A Scalable, iPS Cell Derived Lymphocyte and Myeloid Multi-Cell-Type Therapeutic Platform for Enhanced Tumor Cell Killing

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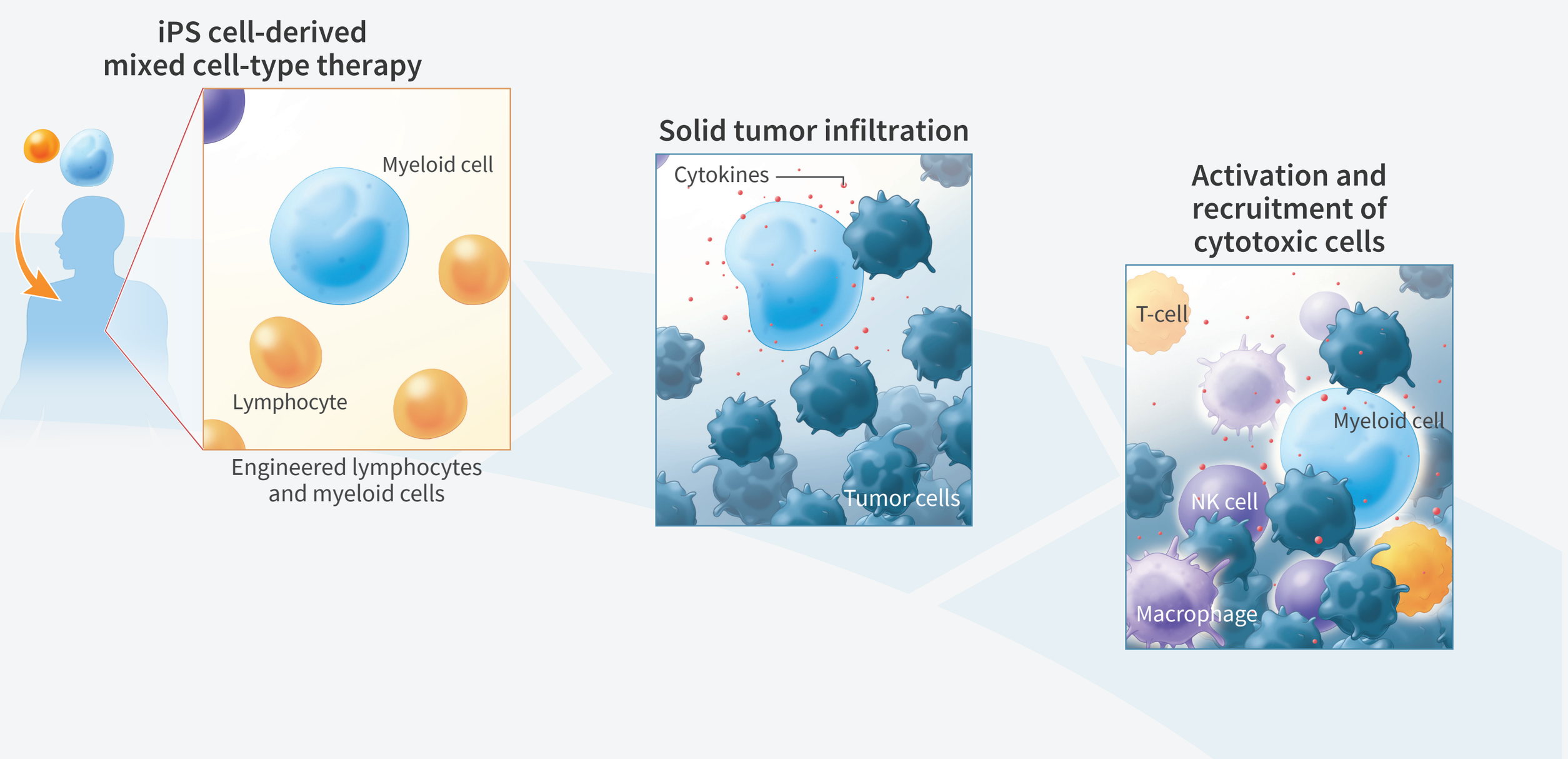
Summary

Induced pluripotent stem (iPS) cell-derived lymphocytes (e.g., T cells and NK cells) have shown clinical promise to treat hematological malignancies due to high cytotoxic effects. More recently, myeloid cell therapies (e.g., monocytes and macrophages) have grown in interest due to their ability to infiltrate and modulate a cancerous solid tumor environment. Despite preliminary success, several challenges remain, including poor infiltration of cytotoxic lymphocytes into solid tumors and insufficient cytotoxicity of myeloid cells. We hypothesized that a multi-cell-type therapy comprising both lymphocyte and myeloid cells may work synergistically to overcome the limitations of each cell type, enhancing the overall cytotoxicity and efficacy. Here we report advances in the generation of a scalable bioreactor-based process for parallel differentiation of mRNA reprogrammed iPS cells into both CD14+ (>95% positive) macrophages and CD56bright/CD16dim NK cells, that can be combined to form a multi-cell type therapy.


iPS cell-derived
mixed cell-type therapy
Myeloid cell
Lymphocyte
Engineered lymphocytes
and myeloid cells
Solid tumor infiltration
Cytokines
Tumor cells
Activation and
recruitment of
cytotoxic cells
T-cell
Myeloid cell
NK cell
Macrophage

Conclusions

Here we describe a scalable, bioreactor based platform for generating iPS cell-derived multi-cell-type cell therapies comprising both lymphoid and myeloid cells. CD14+ macrophage progenitor cells can be maintained for over 70 days in culture and maintain expected surface marker expression, high viability, and cytotoxic effect. CD56+ lymphoid cells can be cryopreserved while maintaining surface marker expression, cytokine release and cytotoxicity. Further, the iPSC derived CD14+ and CD56+ had higher SKOV3 killing than the isolated CD14+ and CD56+ PBMC's. This platform may support the development of a multi-cell type therapy. We demonstrate that, much like the natural cellular immune response, these cells act synergistically to kill tumor cells in vitro. By more closely mimicking natural cellular immunity, multi-cell-type cell therapies represent a new class of cell therapies that may play an important role in the development of new medicines to treat cancer.

1 iPSC Lymphoid and Myeloid Differentiation Overview

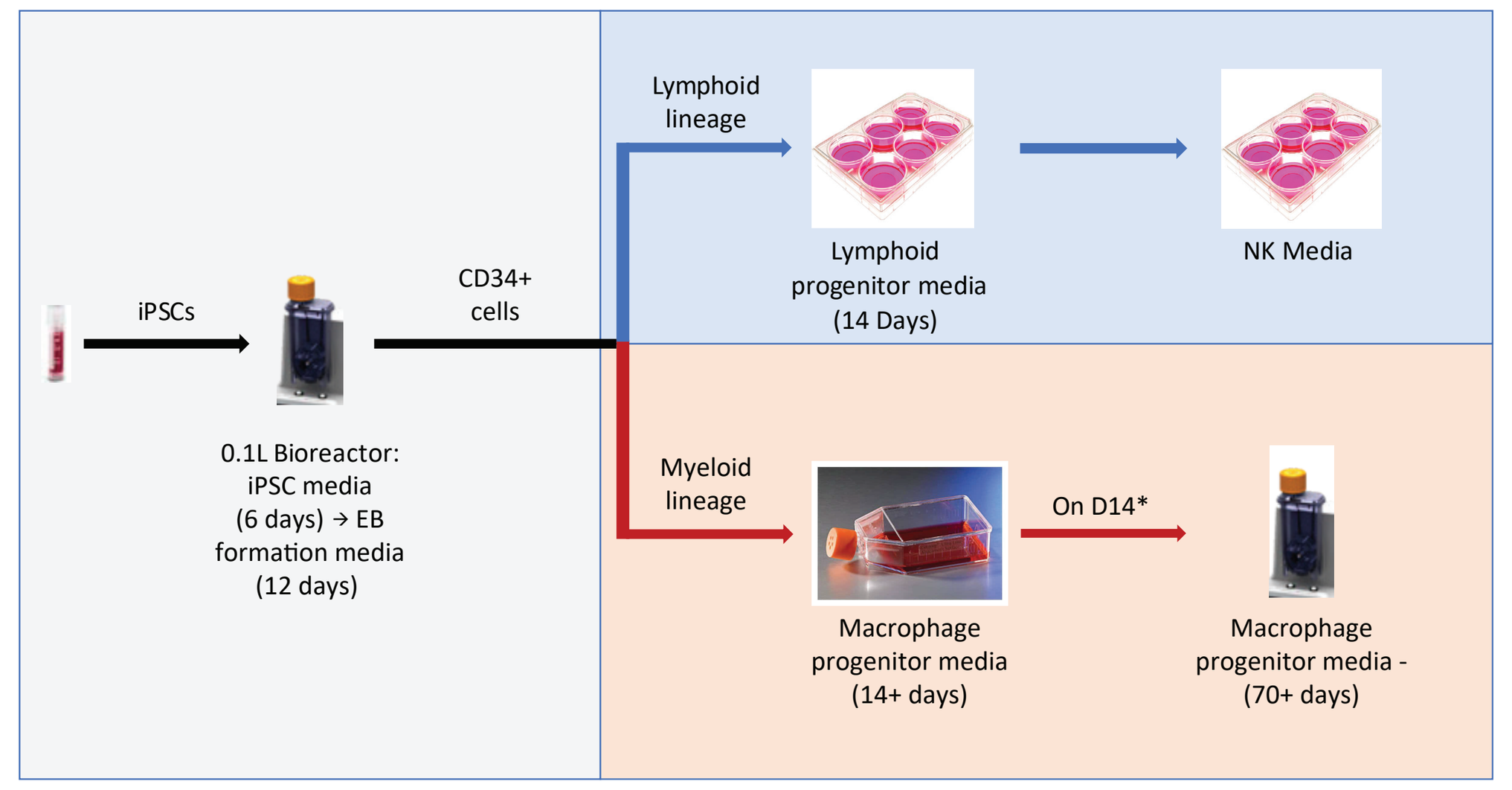
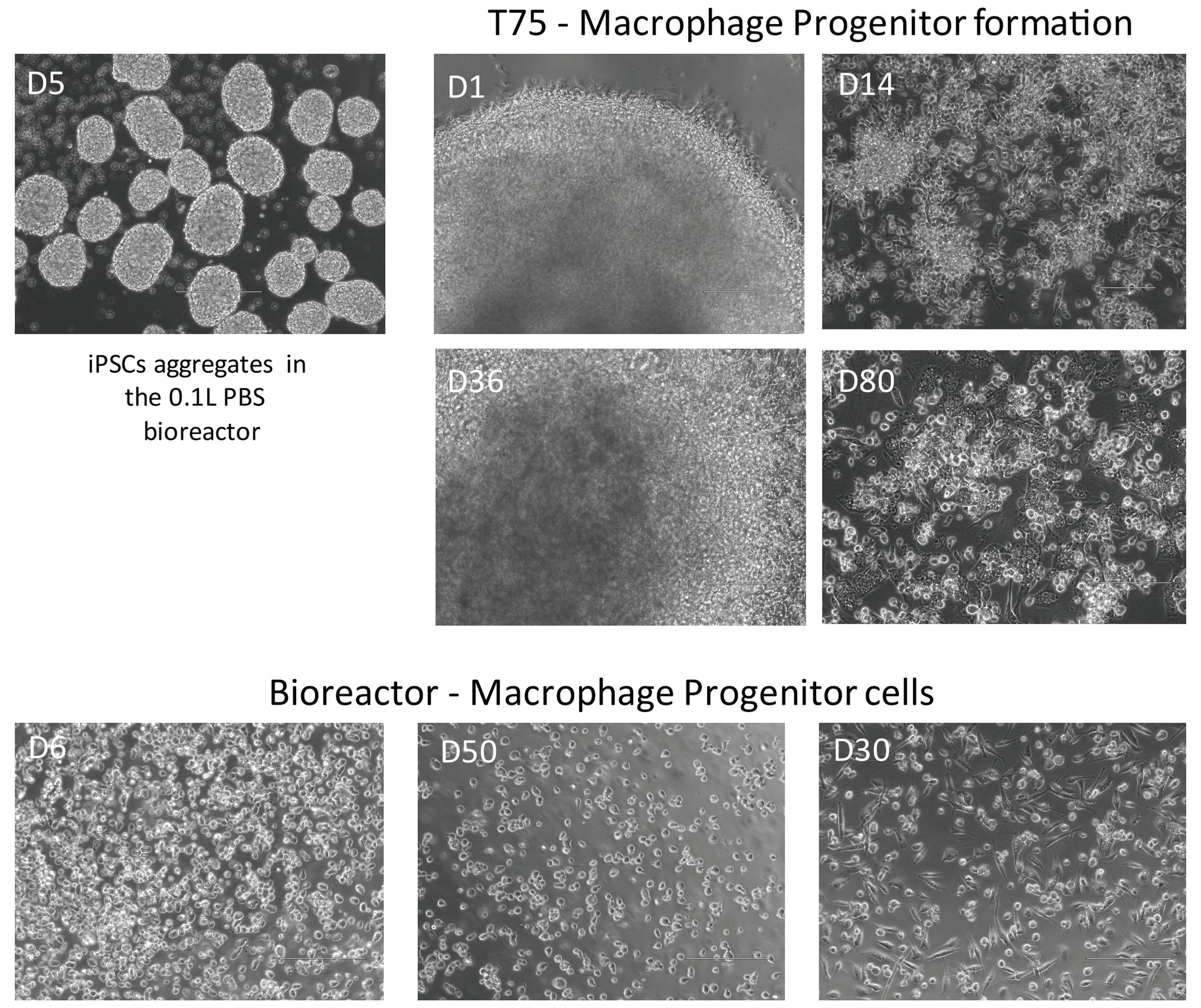


Figure 1. **Overview of scalable iPSC differentiation into lymphoid and myeloid cells.** On D0, iPSCs were thawed directly into a PBS 0.1L vertical wheel bioreactor at 40,000 cells/mL and allowed to recover in mTeSR+. After cells had formed aggregates (> 50 µm), a 100% media change was performed and all aggregates were placed in EB formation media to generate CD34+ embryoid bodies (EB's). After 12 days in EB media (D18), EBs were harvested from the bioreactor and divided into further lymphoid or myeloid differentiation. For lymphoid differentiation, EB' s were dissociated and placed into static 6 well plates. Cells remained in lymphoid progenitor media for 2 weeks followed by NK media for 2 weeks (46 days total). For myeloid lineage were harvested as EBs, and plated into a coated T75 at 1 EB per cm^2 in macrophage progenitor media. After 10 days, CD14+ cells began to bud off of the EBs. After 14 days in the T75, (D32), cells in suspension were harvested from static and seeded into a new PBS 0.1L bioreactor. Cells were maintained in both the T75 and bioreactor for >70 days (>D88) and maintained a high viability (>95%).



Cells were harvested from the bioreactor and placed in a cell bind plate for imaging and functional assessment. Cells adhere within 4 hours.

Figure 2. **Morphology of iPSC to macrophage progenitor cells in the T75 and Bioreactor.** iPSCs formed uniform aggregates. EB's plated into the T75 adhered after 24 hours, and remained highly confluent over the culture period. CD14+ cells budded off of the T75 throughout the culture period. CD14+ cells in the bioreactor maintained their morphology. Cells harvested from suspension culture would adhere quickly to cell bind plates when removed.

2 Macrophage Progenitor Cell Growth and Characterization

Surface marker	Expected	T75			Bioreactor		
		D14	D37	D85	D8	D22	D70
CD5	-	-	-	-	-	-	-
CD11b	+	82	99	98.9	96	75	99
CD14	+	31	99	99	97	99	99
CD19	-	18	0	0	26	0	0
CD33	+	72	94	97	97	88	52
CD45	+	63	98	98	91	98	98
CD56	-	-	0	2	3.6	0	1
HLA-DR	-	0	0	7	0	0	1
TLR4	+	0	84	94	98	85	55

Figure 3. **Flow Cytometry of Macrophage progenitor cells overtime.** The flow data shown was performed with the addition of a human fc blocker. The AttuneNXT was used for data collection. Cells in the T75 had improved surface marker expression after D14, and maintained high levels of expression for key myeloid and macrophage markers (>90% for CD14, CD33, CD45) and were negative for key lymphoid markers (<10% CD5, CD19, CD56, HLA-DR).

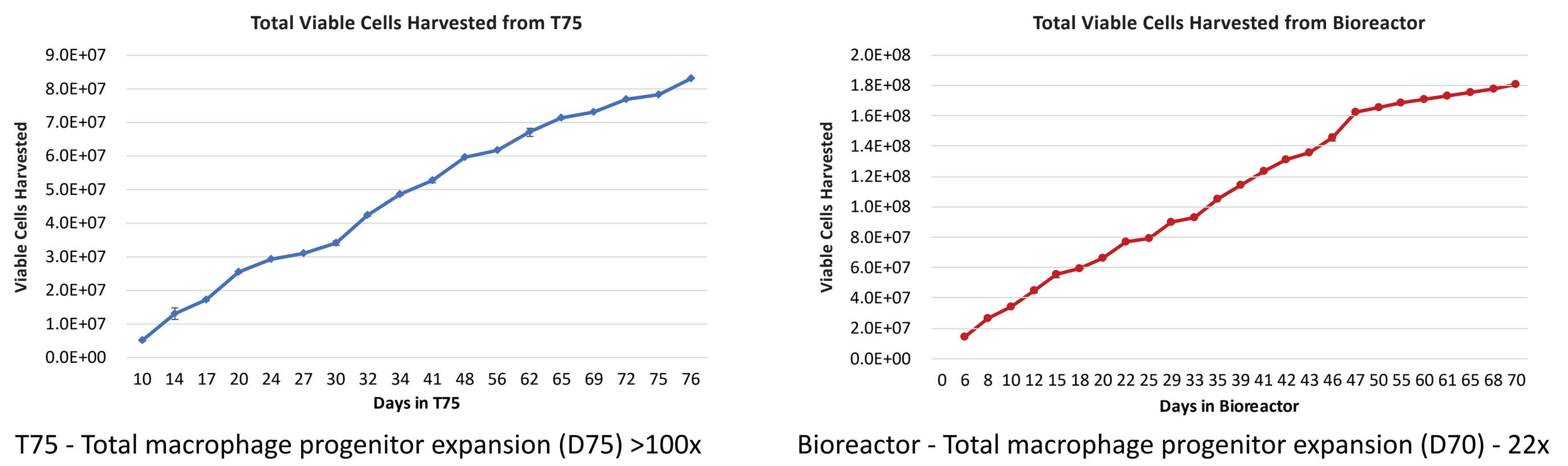

Total Viable Cells Harvested from T75
Viable Cells Harvested
Days in T75
T75 - Total macrophage progenitor expansion (D75) >100x
Total Viable Cells Harvested from Bioreactor
Viable Cells Harvested
Days in Bioreactor
Bioreactor - Total macrophage progenitor expansion (D70) - 22x

Figure 4. **The sum of the viable macrophage progenitor cells harvested from the T75 and bioreactor throughout the culture period.** Cells were harvested throughout the duration of the culture period while being maintained in macrophage progenitor media. Cells were characterized via flow cytometry and cytokine secretion. Functional assessment via cytotoxicity and transfection were performed. The T75 was seeded at ~1 EB/cm2, <1M cells. Throughout the culture period >80 days, over 80M CD14+ cells were harvested from the T75. On D0 of the bioreactor culture, 8M cells were seeded. Over 70 days, 180M CD14+ cells were harvested. Cells maintained high viability (>95%) while in culture. *indicates when a large harvest (>50% of total cells) was performed.

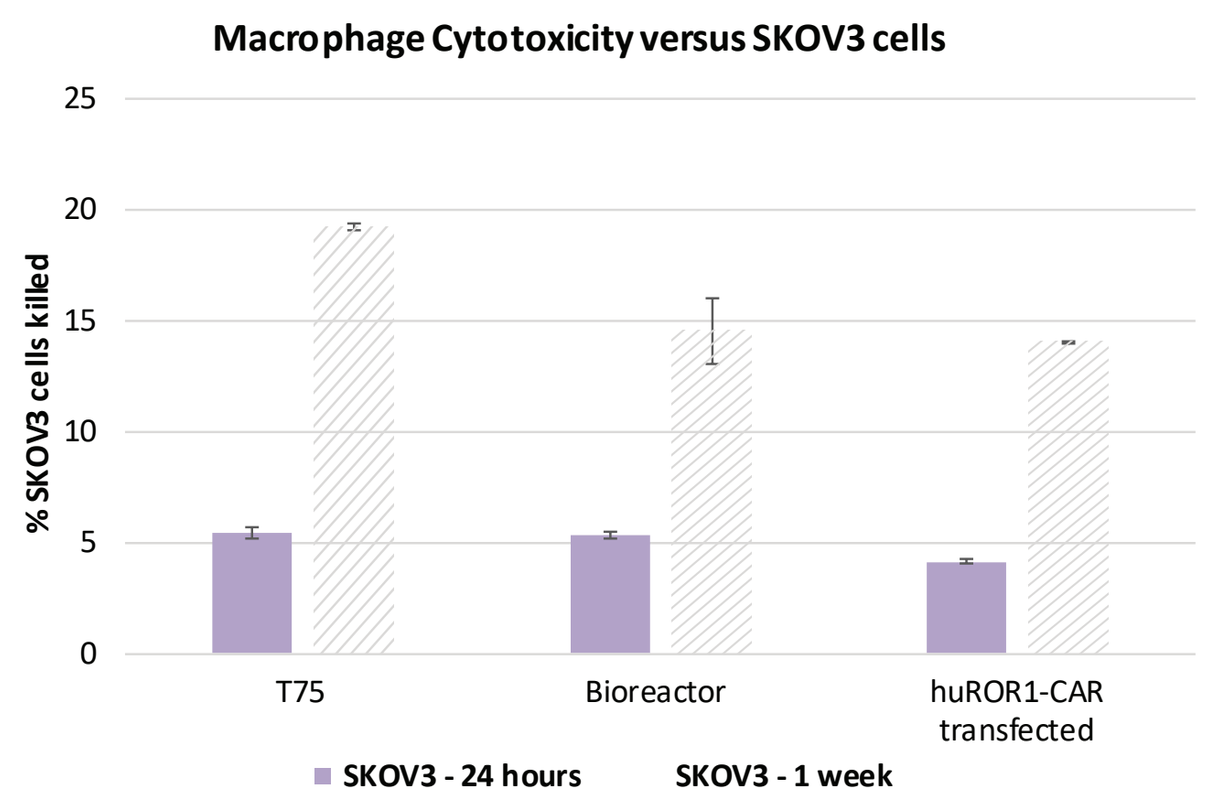


Figure 5. **Assessing baseline macrophage cytotoxicity.** The cytotoxicity of macrophage progenitor cells was measured after a 24 hours and 1 week of co-incubation period with an ovarian cancer cell line (SKOV3) at a 5:1 E:T ratio. The percent dead was determined via a live/dead stain (7-AAD). After 24 hours or 1 week, cells were harvested and stained with CD45 to differentiated between the effector cells (CD45+) and the target cancer cells (CD45-). Cytotoxicity was determined by the percentage CD45- cells (x axis) that were also 7-AAD positive (y axis). Baseline cancer cell death was accounted for by subtracting the average spontaneous cancer cell death from the percent killed in the presence of the macrophages. Macrophages were more effective at killing SKOV3 cells over a longer timepoint. Increased clustering was seen after 72 hours.

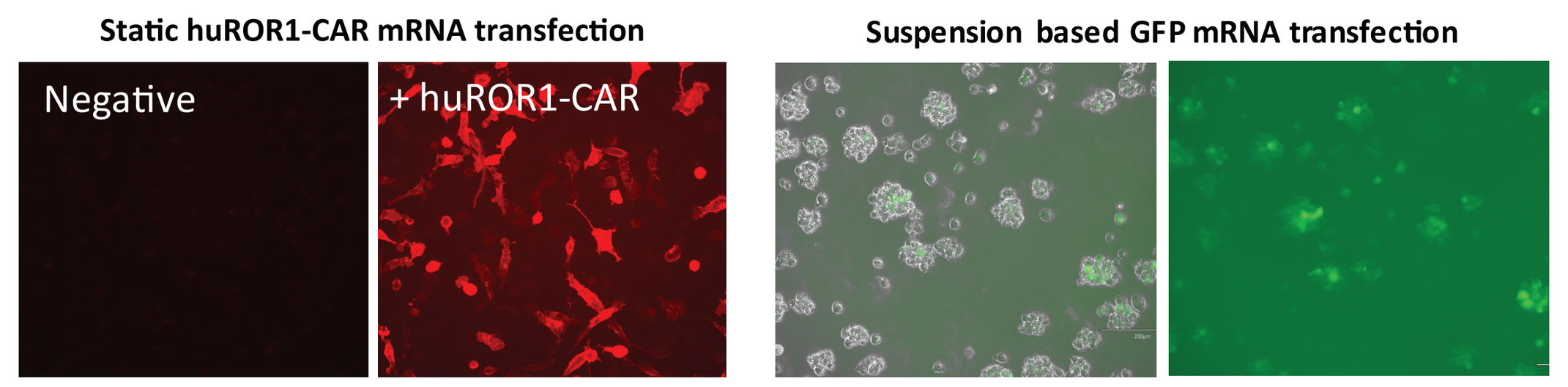


Figure 6. **mRNA Transfection of macrophages.** Cells were transfected with 1ug/1M cells of mRNA encoding a GFP protein or humanized ROR1-CAR protein. Cells were either removed and plated in static and allowed to recover for 48 hours prior to transfection or maintained in suspension culture and the mRNA was delivered via toRNAdo. ROR1-CAR expression was visualized using an RPE- labelled ROR1 recombinant protein.

3 NK Cell Cryopreservation and Characterization

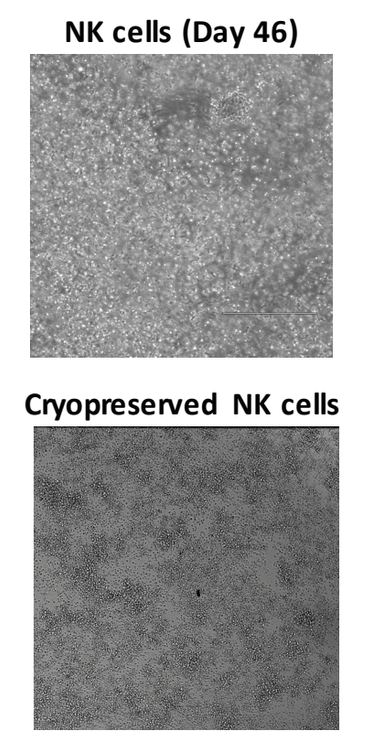


Figure 7. **Morphology of NK cells on the final day of the differentiation protocol versus 24 hours post thaw.** NK cells maintained their uniform, round cell shape and size after being cryopreserved in CryoStor10. Cells were cryopreserved using Cryostor10 (6M/mL). During thaw, cells had 88% viability and a 70% recovery per vial ~4M cells.

	Lymphoid cell expansion
Thaw into Bioreactor	5.7 x 10^6
# of CD34+ cells	2.5 x 10^6
Fold Expansion Lymphoid	33x
Fold Expansion NK Cells	5.5x

Figure 8. **Lymphoid Cell expansion throughout differentiation.** Cells had the highest expansion after seeding the dissociated EB's into static 6 well plates. Cells continued to expand during the static NK stage.

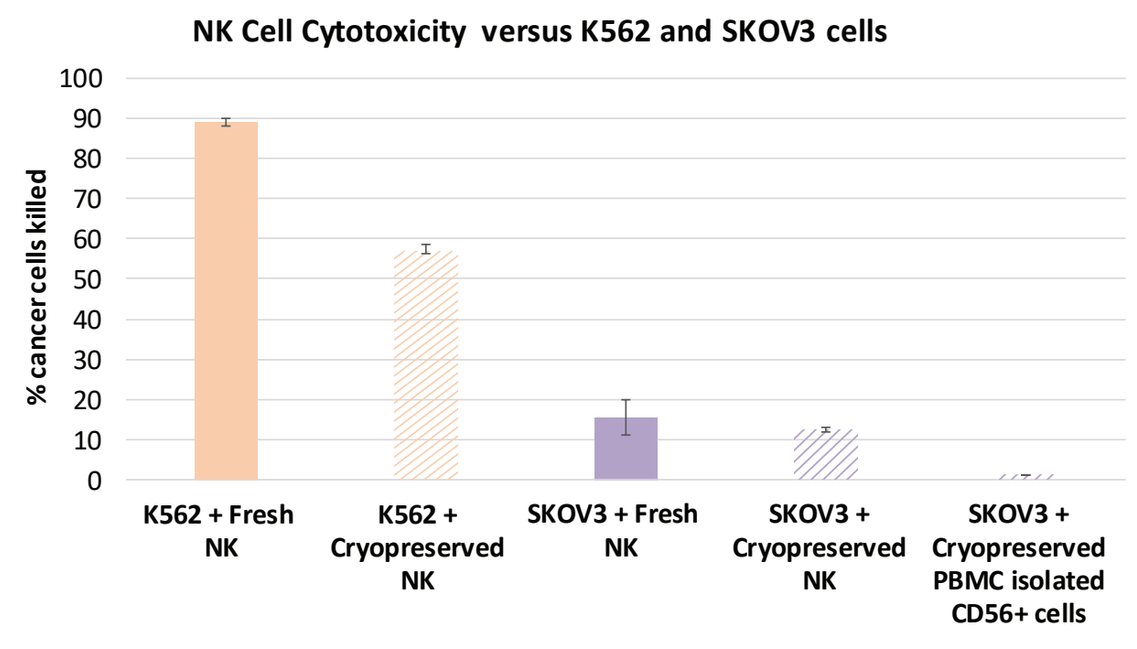


Figure 9. **Effect of cryopreservation on the cytotoxicity of NK cells.** The cytotoxicity of NK cells was measured after a 24 hour co-incubation period with the target cancer cell lines (Lymphoblast K562 cells, ovarian cancer SKOV3 cells) at a 5:1 E:T ratio. The percent dead was determined via a live/dead stain (7-AAD). K562 cells were stained with CFSE prior to incubation. NK cells and SKOV3 cells were stained with CD45. Cytotoxicity was determined by the percentage of either cFSE stained target K562 cells or CD45- SKOV3 cells (x axis) that were also 7-AAD positive (y axis). Baseline cancer cell death was accounted for by subtracting the average spontaneous cancer cell death from the percent killed in the presence of the NK cells. Cryopreservation decreased the cytotoxicity of NK cells, however they were much more cytotoxic than isolated frozen CD56+ cells.

Surface Marker	Expected	Cryopreserved	Fresh
CD56	+	76	74
CD16	-	19	13
CD3	-	18	16
CD62L	+	3	70
CD226	+	2	6
CD4	-	19	39
CD8	-	4	5
TCRgd	-	38	32
CD19	-	3	2
CD34	-	2	3
CD7	+	57	51
CD11b	-	9	24
CD14	-	18	27

Figure 10. **Flow Cytometry of Fresh versus cryopreserved Cytotoxic Lymphocytes.** The flow data shown was performed with the addition of a human fc blocker. The AttuneNXT was used for data collection. Desired NK characterization (CD56bright/CD16dim) was maintained after cryopreservation. TCR and CD7 remained unchanged, as well as maintenance of key negative markers (CD11b, CD14, CD19).

4 Multi-cell Type In-Vitro Assessment

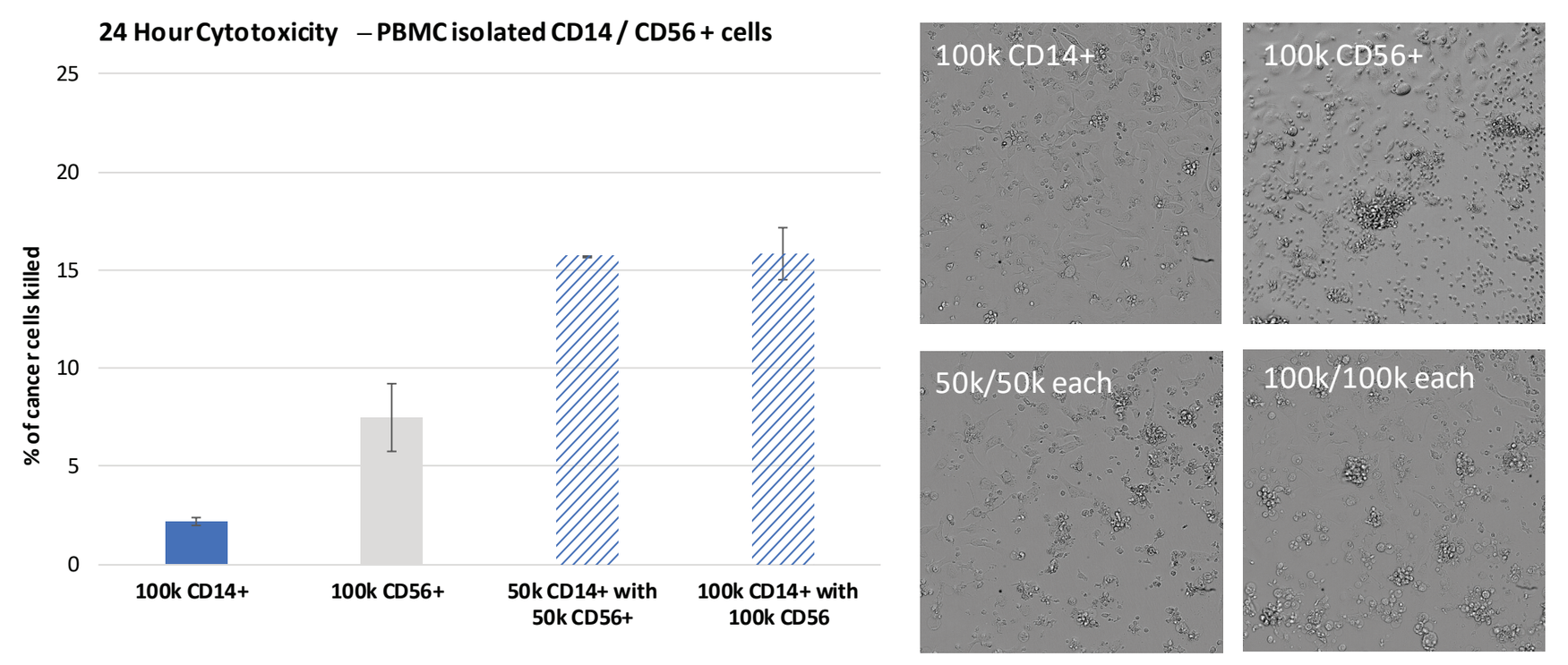


Figure 11. **Isolated hu-PBMC mixed cell type cytotoxicity assay.** CD14+ and CD56+ cells were isolated from fresh huPBMC's and immediately co-incubated with SKOV3 cancer cells. Cells were activated using IL2 and MCSF. After 24 hours, cells were harvested and flowed on the AttuneNXT. The CD14+/CD56+ cells showed synergistic tumor cell killing of SKOV3 ovarian cancer cells (combined: 15.6%; macrophage alone = 2.2% (p<0.01); NK alone = 7.5% (p<0.05); E:T = 5:1). The combined cells showed increased clustering and tumor cell engagement.

Sample	IL-1 beta	IL-4	IL-5	IP-10 (CXCL10)	IL-6	IL-8	IL-10	IL-12p70	IL-13	IL-17A	G-CSF	IFN gamma	GM-CSF	TNF alpha	MIP-1 beta	IFN alpha	MCP-1	TNF beta	IL-18
U2OS only	*	*	*	7.01	*	35.18	*	*	*	*	*	*	*	17.62	*	*	160.63	*	*
SKOV3 only	*	*	*	9.39	1462.69	978.95	*	*	*	*	*	*	6.90	39.97	91.20	*	60.99	*	34.60
NK only	*	*	*	2.87	*	*	*	*	11.74	*	*	*	*	35.29	285.40	*	*	51.30	*
T75 macrophages	13.97	**	53.50	72.08	223.67	6795.19	8.55	*	20.92	21.89	84.82	*	52.30	45.51	2645.49	8.16	29575.91	43.55	164.87
Bioreactor macrophages	5.93	**	**	79.54	33.53	1596.31	5.89	*	13.29	11.68	58.78	*	43.93	42.68	1541.66	*	11533.38	*	74.86
huROR1 mRNA - bioreactor macrophages - suspension transfection	4.76	*	*	55.04	29.99	908.42	7.40	*	**	7.12	33.77	*	25.11	31.79	898.69	*	2801.13	*	45.66
Scramble mRNA - bioreactor macrophages	32.26	190.94	236.51	456.40	7613.95	13272.21	1258.83	37.18	38.93	44.97	232.39	47.91	247.69	173.78	18616.43	23.86	4747.88	37.98	281.34
Bioreactor macrophages 1 week	27.27	340.87	193.83	2840.30	2852.31	8671.05	156.70	37.29	31.15	37.56	235.03	85.46	200.29	93.14	62419.60	8.79	7097.81	52.95	258.74
huROR1 mRNA static transfected macrophages	19.62	90.43	104.41	228.31	724.38	8280.75	25.49	**	21.91	28.25	153.61	*	96.03	85.49	13527.76	21.91	8801.59	16.52	197.46
U2OS + NK	*	58.55	*	222.00	20.44	358.89	*	*	14.67	*	*	*	105.48	35.01	331.43	*	502.04	22.62	45.66
U2OS + T75 Macrophages (D44)	83.38	247.84	299.52	1200.43	**	17573.55	1343.19	34.73	36.53	55.06	309.01	53.50	491.00	581.38	114672.85	87.86	13577.26	49.65	332.70
U2OS + T75 Macrophages (D76)	23.86	63.78	99.03	141.11	876.53	17544.95	30.93	23.87	32.97	34.05	149.90	*	136.10	108.88	6282.06	9.89	27967.64	19.23	269.54
U2OS + Bioreactor Macrophages	16.01	**	67.39	48.05	357.39	3234.37	19.82	10.60	23.09	24.50	112.86	*	158.73	84.50	3039.81	5.75	4868.11	4.27	178.34
U2OS + huROR1 mRNA static transfected macrophages	144.95	295.91	387.15	2701.16	9765.90	23963.57	6433.13	43.14	40.74	86.38	373.51	47.23	394.72	283.72	105413.81	1383.84	23908.34	50.22	449.72
U2OS + Bioreactor macrophages - 1 week	71.15	476.92	131.12	8063.77	8794.44	17114.12	180.29	27.72	32.97	32.24	206.95	81.90	148.77	103.88	6848.46	14.18	24920.03	54.02	355.19
U2OS + NK + T75 macrophages	16.24	133.17	107.87	320.53	1070.83	15452.65	15.21	13.49	34.76	31.32	157.18	*	167.31	126.20	4925.75	7.77	2708.34	32.11	243.70
U2OS + NK + Bioreactor macrophages	18.84	116.03	87.00	341.66	458.18	14437.02	14.36	20.82	36.53	29.44	138.34	*	151.95	129.50	6089.21	6.92	2807.49	28.93	239.52
SKOV3 + NK	5.36	*	82.79	19.02	7730.28	16886.05	3.71	*	55.20	10.29	54.39	*	92.46	113.70	954.34	**	360.89	52.59	212.76
SKOV3 + T75 macrophages (D44)	48.19	189.08	184.95	658.66	22604.37	22824.74	840.20	22.40	28.27	32.16	247.55	*	184.65	227.09	40820.48	20.36	3388.97	32.42	305.27
SKOV3 + huROR1 mRNA static transfected macrophages	38.67	157.06	209.44	551.43	2497.16	21292.85	3696.91	*	22.03	38.42	254.65	*	193.99	104.74	21393.57	79.27	3550.71	28.87	292.00
SKOV3 + huROR1 mRNA suspension transfected macrophages	34.42	184.69	233.27	569.42	7057.42	14570.02	2679.69	22.40	31.12	37.50	232.38	*	234.39	137.12	19023.01	23.09	4672.74	32.97	275.45
SKOV3 + bioreactor macrophages - 1 week	36.94	380.70	85.08	2419.65	4084.11	6521.05	110.66	**	17.30	17.96	113.15	90.94	52.30	76.53	1883.44	13.72	1302.37	23.56	277.46
SKOV3 + NK + Bioreactor macrophages	52.34	335.91	229.07	2297.11	63290.77	13097.17	1508.43	27.60	227.83	41.62	213.33	177.49	251.87	265.77	19860.92	33.60	2613.45	54.59	497.81
SKOV3 + NK + huROR1 suspension transfected macrophages	44.66	212.32	192.49	683.70	13365.18	7433.93	1678.92	24.39	185.37	43.39	200.64	167.79	298.29	222.45	12416.57	19.07	2905.35	42.15	395.25

Figure 12. **iPSC derived immune cell clustering.** iPSC derived NK and macrophages were seeded with U2OS and SKOV3 cells at a 5:1 E:T ratio. After 24 hours, there was increased cell clustering when the two cell types were combined. NK cells were thawed and seeded directly into the assay. Cells were harvested fresh from the T75 (D83) and bioreactor (D68). When the two cell populations were combined, there was increased cell clustering in both U2OS and SKOV3 cells.

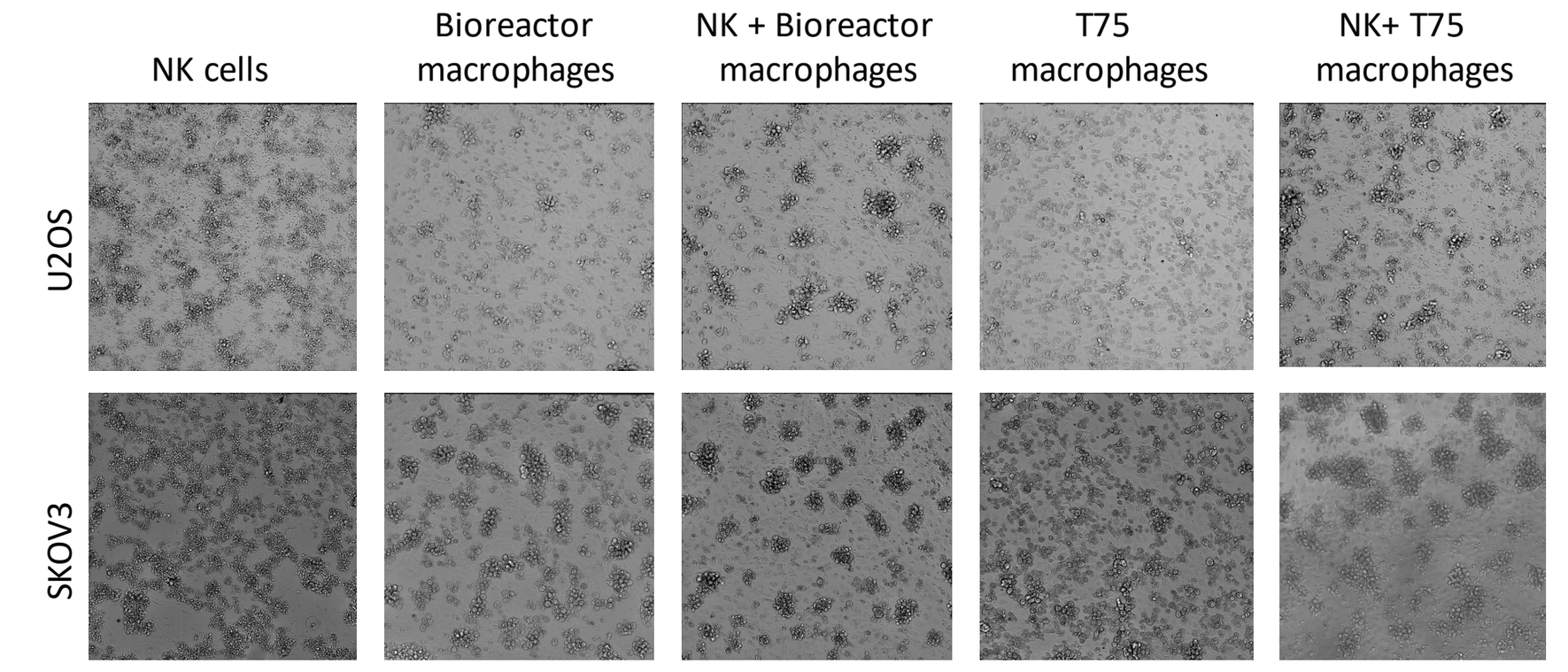


Figure 13. **iPSC derived immune cell clustering.** iPSC derived NK and macrophages were seeded with U2OS and SKOV3 cells at a 5:1 E:T ratio. After 24 hours, there was increased cell clustering when the two cell types were combined. NK cells were thawed and seeded directly into the assay. Cells were harvested fresh from the T75 (D83) and bioreactor (D68). When the two cell populations were combined, there was increased cell clustering in both U2OS and SKOV3 cells.