bodies that were both Annexin V+ and Propidium Iodide+ was quantified as apoptotic K-562.

Cell viability assays in patient-derived organoids

CellTiter-Glo® 3D Cell Viability Assays were used to determine the viability of ovarian cancer PDOs under different media conditions. Briefly, PDOs were grown for 6 days and extracted intact from the Cultrex® BME by incubating them in cold Corning® Cell Recovery Solution (Fisher Scientific, Cat. No. CB-40253) containing 10 µg/mL DNase I (Sigma-Aldrich, Cat. No. DN25), and 10 µm Y-27632 ROCK inhibitor (AbMole, Cat. No. M1817) for 30 minutes at 4°C in a rocking platform. After incubation, the organoids were collected and washed three times with 2-3 x the volume using cold Gibco™ Advanced DMEM/F12 (ThermoFisher Scientific, Cat. No. 12634-028) containing 1% BSA (Gibco, Cat. No. 15260-037), 20 µg/mL DNase I (Sigma-Aldrich, Cat. No. DN25) and 10 µm Y-27632 ROCK inhibitor (AbMole, Cat. No. M1817). The centrifugation was done at 300 x g for 5 minutes at 4°C. After washes, the organoid pellet was resuspended in cold Gibco™ Advanced DMEM/F12 (ThermoFisher Scientific, Cat. No. 12634-028), 1 x Gibco™ GlutaMAX (ThermoFisher Scientific, Cat. No. 35050061), 1 x Penicillin Streptomycin (10,000 U/ml) (Life Technologies, Cat. No. 15140122), 10 mM HEPES (ThermoFisher Scientific, 15-630-080), 100 µg/mL Primocin® (InvivoGen, Cat. No. ant-pm-1) containing 10 µm Y-27632 ROCK

inhibitor (AbMole, Cat. No. M1817). An aliquot from the organoid suspension was used for counting with Keyence hybrid cell count analysis software using phase contrast images from triplicate wells taken with a Keyence BZ-X810 microscope for analysis (Figure S2). Organoids were then resuspended in each media condition containing 5% Cultrex® Reduced Growth Factor BME, Type 2, PathClear™ (Cultrex® BME) (R&D Systems, Cat. No. 3533-010-02) and plated at 1,000 organoids per well in cell culture 384-well microplates (Greiner Bio-One Cat. No. 781091) pre-coated with 10% Cultrex® BME. Four technical replicates per media condition were plated using an Assist Plus pipetting robot (Integra, Cat. No. 4505) with a pre-programmed plating protocol (VIALAB software). Organoids were plated in two replicate plates for 6 h and 20 h readout time points and placed in the incubator at 37 °C. To determine the baseline value of the plated organoids per well, organoids were also plated in a third plate (i.e., control) with nine technical replicates. The control plate was incubated for 1 h at 37 °C. After the corresponding incubation time points, organoid viability was quantified with CellTiter-Glo® 3D Cell Viability Reagent following the manufacturer's directions. Luminescence values were captured using a Clariostar Plus plate reader (BMG Labtech, Cat. No. 04300-501). To determine organoid viability, the fold change at 6 h and 20 h compared to the control plate was calculated and compared across media conditions in three independent experiments.

Natural killer cells and tumor organoids staining.

To set up a co-culture experiment, iNKs were collected from overnight culture after thawing as described above, washed in DPBS (spin 300 x g for 5 minutes at room temperature), and counted prior to labeling. iNKs were then labeled with 20 µM eBioscience™ Cell Proliferation Dye eFluor™ 450 (ThermoFisher Scientific, Cat. No. 65-0842-85) at 2,000 iNKs/µl DPBS for 10 minutes at 37 °C. The staining reaction was quenched with 5 times the volume of the staining suspension using chilled RPMI (Life Technologies, Cat. No. 61870036) containing 10% heat-inactivated fetal bovine serum (HI-FBS; Gibco Cat. No. 10082147) (RPMI/10% HI-FBS) for five minutes on ice. Stained iNKs were then spun down at 450 x g for 5 minutes at room temperature and washed three times

with RPMI/10% HI-FBS. The labeled iNK cell pellet was then resuspended in iNK co-culture media, consisting of the iNK differentiation media containing 2 x concentration of growth factors and cytokines as follow: 40 ng/ml recombinant human SCF (PeproTech, Cat. No. 300-07), 40 ng/ml recombinant human IL-7 (PeproTech, Cat. No. 200-07), 20ng/ml recombinant human IL-15 (PeproTech, Cat. No. 200-15), and 20 ng/ml recombinant human Flt3-Ligand (PeproTech, Cat. No. 300-19), counted, and kept on ice until plating.

For PDOSs staining, organoids were expanded for 6 days in standard culture conditions and extracted intact as fully formed organoids. To extract the organoids from the Cultrex® BME domes, the culture media was aspirated and replaced with cold Corning® Cell Recovery Solution (Fisher Scientific, Cat. No. CB-40253) containing 10 µg/mL DNase I (Sigma-Aldrich, Cat. No. DN25), and 10 µM Y-27632 ROCK inhibitor (AbMole, Cat. No. M1817). We then incubated the plate at 4°C for 30 min. in a rocking platform. Organoids were then collected and washed three times with 2-3 x the collected volume using cold Gibco™ Advanced DMEM/F12 (ThermoFisher Scientific, Cat. No. 12634-028) containing 0.1% BSA (Gibco, Cat. No. 15260-037), 20 µg/mL DNase I (Sigma-Aldrich, Cat. No. DN25) and 10 µM Y-27632 ROCK inhibitor (AbMole, Cat. No. M1817). Organoids resuspended in the washing media were placed at 37 °C for 10 minutes, then spun down at 200-250 x g, 4-8°C for 5 min. After washing, the organoid pellet was resuspended in the same media without BSA for counting, for which we used Keyence hybrid cell count analysis software. For counting, an aliquot of the organoid suspension was plated in 96-well plates in triplicate, and phase contrast images were taken with a Keyence BZ-X810 microscope for analysis (Figure S2). Organoids were then labeled with 15 µM CellTracker™ Orange CMRA Dye (ThermoFisher Cat. No. C34551) at a concentration of 50×10^3 organoids/mL in an Eppendorf® Protein LoBind tube (Millipore Sigma, Cat. No. EP0030122356) at 37 °C for 45 minutes, with gentle mixing every 15 minutes. At the end of 45-minute incubation, organoids were spun down at 200 x g for 1 minute at room temperature and washed three times with 1.5 x the staining volume using tOVA media. Labeled organoids were resuspended in tOVA media, filtered through a 70

µm Fisherbrand™ Sterile Cell Strainer (Fisher Scientific, Cat. No. 22-363-548), counted as described above, and kept on ice until plating.

Patient-derived ovarian cancer organoids and iNKs co-culture

For co-culture experiments, we used PhenoPlate™ 96-well microplates (Revvity, Cat. No. 6055300) with freshly coated wells using 100 µL of 1:1 (v/v) DMEM (ThermoFisher Scientific, Cat. No. 10569010): Ham's F 12 (Life Technologies, Cat. No. 31765035) containing 10% Cultrex® BME for at least 2 hours at 37°C before plating. The coating media was aspirated from the assay plates right before plating. In this study, we used 20:1 Effector (iNKs) to Target (organoid units) (E:T) ratio per well. To standardize the plating and minimize variability across experiments, the labeled iNKs and PDOs suspensions were brought up to 300K/ml and 15K/ml, respectively, using the same media they were resuspended in. For PDOs, we also added Cultrex® BME at 10% final concentration, DNase I (Sigma-Aldrich, Cat. No. DN25) at 20 µg/mL final concentration, and CellEvent™ Caspase-3/7 detection reagent (ThermoFisher Scientific, Cat. No. C10423) at 10 µM final concentration. iNKs and PDOs solutions were then mixed at a 1:1 (v/v) ratio, placed on ice, and gently pipetted up and down twice prior to plating. 200 µL of the iNK/PDO solution was added to each well, resulting in 30,000 iNKs /1,500 PDOs per well. For monoculture controls, the PDOs solution was mixed with iNK co-culture media containing 2 x concentration of growth factors and cytokines as described above (i.e 40 ng/ml recombinant human SCF (PeproTech, Cat. No. 300-07), 40 ng/ml recombinant human IL-7 (PeproTech, Cat. No. 200-07), 20ng/ml recombinant human IL-15 (PeproTech, Cat. No. 200-15), and 20 ng/ml recombinant human Flt3-Ligand (PeproTech, Cat. No. 300-19). The assay plates were then spun down at 50 x g for 1 minute at room temperature and placed in the Opera Phenix™ high-content imaging screening system (Revvity) for imaging. Of note, there are 30-45 minutes lapse from the time of iNKs/PDOs mixing to the first imaging time point shown.

Live-cell imaging of PDOs and iNKs co-cultures

Plates were imaged using the Opera Phenix™ high-content imaging screening system (Revvity) at 37°C and 5% CO₂. Images were acquired every 15 minutes for up to 11 hours. Assay plates were stored in an automated incubator at 37°C and 5% CO₂ (Cytomat, ThermoFisher) between each acquisition time point. Images were acquired in confocal mode with a 10x (Air, NA 0.3) or 20x objective (Water, NA 1.0) using the following excitation wavelengths: 388 nm to acquire eFluor-iNKs, 488 nm to acquire Caspase-3/7 reagent, and 561 nm to acquire CMRA-PDOs, respectively. The entire well was imaged using (21 fields in total) with Z-stack images acquired within a range of 103.6 µM, with 7.4 µM between each plane for a total of 15 planes. Analyses were performed using the Opera Phenix™ Harmony software (version 4.9).

Image analysis

Image analysis was performed using the Harmony High-Content Imaging and Analysis Software (version 4.9). Z-stack images were overlaid to create a max projection. Organoids were defined as objects with a surface area over 314 µm² (corresponding to > 20 µm in diameter) exhibiting CMRA fluorescence at a minimum of 1000 intensity units (CMRA positive). Caspase-3/7 activation was defined as a minimum signal of 2500 intensity units. To quantify organoid treatment response, the percentage of the total organoid area (i.e., CMRA positive) for each individual organoid overlapping with a positive Caspase-3/7 signal was quantified at each time point. 3D images were rendered and processed into a time-lapse video with Imaris software (Oxford instruments).

Statistics

Results are presented as means ± SD. The number of independent biological replicates is indicated by “n” in each figure panel and the corresponding figure legend. The statistical significance was determined by a two-tailed unpaired t-test, Mann-Whitney test or two-way ANOVA Sidak’s multiple comparisons test as indicated in each figure legend (GraphPad Prism, version 10.3). Results were considered significant at p -value < 0.05 (p < 0.05 is indicated as *; p

< 0.002 is indicated as **; $p < 0.0002$ is indicated as ***; $p < 0.0001$ is indicated as ****). ns = not significant.

Bayesian modeling of organoid apoptosis

To investigate resistant and sensitive organoid populations, we used brms 2.22.0 to fit a Bayesian two-component Beta mixture model, where the apoptosis outcome is distributed as a mixture of two Beta distributions and the mixture proportion θ_2 is predicted based on the patient covariate p_i .

$$y_i \sim \theta_1 \cdot \text{Beta}(\mu_1, \phi_1) + \theta_2 \cdot \text{Beta}(\mu_2, \phi_2) \quad (1)$$

$$\theta_2 = \alpha + p_i \cdot \beta_{\theta_2} \quad (2)$$

The Beta distribution was parametrized using a mean (μ) and a precision (ϕ) parameter as implemented in brms. The softmax transformation was applied to the linear predictor terms for the mixture proportions to ensure that θ_1 and θ_2 were valid probabilities summing to 1. The priors on the mixture model parameters were:

$$\mu_1 \sim \mathcal{N}(0.1, 0.5) \quad (3)$$

$$\mu_2 \sim \mathcal{N}(0.5, 0.9) \quad (4)$$

$$\phi_1 \sim \text{Gamma}(0.01, 0.01), 1.5 \leq \phi_1 \leq 3 \quad (5)$$

$$\phi_2 \sim \text{Gamma}(0.15, 0.01), \phi_2 \geq 5 \quad (6)$$

$$\alpha \sim \text{Logistic}(0, 1) \quad (7)$$

$$\beta_{\theta_2} \sim \text{Uniform}(-\infty, \infty) \quad (8)$$

The softmax transformation was applied on the linear predictor terms for the mixture weights to ensure that θ_1 and θ_2 are valid probabilities in the interval (0,1) that sum to 1. Before model fitting, to avoid apoptotic surface area values of 0 and 1, values were normalized to the exclusive 0-1

range²⁸. The model was fit using 4 chains with 4,000 iterations per chain and convergence was determined based on R-hat values <1.01 ²⁹ and effective sample size (ESS) ≥ 1000 ³⁰. Point estimates and credible intervals (CI) for parameters were calculated based on the fitted model. We used posterior predictions from the fitted model to calculate the difference in non-apoptotic organoids between the two patients using the `compare_levels` function from the `tidybayes` package³¹. We calculated posterior probabilities of mixture component memberships across observations with `brms` and determined a threshold for membership in the non-apoptotic organoid group. This threshold was then applied across experimental timepoints to compare mean apoptosis between non-apoptotic and susceptible apoptotic organoids.

To fit the percentage of apoptotic organoids over time based on this threshold and identify the asymptote representing the persistent percentage of non-apoptotic organoids, we compared logistic, Hill and Gompertz models (Text S6; Figures S7-9; Data S4).

Data availability

The datasets generated and analyzed during the current study are available via GitHub (https://github.com/NYSCF/ovca_ink_analysis).

Code availability

The code used for Bayesian modeling with `brms` and visualization of apoptosis in organoids is available via GitHub (https://github.com/NYSCF/ovca_ink_analysis).

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Author Contributions

Conceptualization: LM, YW, AS, SS. Tumor sample processing and organoid derivation: JH. Organoid culture and biobank: MM, TV, WS. iNK production, culture, characterization, and biobanking: YW, JE, MM. iNK K562 functional assays: YW, JE, NH. Co-culture optimization: MM, JE. Organoid collection, quantification, and labeling: MM, WS, JSC. iNK labeling: YW and JE. Co-culture and live cell imaging: JE, WS, JSC, MM. Quantification pipelines: LM, YW, MS, TR, JE, JSC, WS. Data analysis: AS, LM, MM, SS, YW, JE. Supervision: LM, YW, SS, FJM. Resources and funding acquisition: LM. Writing original draft: LM, AS, YW. Writing-review and editing: LM, YW, SS, AS, MM, FJM, JSC, WS. All authors have approved the manuscript.

Corresponding authors

Correspondence to Laura A. Martin (Laura.AndresMartin@jax.org), Yinan Wang (Yinan.Wang@jax.org) or Stefan Semrau (Stefan.Semrau@jax.org).

Competing interests

All authors declare no financial or non-financial competing interests.

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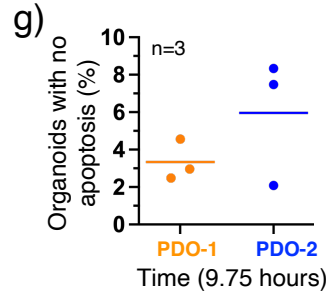
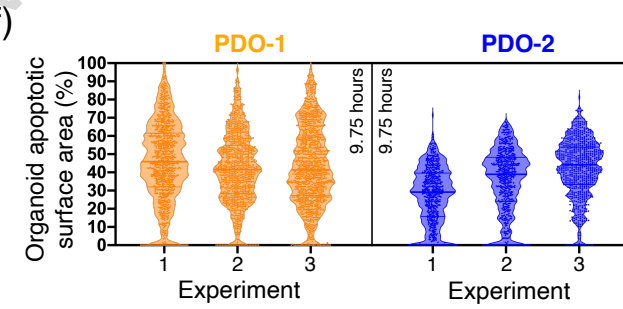
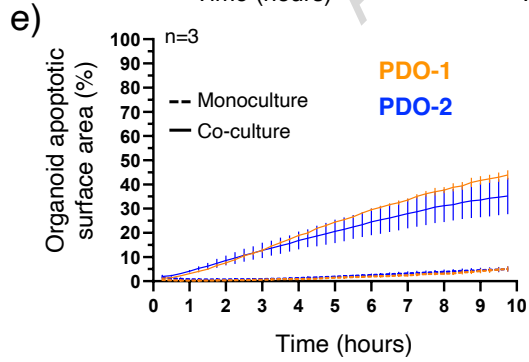
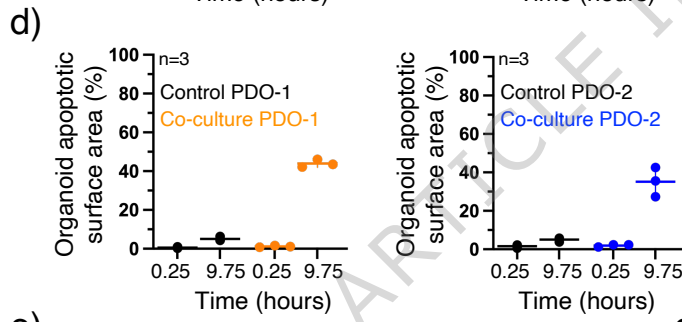
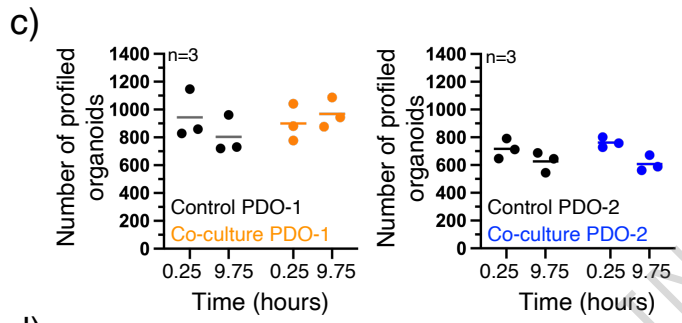
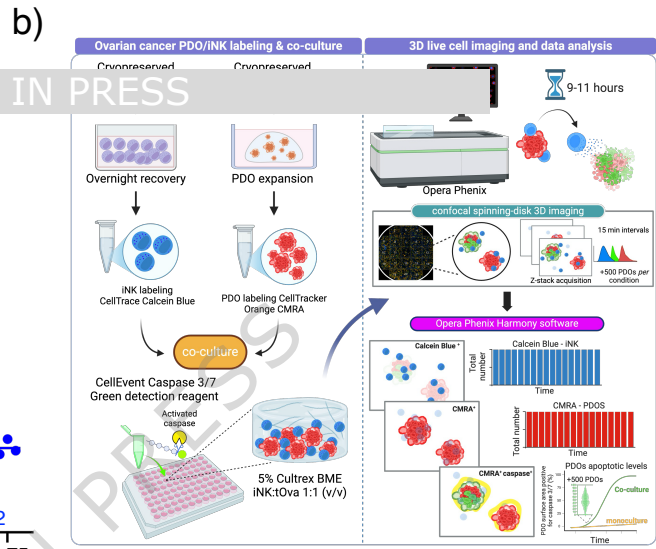
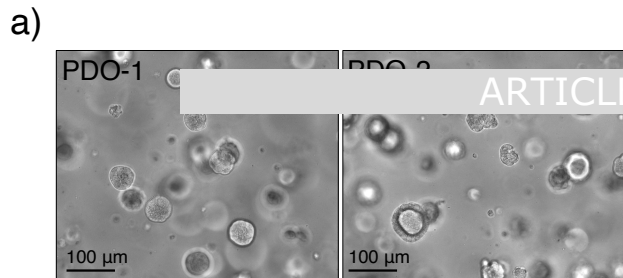
Figure 1. Patient-derived ovarian cancer organoids and iNK cells can be maintained in co-culture media conditions. (a) Representative bright-field images showing organoid morphology of PDO-1 and PDO-2. Scale bar, 200 μ m. (b) iNKs generation protocol and representative flow cytometry plots at day 6 showing the percentage of CD34⁺ hematopoietic progenitors, and day 34 showing the percentage of CD56⁺CD45⁺ cells and expression of canonical NK cell surface markers (NKp30, NKp44, and NKp46 are shown). (c) Pooled flow cytometry data showing robust production of iNK cells (mean $\pm$ SD; n = 10 independent differentiation rounds from one iPSC line). (Left) percentage of CD56⁺CD45⁺ cells at day 34. (Right) percentage of CD56⁺CD45⁺ cells expressing the indicated NK cell surface marker. Each dot represents one independent experiment. (d) Fraction of apoptotic cells (Annexin V and Propidium Iodide /Annexin V double positive) in cytotoxic assays against K562 cells using a dilution series of effector (iNK) to target (K562) ratio (E:T) from 0.01:1 to 10:1 for 4 h (mean $\pm$ SD; n = 3 independent experiments). (e) Fraction of Propidium Iodide + /Annexin V + double positive target K562 cells in cytotoxic assays comparing freshly differentiated iNKs to cryopreserved iNKs after overnight recovery. The E:T ratios are indicated. Each dot corresponds to one independent round of differentiation (median; n $\geq$ 3 independent rounds of differentiation: fresh iNKs n=3; frozen iNKs n=5; non-parametric Mann-Whitney test). (f) Survival of iNK cells in different media conditions at 12 h and 24 h in comparison to iNK media control. The plots represent pooled flow cytometry experiments to quantify the proportion of CD56⁺CD45⁺ cells (left) and their viability (right) (mean $\pm$ SD; n = 3 independent experiments; two-way ANOVA, Sidak's multiple comparisons test, single pooled variance). (g) Fold change of pooled CellTiter Glo® 3D signal in PDOs under different media conditions as indicated at 6 h and 20 h relative to standard organoid media (mean $\pm$ SD; n = 3 independent experiments; two-tailed, unpaired t-test). *p<0.05; **p<0.002; ***p<0.0002; ****p<0.0001; ns = not significant.

Figure 2. Inter- and intratumor response heterogeneity to iNKs cytotoxic activity. (a) Representative bright field images showing PDO-1 and PDO-2 cultures at the time of collection for co-culture experiments. Scale bar, 100 μ m (b) Schematic representation of the 3D live cell imaging killing assay in PDO/iNK co-cultures (c) Total number of individual organoids captured per experiment and (d) average organoid area positive for caspase-3/7 in each individual organoid per condition (monoculture control vs. co-culture) in PDO-1 and PDO-2. The start and endpoint of the assay are shown. Each data point represents one independent experiment [(c) mean; (d) mean $\pm$ SD]. (e) time resolved organoid death quantification from 3D live cell imaging assays in PDO-1 (blue) and PDO-2 (orange) in monoculture controls (dashed lines) vs. co-cultures (solid lines) (15 minutes intervals; pools of 3 independent experiments; mean $\pm$ SD). (f) violin plots of apoptotic area in individual organoids, showing heterogeneous susceptibility to iNKs in PDO-1 and PDO-2 after 9.75h in co-culture. (g) fraction of organoids with no apoptotic events at the end of the assay (mean). Each data point represents one independent experiment. The specific percentage is shown in the table.

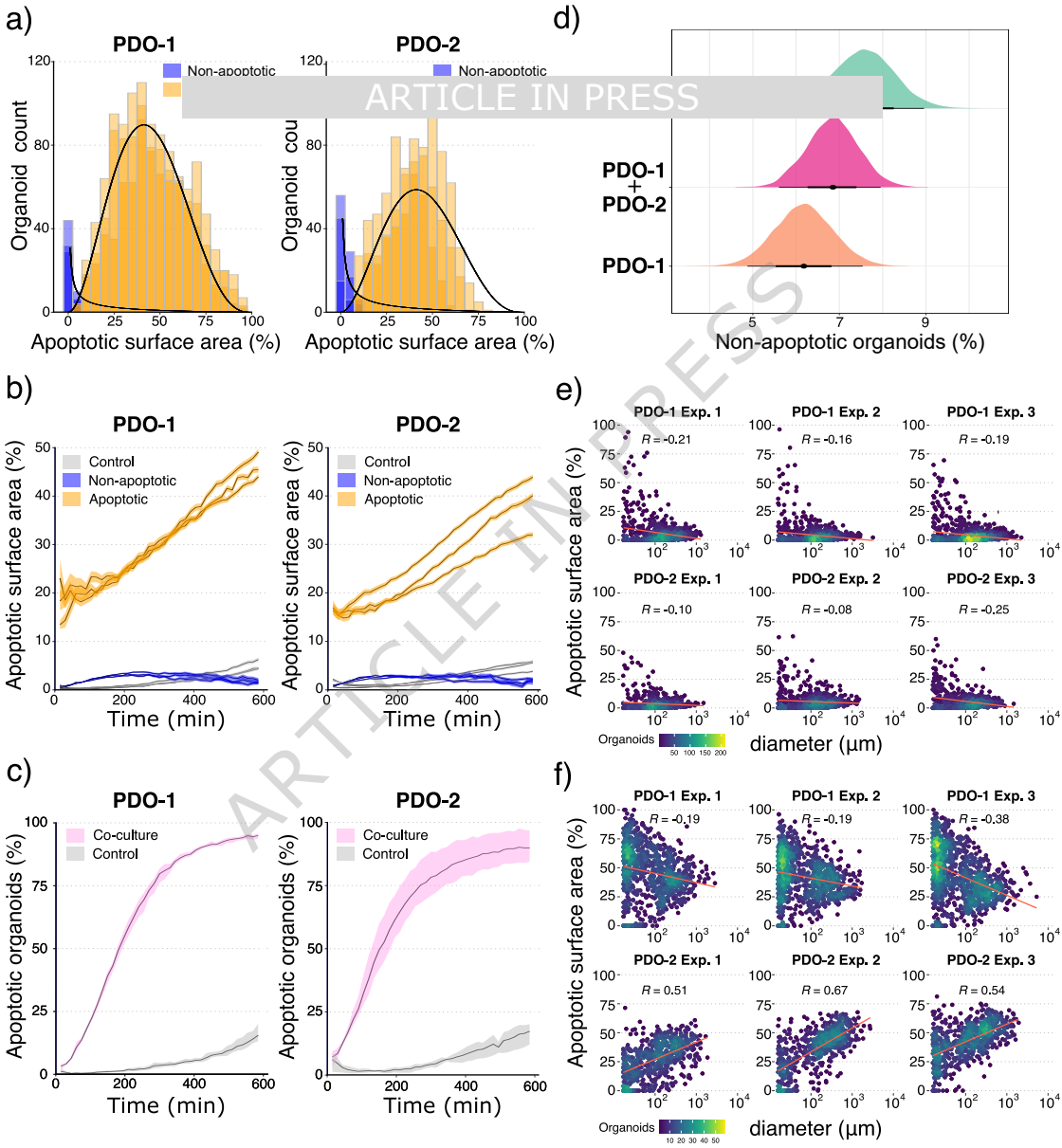
Figure 3. Ovarian cancer PDO cultures contain organoids resistant to iNK cytotoxic activity. (a) Organoid apoptotic area in PDO-1 and PDO-2 at the final time point. Density curves for a fitted beta mixture model are shown, where the two components correspond to non-apoptotic organoids (small apoptotic area, blue) and apoptotic organoids (large apoptotic area, orange). Three independent experiments are shown for each PDO culture depicted with different color intensities. (b) Mean apoptotic area over time in PDO-1 and PDO-2. iNK-treated organoids are split into resistant (blue) and sensitive groups (orange). Three independent experiments are shown for each PDO culture including the control monoculture (grey). Error bands correspond to the mean $\pm$ 2 SEM across all individual organoids quantified in each time point). (c) Fraction of

apoptotic organoids over time in PDO-1 and PDO-2; Error bands represent the minimum and maximum percentage across three independent experiments. (b,c) Organoid populations (non-apoptotic and apoptotic) are defined based on an 8.6% threshold of apoptotic area inferred using a Bayesian beta mixture model. (d) Density plots of the percentage of non-apoptotic organoids in each PDO inferred using a Bayesian Gompertz model. The PDO-1+PDO-2 group indicates the values for a model without a coefficient for the patient, where the groups are merged. The median and the 95% and 80% credible intervals are shown by the dot, bold bar and thin bar respectively. (e-f) Organoid apoptotic area versus diameter (μm) across PDOs in three independent experiments in monoculture controls (e) and the iNK co-culture condition (f) at the final time point. Color scale in scatter points indicates point density. The Pearson's correlation (R) for each plot is shown.

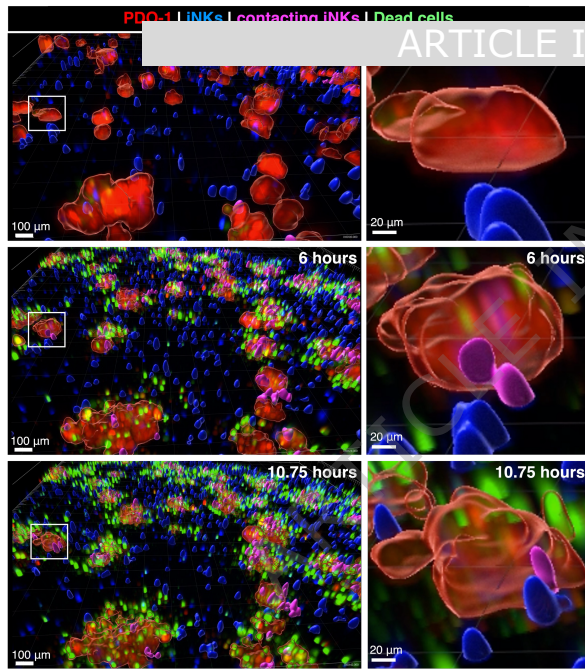
Figure 4. Ovarian cancer PDOs contain organoids with no apoptotic events after active engagement with iNKs. 3D images were rendered and processed with Imaris software (Oxford instruments) using image stacks captured with Opera Phenix™. Apoptosis marked by Caspase 3/7 activation (green) in patient-specific (a) PDO-1 (10x) and (b) PDO-2 (20x) organoids (red) in co-culture with iNKs (blue) at three time points is shown. The enlarged section, to the right of each field panel shows a representative iNK-resistant organoid at three indicated timepoints (PDO-1: 0h, 6h, 10.75h; PDO-2: 0h, 9h, 15h) from a representative experiment. Cells colored in magenta correspond to iNKs contacting with organoids defined as those located at a 0 μm distance from the corresponding organoid. Scale bars, 100 μm (field panels) and 20 μm (enlarged panels).



	PDO-1	PDO-2
Exp 1	4.56	8.33
Exp 2	2.96	7.47
Exp 3	2.48	2.09



a)



b)

