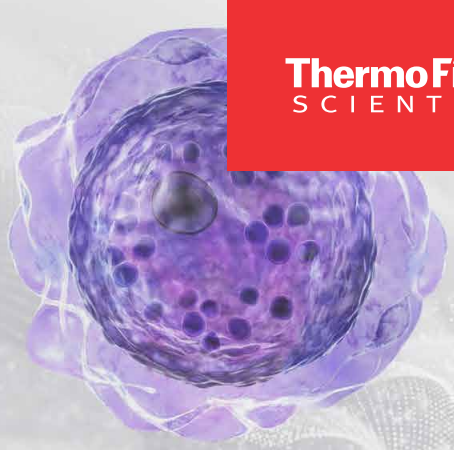

Cell therapy

Functional and phenotypic characterization of human NK cells post-expansion

Assessment of phenotype and killing potential of *ex vivo*–expanded NK cells grown in CTS NK-Xpander Medium

Introduction

Natural killer (NK) cells are a potent component of the innate immune system, acting in an antigen-independent manner to kill virally infected or malignant cells. NK cells have many advantages over other cellular therapies, including better safety and potential for off-the-shelf manufacturing. Several clinical trials have shown NK cells' great potential for treating cancers [1]. Thus, NK cells are a promising approach that bypasses the necessity for time-consuming, patient-specific manufacturing of personalized cell therapeutics.

However, researchers are still seeking the ideal conditions needed to generate large numbers of functional NK cells for therapy. Additionally, the parameters currently used to determine NK cell potency are not consistently predictive. NK cells with similar phenotypic markers can serve distinct functional roles, and conversely, NK cells with similar functionalities can display varying phenotypic markers, thus pointing to discrepancies between NK cell phenotype and function [2].

Current approaches for uncovering fingerprints with the potential to profile NK cell function include immunometabolism, transcriptomics, and epigenomics. Due to the importance of functionality, we focus here on profiling NK cell donors by relative killing potential.

Here we describe NK cell expansion using Gibco™ CTS™ NK-Xpander™ Medium in static bioreactors. These expanded NK cells are functional and maintain cytokine expression, degranulation, and cytotoxicity when cocultured with a K562 cancer cell line or with patient-derived tumoroids. Retaining cytotoxic function is crucial for clinical applications, and while K562 screening is widely considered to be a gold standard, patient-derived tumoroids provide a physiologically relevant cell-based screening tool to assess killing potential of NK cells expanded *ex vivo* (Figure 1).

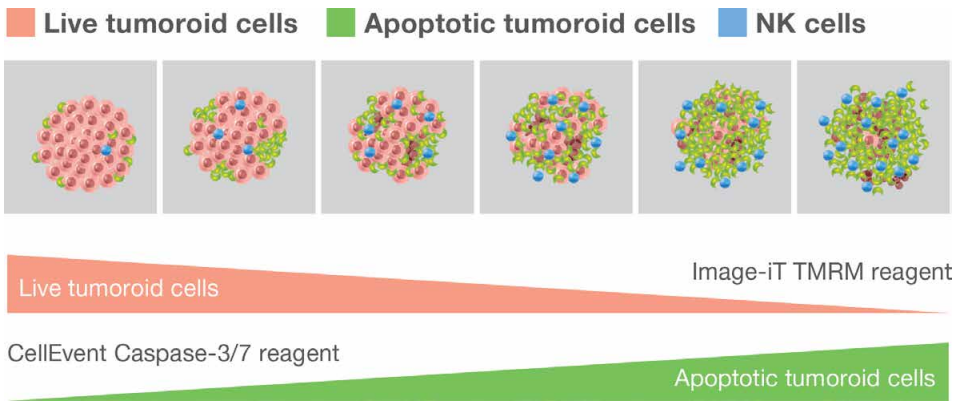

Figure 1. The functional killing capacity of NK cells expanded in CTS NK-Xpander Medium can be tested in patient-derived tumoroid models to create a physiologically relevant NK cell screening tool. As the proportion of NK cells increases, so does the proportion of tumoroid cells that become apoptotic.

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Materials and methods

NK cell expansion

NK cells were enriched from cryopreserved peripheral blood mononuclear cells (PBMCs) via negative selection and plated in a Thermo Scientific™ Nunc™ non-treated 48-well plate at 50,000 cells per well in 500 µL of CTS NK-Xpander Medium containing 5% human AB (hAB) serum and 500 U/mL of Gibco™ Peprotech™ Recombinant Human IL-2 (see product insert for detailed instructions on media preparation and culture conditions).

NK cells were expanded from day 0 to 21, scaling from a Nunc non-treated 48-well plate to a 40 mL G-Rex™ 6 Well Plate (Wilson Wolf). Approximately 6×10^6–8×10^6 cells were pooled and transferred to a 40 mL, 6-well G-Rex plate on day 7 or 9 based on cell expansion. Fresh CTS NK-Xpander Medium containing 5% hAB serum and IL-2 was added and cells were expanded until day 21. NK cell expansion was monitored on the Vi-CELL™ BLU Cell Viability Analyzer (Beckman Coulter).

NK cell purity and phenotypic characterization

Expanded cells were stained for the NK cell markers CD56 and CD3, and were assessed for expression of markers for activation (CD16, DNAM-1, NKG2D, and NKp46) and inhibition (CD158, PD-1, and TIGIT) using appropriate antibodies and the Invitrogen™ Attune™ NxT Flow Cytometer on days 0, 14, and 21.

NK cell function

Intracellular cytokine stain for cytokine detection

Expanded NK cells were co-incubated with K562 target cells in a range of effector-to-target (E:T) ratios in Gibco™ IMDM with 10% FBS and Invitrogen™ eBioscience™ Brefeldin A Solution for 4 hours. Following incubation, cocultures were stained with Invitrogen™ LIVE/DEAD™ Fixable Dead Cell Stains and with labeled antibodies against surface markers CD56 and CD3. After fixation and permeabilization, cells were stained with anti-TNF-α or anti-IFN-γ antibodies and analyzed on the Attune NxT Flow Cytometer (Figure 2).

Degranulation and K562 cytotoxicity

Expanded NK cells were co-incubated with GFP-positive K562 target cells for 2 hours (E:T ratios of 0.625:1, 1.25:1, 2.5:1, and 5:1) in IMDM with 10% FBS. Following incubation, degranulation was assessed via CD107a expression on $CD56^+$ NK cells on the Attune NxT Flow Cytometer. Cytotoxicity was assessed by measuring K562 cell death, using the LIVE/DEAD Fixable Dead Cells Stains, on the Attune NxT Flow Cytometer (Figure 2).

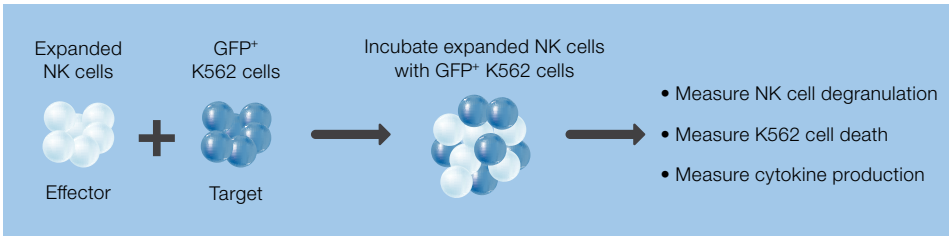

Figure 2. NK cell function was assessed by measuring degranulation—the ability of the NK cells to kill K562 target cells and produce cytokine.

Tumoroid cytotoxicity

Breast and endometrial cancer tumoroids were grown using the Gibco™ OncoPro™ Tumoroid Culture Medium Kit, following the suspension culture method guidelines in the user guide. After reaching ~200–300 µm in diameter, tumoroids were dissociated, seeded in 96-well microcavity plates in the presence of Invitrogen™ Image-iT™ TMRM Reagent for labeling, and cultured for 3 days to promote tumoroid formation. Tumoroids were then washed and co-incubated with expanded NK cells at various E:T ratios ranging from 0:1 to 1:0, in a 1:1 mix of OncoPro medium supplemented for breast and endometrial culture (without addition of Gibco™ Geltrex™ matrix or ROCK inhibitor Y-27632) and CTS NK-Xpander Medium with 5% hAB serum. This 1:1 mixture was supplemented with 500 U/mL IL-2 and 10 µM Invitrogen™ CellEvent™ Caspase-3/7 Green Detection Reagent for the extent of coculture. Cocultures were imaged every 4 hours for 72 hr.

For additional information on generating a tumoroid line, please review the application note **Tumoroid line derivation using OncoPro Tumoroid Culture Medium**.

Results

NK cell expansion

NK cells were enriched from cryopreserved PBMCs using a negative selection kit and were expanded for 21 days in CTS NK-Xpander Medium supplemented with 5% hAB serum and 500 U/mL IL-2. Enriched NK cells were plated in a Nunc non-treated 48-well plate and expanded to a 6-well G-Rex plate.

Total cell number and viability were measured on the Vi-CELL BLU Cell Viability Analyzer. Figure 3 shows NK cells expanded an average of 185-fold after 21 days for 3 donors, with greater than 90% viability. The expanded NK cells increased in purity during expansion and were >95% $CD56^+$ $CD3^-$ by day 7 when starting from an enriched NK cell population (Figure 3).

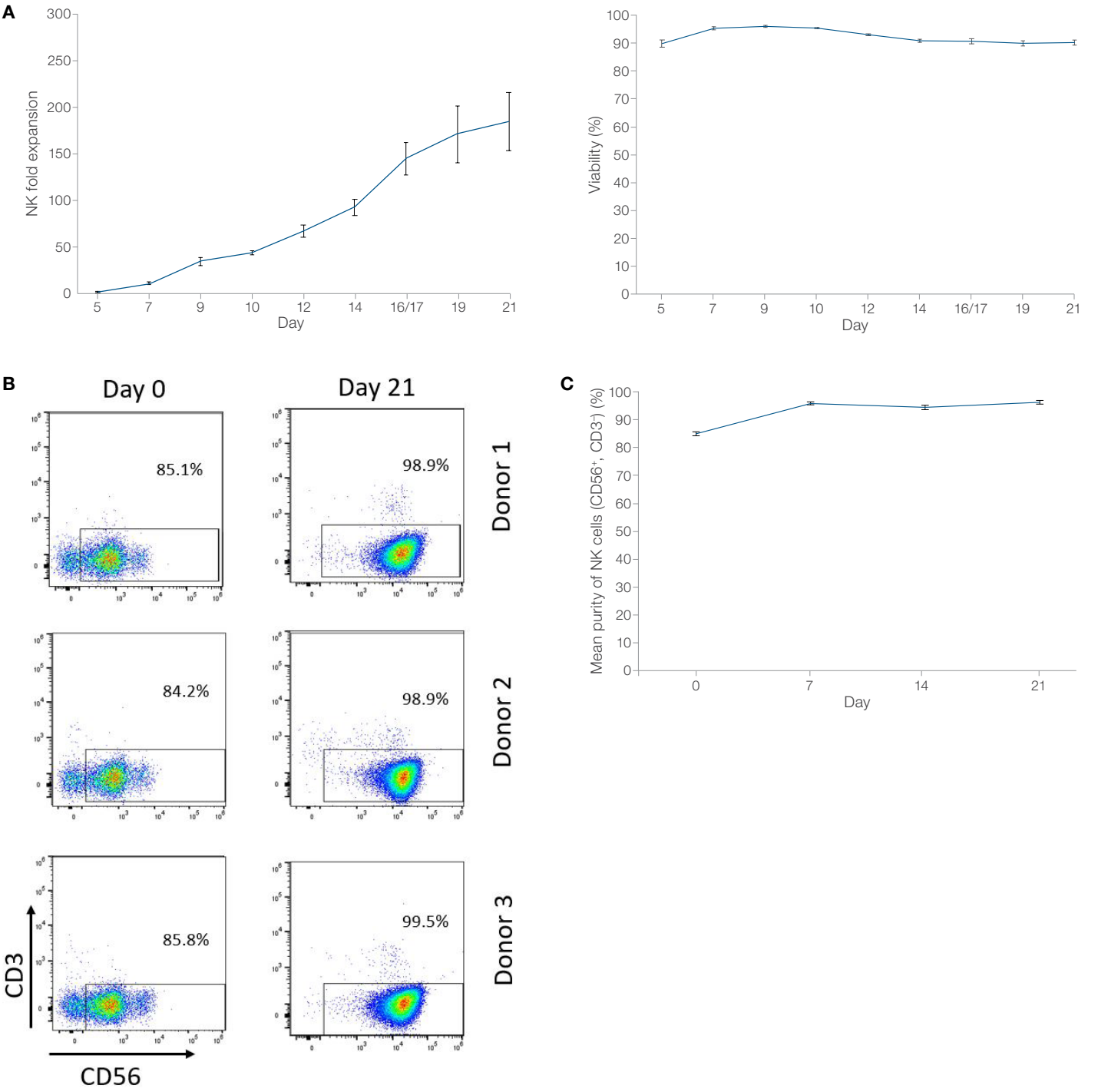

Figure 3. CTS NK-Xpander Medium expands NK cells with high viability and purity. NK cells from 3 different donors were cultured in CTS NK-Xpander Medium for 21 days. Data shown are averages. **(A)** Cells expanded an average of 185-fold after 21 days with viability exceeding 90% throughout the culture period. **(B)** Dot plots for NK cells from 3 donors at day 0 and day 21. **(C)** The purity of expanded NK cells reached greater than 90% by day 7 in culture. (Data shown are averages.)

Phenotyping

Expanded NK cells were assessed for surface markers of maintenance of activation (CD16, DNAM-1, NKG2D, and NKp46) and inhibition (CD158, PD-1, and TIGIT) using appropriate antibodies, and were analyzed on the Attune NxT Flow Cytometer on days 0, 14, and 21. The selected activation markers are important for various aspects of NK cell cytotoxicity, while the inhibition markers are related to regulation of NK cell activation (CD158/KIR) and targets for checkpoint blockade immunotherapy (PD-1 and TIGIT). Expression of CD16 decreased slightly; however, NKG2D and NKp46 expression increased slightly. DNAM-1, CD158, PD-1, and TIGIT showed relatively steady levels of expression over the expansion period (Figure 4).

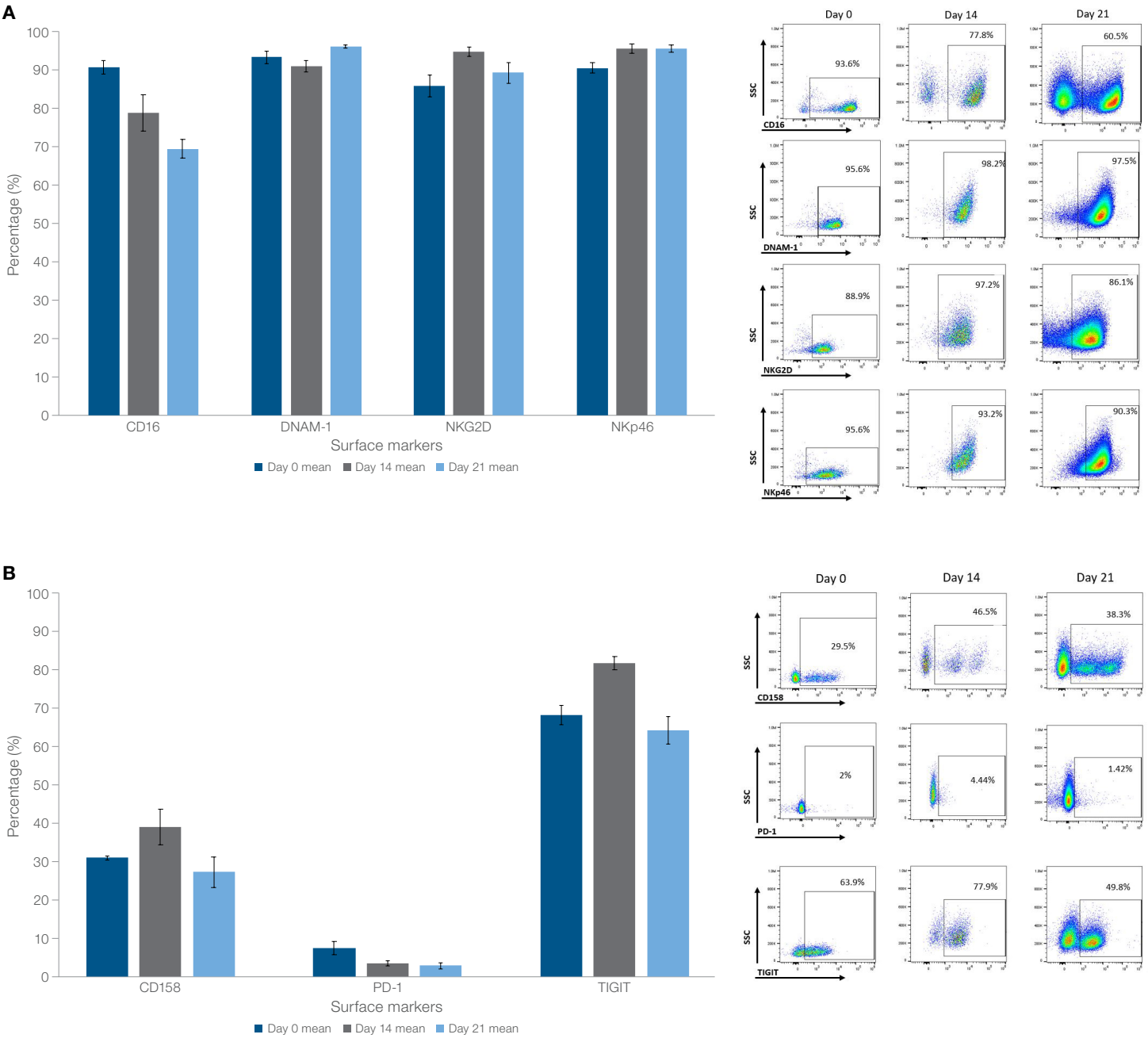

Figure 4. Impact of expansion of NK cells in CTS NK-Xpander Medium on cell surface marker expression. Cell surface marker expression of NK cells (CD56$^+$) expanded over 21 days was evaluated. Average expression and representative dot plots for **(A)** activation markers and **(B)** inhibition markers.

NK cell function

For effective immunotherapy, NK cells must maintain their functionality post-expansion. NK cell function can be measured in a number of ways, including cytokine production, degranulation, and direct cytotoxicity. NK cells grown in CTS NK-Xpander Medium retained their dose-dependent ability to produce TNF-α (Figure 5) and IFN-γ (Figure 6), and to degranulate (Figure 7), and they displayed cytolytic function (Figure 8) when cocultured with K562 target cells. These are the key indicators of NK cell function *in vitro*.

Cytokine production

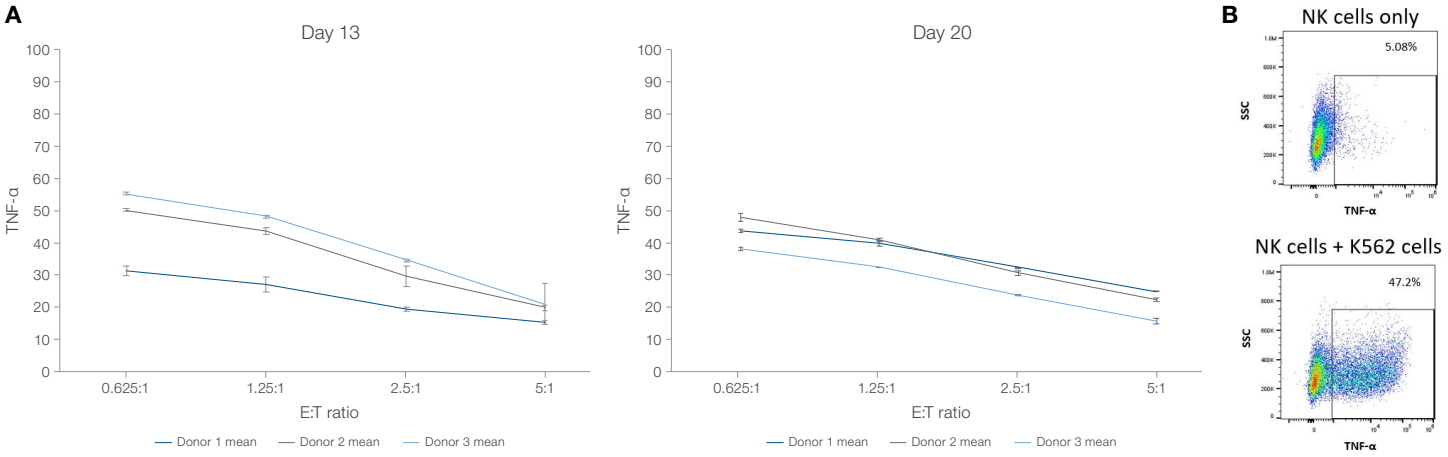

Figure 5. NK cells expanded in CTS NK-Xpander Medium produce TNF-α in a dose-dependent manner. (A) Percentage of cells producing TNF-α at varying ratios of NK cells to K562 cells at day 13 and day 20. **(B)** Representative dot plots for NK cells only and for K562 cells cocultured with NK cells.

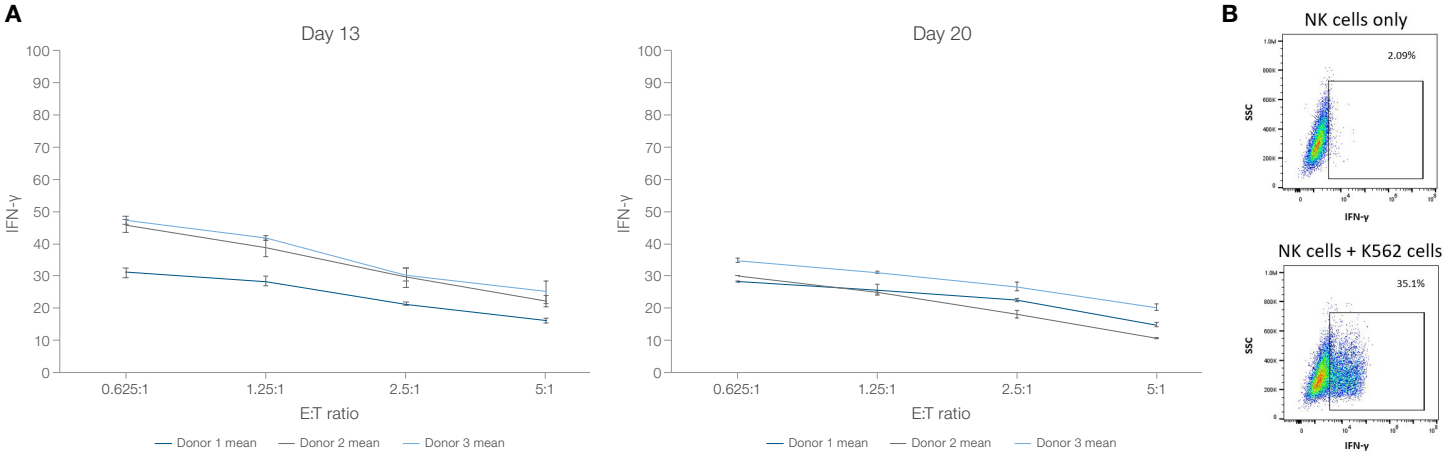

Figure 6. NK cells expanded in CTS NK-Xpander Medium produce IFN-γ in a dose-dependent manner. (A) Percentage of cells producing IFN-γ at varying ratios of NK cells to K562 cells at day 13 and day 20. **(B)** Representative dot plots for NK cells only and for K562 cells cocultured with NK cells.

Degranulation

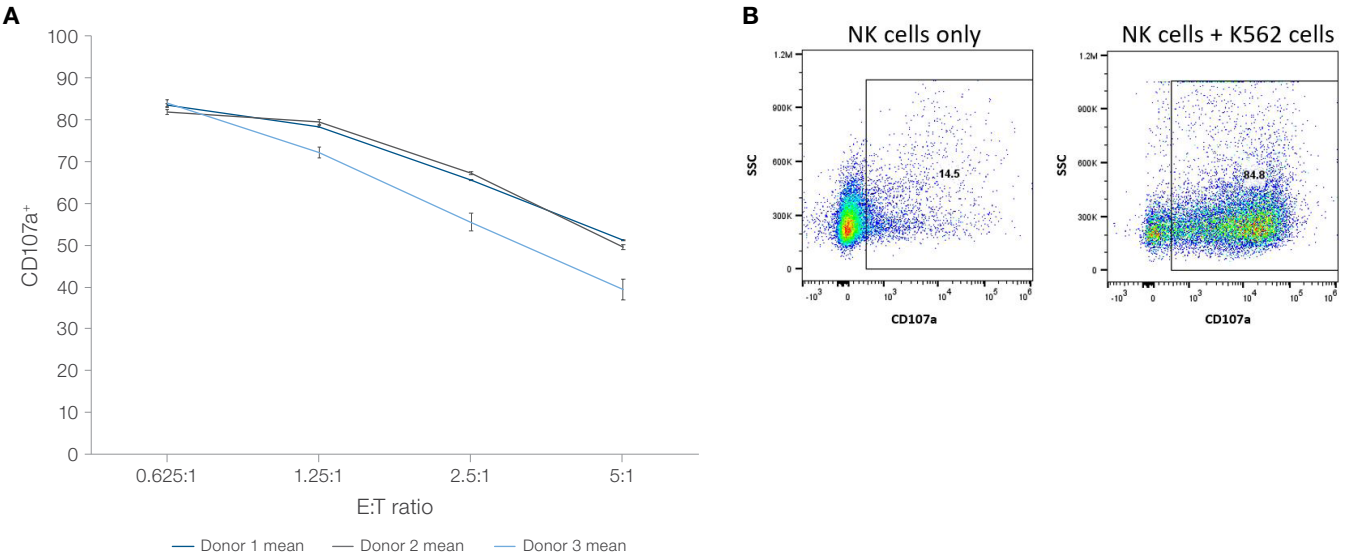

Figure 7. NK cells expanded in CTS NK-Xpander Medium degranulate in a dose-dependent manner, as demonstrated by surface CD107a expression. (A) Percentage of NK cell degranulation ($CD107a^+$) at varying ratios of NK cells to K562 cells at day 21. **(B)** Representative dot plots for NK cells only and for K562 cells cocultured with NK cells.

K562 cytotoxicity

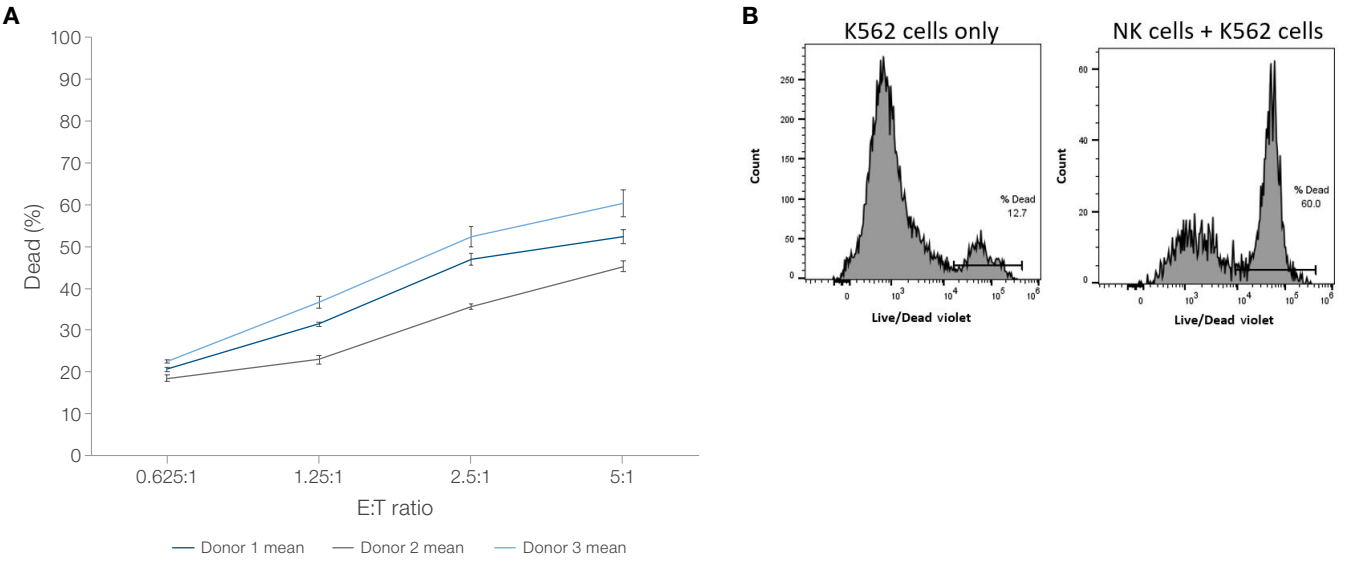

Figure 8. NK cells expanded in CTS NK-Xpander Medium kill K562 target cells in a dose-dependent manner. (A) Percentage of K562 cell killing at various ratios of NK cells to K562 cells at day 21. **(B)** Representative histograms for NK cells only and for K562 cells cocultured with NK cells at a 5:1 E:T ratio. Violet dye from a LIVE/DEAD Fixable Dead Cell Stain Kit was used for this assay.

Tumor-specific killing

Tumoroids, or cancer organoids, are patient-derived cancer cells that are grown as 3D, self-organized multicellular structures. Scientific literature suggests tumoroids are a promising tool for drug development studies and precision medicine applications because, compared to traditional immortalized cancer cell lines, they better represent patient characteristics, support tumor heterogeneity, and offer more clinically predictive results. Here we tested the ability of NK cells expanded in CTS NK-Xpander Medium to kill breast cancer and endometrial cancer tumoroids cultured in OncoPro medium. The NK cells expanded in CTS NK-Xpander Medium killed patient-derived 3D breast cancer (Figure 9) and endometrial cancer (Figure 10) tumoroids in a dose- and donor-dependent manner, showcasing a functional assay with more physiological relevance than standard K562 killing assays. The tumoroid-killing kinetics were slower than what is typically observed in the K562 cytotoxicity assay (approximately 24 hours vs. 2 hours). This is likely due to a range of factors, including the 3D structure of the tumoroids, tumor type, and differences in the activation and inhibition ligands expressed by the tumoroids.

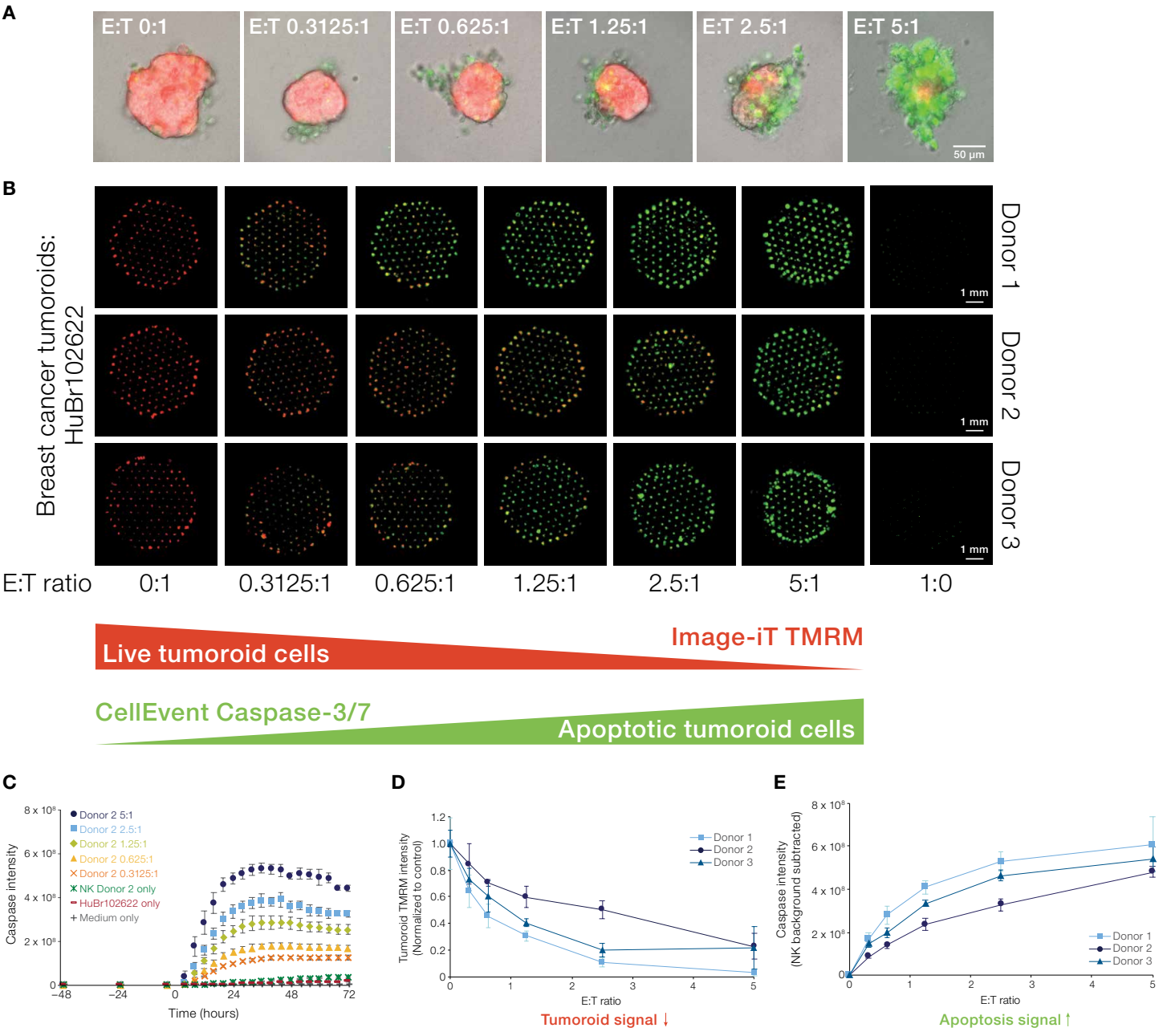

Figure 9. NK cells expanded in CTS NK-Xpander Medium kill breast cancer tumoroids in a dose-dependent manner. (A) Immunofluorescence of individual breast cancer tumoroids (red, with apoptotic cells depicted in green). **(B)** Whole-well scan depicting multiple tumoroids (stained with Image-iT TMRM reagent, red) and apoptotic cells (stained with CellEvent Caspase-3/7 reagent, green) after overnight incubation at various E:T ratios. Quantitative analysis of **(C)** a 72-hour time course of apoptosis signal, and **(D)** tumoroid and **(E)** apoptosis signals after 24 hours.

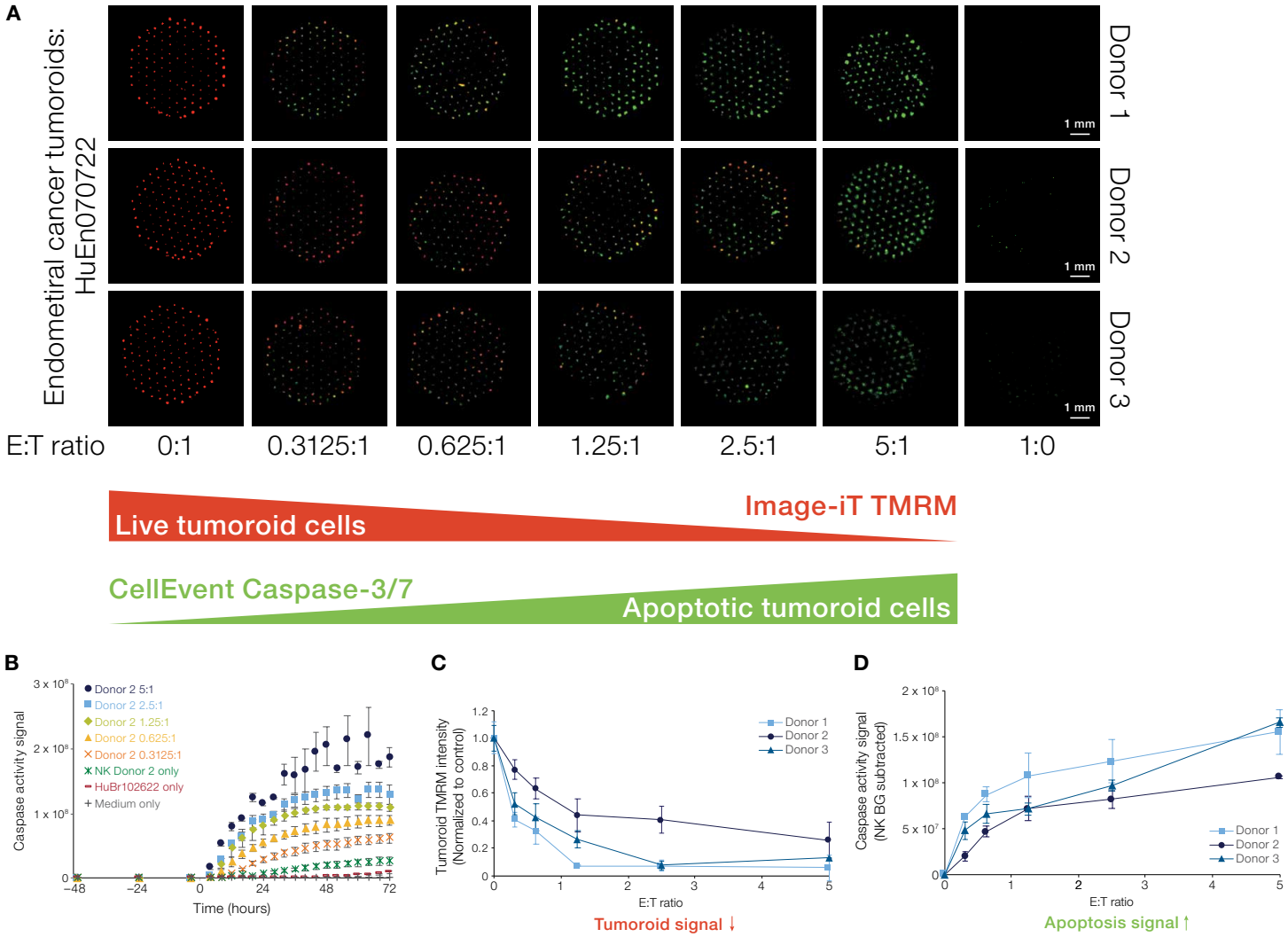

Figure 10. NK cells expanded in CTS NK-Xpander Medium kill endometrial cancer tumoroids in a dose-dependent manner.
(A) Whole-well immunofluorescence images of endometrial cancer tumoroids (stained with Image-iT TMRM reagent, red) and apoptotic cells (stained with CellEvent Caspase-3/7 reagent, green) after overnight incubation at various E:T ratios. Quantitative analysis of **(B)** a 72-hour time course of apoptosis signal, and **(C)** tumoroid and **(D)** apoptosis signals after 24 hours.

Summary

To realize the promise of off-the-shelf NK cell immunotherapy, more effective cell expansion and characterization tools are needed. Here we have showcased a method for creating physiologically relevant cancer models using patient-derived tumoroids generated with the OncoPro Tumoroid Culture Medium Kit, to test functions of expanded NK cells. Importantly, CTS NK-Xpander Medium can yield clinically relevant NK cell numbers without negatively impacting phenotype and function. This can represent an essential advance toward achieving truly effective NK cell immunotherapy.

Ordering information

Description	Quantity	Cat. No.
Culture media		
CTS NK-Xpander Medium	500 mL bottle	A5019001
	5 L bag	A5019002
Human Serum, Type AB, Fisher BioReagents	100 mL	BP2525-100
PeproTech Human IL-2 Recombinant Protein	1 mg	200-02-1MG
Nunc Non-treated Multidishes (48-well)	Case of 75	150787
OncoPro Tumoroid Culture Medium Kit	1 kit	A5701201
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix	1 mL	A1413201
Heat Stable FGF-10 Recombinant Protein	5 µg	PHG0371
Beta-Estradiol, Fisher Scientific	1 mg	501848155
Tumoroid cell lines are available through **custom services**		
Cell characterization tools		
Attune NxT Acoustic Focusing Cytometer	1 instrument	A24858
CD56 (NCAM) Monoclonal Antibody (CMSSB), PE, eBioscience	500 µL	12-0567-42
CD3 Monoclonal Antibody (OKT3), FITC, eBioscience	500 µL	11-0037-42
TIGIT Monoclonal Antibody (MBSA43), APC, eBioscience	500 µL	17-9500-42
CD279 (PD-1) Monoclonal Antibody (eBioJ105 (J105)), Super Bright 702, eBioscience	500 µL	67-2799-42
CD158a/h/g Monoclonal Antibody (HP-MA4), PE-Cyanine 7, eBioscience	500 µL	25-1589-41
CD226 (DNAM-1) Monoclonal Antibody (11A8.7.4) PE-Cyanine 7, eBioscience	500 µL	25-2269-42
CD335 (NKp46) Monoclonal Antibody (9E2), APC, eBioscience	500 µL	17-3359-42
CD314 (NKG2D) Monoclonal Antibody (1D11), PerCP-eFluor 710, eBioscience	500 µL	46-5878-42
CD16 Monoclonal Antibody (eBioCB16) (CB16)), APC-eFluor 780, eBioscience	500 µL	47-0168-42
TNF-alpha Monoclonal Antibody (MAb11), PE-Cyanine 7, eBioscience	500 µL	25-7349-82
IFN-gamma Monoclonal Antibody (4S.B3), APC, eBioscience	500 µL	17-7319-82
eBioscience Brefeldin A Solution (1,000X)	1 mL	00-4506-51
Fc Receptor Binding Inhibitor Polyclonal Antibody, eBioscience	1 mg/mL	14-9161-73
eBioscience Intracellular Fixation and Permeabilization Buffer Set	1 kit	88-8824-00
Image-iT TMRM Reagent (mitochondrial membrane potential indicator)	5 x 100 µL	I34361
CellEvent Caspase-3/7 Detection Reagents	100 µL	C10423
LIVE/DEAD Fixable Dead Cell Stain Kit, for 405 nm excitation (violet)	200 assays	L34955
CD107a (LAMP-1) Monoclonal Antibody (eBioH4A3), PE-Cyanine 7, eBioscience	100 tests	25-1079-42

References

1. Shapiro R et al. (2023) The natural killer cell immunotherapy platform: An overview of the landscape of clinical trials in liquid and solid tumors. *Seminars in Hematology* 60:1. **https://doi.org/10.1053/j.seminhematol.2023.02.002**.
2. Foley B et al. (2021) Making a killer: Selecting the optimal natural killer cells for improved immunotherapies. *Frontiers in Immunology* 12:765705. **https://doi.org/10.3389/fimmu.2021.765705**.

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