REVIEW ARTICLE

Directed Differentiation of Natural Killer Cells from Human Induced Pluripotent Stem Cells: From Developmental Cues to *In vitro* Production

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ABSTRACT

Natural killer (NK) cells are currently explored for cell therapy due to their innate tumour-killing properties. Their potential for adoptive immunotherapy has garnered significant attention, especially as an allogeneic cell source for cancer treatment due to their graft-versus-leukaemia effect and the safety profile. However, challenges involving ex vivo expansion of primary NK cells have hindered, to a certain extent, their clinical application. Hence, alternative cell source which can preferably be provided off-the-shelf are needed. Human-induced pluripotent stem cells have emerged as the promising cell source for producing homogenous NK cells under highly defined conditions for clinical treatment. In view of this, the understanding of the differentiation signalling cues underlying the development of NK cells is important to ensure highly efficient production of NK cells can be achieved in vitro. In this review, we briefly describe the recent chimeric-antigen receptor (CAR) technology used in engineering tumour-specific T cells and their current limitations, as well as summarizing the role of NK cells as an alternative cell source in adoptive immune cell therapy, the differentiation signalling from the view of NK cell development in human, comparing the current differentiation protocols using human induced pluripotent stem cells.
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INTRODUCTION

Cancer immunotherapy utilises specific immune cells to kill tumours, through reactivation of active or passive anti-tumour immune responses. Active immunotherapy employs cytokines to modulate host immunity to assault tumour cells, whereas passive immunotherapy utilises exogenous agents, including monoclonal antibodies or adoptive cell therapy with genetically modified chimeric antigen receptor (CAR) T or Natural killer (NK) cells, to induce anti-tumour effects. The most common immune cells used in CAR engineering and treatment is T lymphocytes. Notably, CAR-T cell therapy has been gaining traction with proven clinical success in treating B-cell malignancies (1). However, the widespread application of T-cell therapy is hindered by safety concerns. T-cell therapy requires autologous or HLA-matched donor cell source to prevent host-mediated immune rejection, this contributes to the high cost of treatment (2). Despite using autologous cell sources, high toxicity has been reported from clinical trials which include inducing cytokine release syndrome and neurotoxicity (3).

Natural killer (NK) cells are innate lymphoid cells that can effectively target and eliminate cancerous and virus-infected cells without prior sensitization, or activated to become responsive to, specific antigens. This unique characteristic positions NK cells as a promising source for innate tumour-killing therapies with a favourable safety profile. Their activation is governed by a balance of signalling from activating and inhibitory receptors, particularly through the natural-killer group 2, member D (NKG2D) receptor and various natural cytotoxicity receptors (4). Notably, NKG2D ligands are upregulated in cells undergoing DNA damage, metabolic stress, or oncogenic transformation (5). Furthermore, NK cells can

target tumours that lack specific inhibitory molecules, such as killer cell inhibitory immunoglobulin-like receptors (KIR) and D94/NKG2A, which are often lost during tumour progression (6, 7)

Due to their inherent tumour-killing capabilities, NK cells offer a viable alternative for chimeric antigen receptor (CAR) therapy, enabling the potential for "off-the-shelf" treatment options. Unlike T cell therapy, NK cells can be administered safely without the need for HLA matching between donor and recipient. Evidence shows that allogeneic NK cell administration lower risk of graft-versus-host responses, cytokine release syndrome, or neurotoxicity, which are common complications associated with T cell therapies (6, 7). Furthermore, single antigen-targeting CAR constructs often develops immune resistance in tumours, leading to the antigen escape phenomenon that limits the efficacy of CAR-T therapy (3). To mitigate potential relapse, combinations with other therapies or multi-specific antigen targeting may be required, which could then increase the treatment costs (8). In contrast, NK cells can exert killing through multifactorial activation through both CAR-dependent and independent pathways, thus enhancing their therapeutic efficacy as compared to using T cells (9). Moreover, NK cells typically exhibit limited *in vivo* persistence, remaining viable for only several weeks post-administration(10). While this short persistence reduces the risk of long-term cytotoxicity and enhances safety, it also necessitates multiple dosing and limits effectiveness against solid tumours (11). This stands in stark contrast to T cells, which can persist in patients for months to years, as memory T cells can develop *in vivo* (12). This characteristic gives NK cells an advantage over T cells, as they leave no engineered cells behind in the host.

Nonetheless, while the manufacturing processes for T cells are well-established, large-scale production of NK cells presents significant challenges (13). Although recent research has shown that metabolic reprogramming through the deletion of the CISH gene, can enhance NK cell proliferation and tumour-killing capabilities (10). Nonetheless, NK cells generally exhibit a slower proliferation rate compared to T cells, characterized by a longer doubling time and a lower expansion-fold threshold (14).

SOURCE OF NK CELLS

Several possible sources to obtain NK cells for immunotherapy may include peripheral blood mononuclear cells (PBMCs), umbilical cord blood (UCB), the immortalised NK-92 cell line, or from human induced pluripotent stem cells. PBMCs are the primary, easily accessible source of NK cells. However, they only represent 10-15% of the total PBMCs. Hence, extensive *in vitro* expansion is required to generate sufficient cell number for therapy (14), which can be limited by the age (15) and the possibility of reduced functionality due to comorbidities in patients (16). Alternatively, younger cell source such as umbilical cord blood (UCB) contains a higher proportion of NK cells (>20%) with comparatively higher proliferation rate can be considered (17). The presence of UCB banking is also helpful for HLA screening of donors for specific NK receptor profiles (18). However, UCB-derived NK cells have different NK subpopulations compared to PBMC and exhibit lower cytotoxicity (19).

Another source of NK cells is the immortalised cell lines, the NK-92 line from a male patient with non-Hodgkin's lymphoma (20). Among commonly cultured NK cell lines, NK-92 cells can be readily genetically engineered and can be screened using IL-2 as the selection marker (20). NK-92 has also proved to be suitable for CAR engineering and shows a favourable safety profile *in vivo*(6). However, the use of NK-92 cells originally from lymphoma patients for therapy requires prior irradiation with 10 Gy to prevent *in vivo* proliferation of the cells that could lead to a higher risk of developing secondary lymphoma and immune rejection after administration (21). Such irradiation procedure, on the other hand, could reduce cell viability and cytotoxicity (20). Ongoing strategies for optimizing the cell cytotoxicity for therapy, such as using anti-proliferative pretreatment, genetic deletion of Fas or NK activation ligands (22) or the use of low-energy electron irradiation (23) have also been explored.

The invention of human induced pluripotent stem cells (hiPSCs) offer a new source of cells for making NK cells. HiPSCs are generated through cellular reprogramming that converts adult somatic cells into a pluripotent state similar to the embryonic stem cells, using the defined embryonic transcription factors Oct-3/4, Sox-2, Klf-4, and c-Myc (24). Due to its pluripotent plasticity, hiPSCs can be directed to differentiate into NK cells through applying developmentally relevant cytokines and growth factors (25). This offers the advantage of realising large-scale production of homogeneous NK cell populations (26), (27). Currently, several phase I clinical trials are underway involving iPSC-derived NK cells, utilising anti-CD19 CAR-engineered iNK cells for treating B-cell malignancies (28, 29). Another phase I trial with anti-GPC3 CAR-ILC/NK cells to treat ovarian cell carcinoma are still ongoing (30).

Advances in hiPSC generation has enabled derivation of clinical grade cells from a fully cGMP-compliant process with completely defined and animal-free conditions (31). Nonetheless, notable differences in epigenetic signatures have been observed between iPSCs and embryonic stem cells (ESCs) and between different cell sources for iPSCs(32), which influences the differentiation efficiency towards specific lineage (33). Various iNK generation protocols have utilized hiPSCs which were reprogrammed from blood, which

has higher tendency of committing haematopoietic lineage (33). UCB mononuclear cells represent younger cell source for iPSC generation because they have lesser acquired genetic mutations in the nucleus and mitochondria (34, 35) and is more likely to be free from infection and influence from any comorbidities than the adult cell source.

DIFFERENTIATION OF NK CELLS FROM HEMATOPOIETIC STEM CELLS

To differentiate NK cells from hiPSCs, the understanding of the signalling pathway controlling the NK cell development is pivotal. Prior to the invention of hiPSCs, differentiation of NK cells were mainly performed from the hematopoietic stem cells (HSCs), the multipotent stem cells that reside in the perivascular niche in the bone marrow adjacent to sinusoids, which capable of differentiating into myeloid and lymphoid lineages. HSCs are heterogeneous, with their lifespan influencing their self-renewal capabilities and differentiation (36). The self-renewal of HSCs in the BM depends on quiescent long-term HSCs (LT-HSCs), which can convert to short-term HSCs (ST-HSCs) under stress conditions, resulting in increased differentiation ability (37).

Factors that support the maintenance of haematopoietic stem cell (HSC) stemness and self-renewal are crucial for identifying and culturing the cells when deriving them from pluripotent stem cells. In humans, different cell types in the bone marrow contribute to the maintenance and self-renewal of HSCs through various cytokines and chemokines, including Chemokine (C-X-C motif) ligand 12 (CXCL12) and stem cell factor (SCF). Most HSCs are in contact with perivascular CXCL12-abundant reticular cells surrounding the endothelial cells in the bone marrow, where CXCL12 is a critical regulator of HSC quiescence and retention(38). Consequently, deletion of CXCR4, the receptor for CXCL12, results in a significant reduction of the HSC pool (38). SCF is also crucial for HSC maintenance, secreted by endothelial cells, perivascular CXCL12-abundant reticular cells, and osteoblasts (39). Importantly, depletion of SCF from endothelial and perivascular stromal cells reduces HSC numbers in the bone marrow. SCF also plays a role in the maturation of pre-HSCs into definitive HSCs(40). Additionally, other regulatory factors released by bone marrow, such as BMP4, VEGF, angiogenin, angiopoietin, FGF, IL-6, TGF-β, Wnt ligands, and Notch ligands, have been implicated in modulating the HSC niche but are dispensable for normal haematopoiesis (41).

The differentiation of NK cells from HSCs is divided into six distinct stages (Table I) (42-44), based on the expression of cell surface, with NK intermediates progressively losing multipotency (45) . The differentiation of HSCs to CD34+CD45RA+ common lymphoid progenitors (CLPs) represent a critical commitment to the lymphoid lineage including T cells, B cells, and NK cells (46). Stage 1 cells, characterized as CD34+CD45RA+CD117−CD94− multipotent pro-NK cells, can rapidly progress to the stage 2 phenotype with stimulation from FL, IL-3, and IL-7 (45). The emergence of IL-1R1 and low levels of CD122 (IL-2Rβ) indicates the transition to NK precursors (NKPs) stage 2b, which are responsive to IL-15 for further development(45). Transition to immature NK cells (stage 3) is marked by the expression of NKG2D, a major NK activating receptor that targets NKG2D ligands on stressed and tumour cells(47).

Table I: The surface markers of NK cell developmental instermediates (NKDIs) in secondary lymphoid tissues (SLTs) throughout the stages.

	Pro-NK	NK Precursor		Immature NK	Mature NK ($CD56^{bright}$)		Mature NK ($CD56^{dim}$)	Terminal/ adaptive NK
Surface markers	Stage 1	Stage 2a	Stage 2b	Stage 3	Stage 4a	Stage 4b	Stage 5	Stage 6
CD34	+	+	+	-	-	-	-	-
CD10	+	-	-	-	-	-	-	-
CD117/c-KIT	-	+	+	+	+/low	low/-	low/-	-
IL-1R1	-	-	+	+	+/low	low/-	low/-	low/-
CD122/IL-2Rβ	-	-	low	low	+	+	+	+
NKG2D	-	-	-	+/-	+	+	+	+
NKG2A/CD94	-	-	-	-	+	+	+/-	+/-
NKP80	-	-	-	-	-	+	+	+
CD16	-	-	-	-	-	-	+	+
CD56	-	-	-	-	bright	bright	dim	dim
CD57	-	-	-	-	-	-	-	+

Abbreviations: (+) – positive, (-) – negative, IL – interleukin, NK – natural killer, NKG2A – natural killer group 2 member A, NKG2D – natural killer group 2 member D

The final transition into mature NK cells is indicated by CD56 expression. Mature NK cells are categorised into $CD56^{bright}CD16-$ (stage 4) and $CD56^{dim}$ CD16+ subpopulations (stage 5). $CD56^{dim}$ NK cells consist of 90% of NK cells in the PB, while $CD56^{bright}$ subset consists of the remaining 10% (48). $CD56^{dim}$ subset produces stronger cytotoxicity compared to $CD56^{bright}$ and expresses higher KIRs and CD16 on the cell surface.(49). Meanwhile, the $CD56^{bright}$ subset produces considerable amounts of cytokines and chemokines and is known to play an immunoregulatory role (48). $CD56^{bright}$ subset produces cytokines including interferon-γ (IFN-γ), tumour necrosis factor β (TNF-β), and granulocyte-macrophage colony-stimulating factor (GM-CSF); and chemokines including CCL3, CCL4, CCL5, XCL1, CXCL8 (50). The $CD56^{bright}$ subset is proliferative and has been identified as the precursor to $CD56^{dim}$ cells, $CD56^{bright}$ cells acquire $CD56^{dim}$ phenotype upon cytokine activation(51). $CD56^{bright}$ NK cells primarily populate SLTs, where small populations of $CD56^{bright}$ cells are released into circulation and maintain the large numbers of $CD56^{dim}$ cells in peripheral tissues (52).

Terminally matured NK cells express CD57 and have higher cytotoxicity and lower proliferation compared to the $CD56^{dim}$ NK subset (53). In addition, NK cells undergo a stepwise decrease of NKG2A expression and acquire KIRs in terminal maturation, a crucial step in NK cell education which determines responsiveness (54). The final stage of NK differentiation represents the formation of memory NK cells (43). However, memory NK cells have elusive phenotypes according to their diverse induction pathways which include induction from viruses, chemical haptens, and cytokines (55). Some memory NK-specific markers have been identified while not being strictly required, including NKG2C and CD57 (55).

THE ROLE OF CYTOKINES IN NK CELL DIFFERENTIATION

Cytokines, including interleukins, growth factors, and interferons, play crucial roles in NK cell differentiation and function, with the interplay of different cytokines being orchestrated at distinct stages. According to the model described by Wu, Tian (56) (Fig. 1A), a cytokine combination of stem cell factor (SCF), FMS-like tyrosine kinase 3 ligand (Klt3L), IL-3, IL-7 and IL-15 is required for the differentiation of mature NK cells from HSCs. In brief, SCF, Klt3L, IL-3 and IL-7 are required in the transition of HSCs to CLPs, while IL-15 is crucial in directing CLPs toward NK maturation(56). This cytokine cocktail was demonstrated to differentiate CD34+ myeloid precursors to mature NK cells *in vitro*(57). Moreover, myeloid precursors at later differentiation stages were also capable of differentiation into NK cells in the presence of hydrocortisone and stromal cells(57). The cytokine signalling mediated by the gamma (γc)

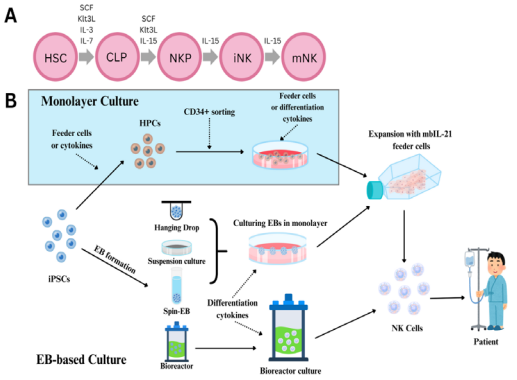

Fig. 1: Schematic diagram depicting various cytokines involved in successive phases in NK cell development and summary of approaches in generation of NK cells from iPSCs.

chain receptors (CD132) is significant for NK cell development. The γc receptor serves as the signalling subunit for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, where the signal is transduced downstream through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway(58). IL-2 and IL-15 are identified as crucial regulators of NK cells. Despite low sequence similarity, both cytokines have high affinity to form heterodimers with IL-2Rβ (CD122) subunit and γc chain via the α-receptor subunit (IL-2α and IL-15α respectively)(59). Both cytokines are also involved in the stimulation and proliferation of NK cells(60). The overlap in regulatory action between the two cytokines is due to their shared signalling receptor; however, they are not functionally redundant(60). Genetic defect of IL-2α (61) in mice model showed phenotypically normal but reduced NK cells while defect of IL-2Rβ (62) or IL-15α (63) showed significant NK cell defect. Such reveals IL-2 being dispensable in NK cell development while IL-15 is strictly required. IL-15 plays a significant role in the survival of NK cells *in vivo*(64, 65). Furthermore, IL-15 and IL-7 participate in the development of NK subsets. In mice, IL-7 is responsible for the development of a thymic CD127 (IL-7α) NK subset which shares similarities with human $CD56^{bright}$ NK cells where it has low cytotoxicity and high cytokines secretion(66). Additionally, IL-7 is known to sustain the survival of $CD56^{bright}$ NK cells in humans(67). IL-7 is also required in the survival and response of NK-22 cells, an IL-22-secreting human NK subset localized in mucosal-associated lymphoid tissues(68).

Another γc receptor subunit IL-21 acts as a regulator for NK cell responses. IL-21 upregulates IFN-γ secretion in NK cells, thereby enhancing the antibody-dependent cellular cytotoxicity effect(69). Furthermore, IL-21 increases the expression of perforin and granzyme effector molecules, and in synergy with IL-2, upregulates NK surface receptors including NKG2A, CD25, CD86 and CD69(70). In murine NK cells, IL-21 is known to induce maturation into large granular lymphocyte phenotype with enhanced effector function(71). In

human NK cells, IL-21 accelerates *in vitro* differentiation of HSCs toward mature CD56^{dim} NK cells with mature KIR repertoire in combination with IL-15, SCF and FLT-3(72). In addition to γc receptor cytokines, SCF and Klt3L have also been implicated in NK development. Both cytokines have overlapping effects in maintaining HSC survival and proliferation and priming of HSCs toward lymphoid lineages(73-75). IL-3 has also been implicated in HSC maintenance and may be required in the differentiation of early NKPs(75).

DIFFERENTIATION OF NK CELLS FROM hiPSCs

Despite the well-established differentiation protocols to guide HSC differentiation into NK cells. Hurdles remains into scaling the production for clinical use due to the low efficiency and progressive loss of multipotency in adult HSCs (76). Recent development in hiPSC differentiation into therapeutic NK cells has shown some success, especially the increase in cell yield and scalability for clinical use(77). Differentiation of NK cells from hiPSCs consists of haematopoietic differentiation (stage 1) and NK differentiation stage (stage 2). Earlier models for NK differentiation from hiPSCs were based on hESCs before the invention of hiPSC. Such models require the use of feeder cells which offers high differentiation efficiency(14). The transition towards feeder-free, animal-free based differentiation systems is crucial towards generating GMP-grade hiPSC-derived NK cells (iNKs) suitable for clinical use. The iNK generation has been approached using monolayer culture and 3D systems using embryoid bodies (EBs) (Fig. 1B).

The first generation of NK cells from pluripotent stem cells were based on monolayer cultures that were reliant on feeder cells at both stages (Table II).In 2005, Kaufman group developed a differentiation method via a two-step monolayer culture consisting of co-culturing ESCs with murine bone marrow stromal cells for haematopoietic differentiation; followed by co-culture HPCs with mouse liver cells for subsequent NK(78). Due to the low haematopoietic differentiation efficiency of the method, magnetic sorting of CD34+CD45+ HPC population was required prior to NK differentiation(78). The cytokine cocktail including IL-15, IL-3, IL-7, SCF, and Flt3L yielded functional, cytotoxic NK cells expressing inhibitory and activating receptors as of mature NK cells(78). Zeng, Tang (79) developed an efficient haematopoietic differentiation of iPSCs by co-culturing with OP9 feeder cells, thereby foregoing the sorting step. The method involved co-culture of HPCs with OP9 cells expressing DLL1 resulting in highly efficient generation of CD56+CD45+ NK population (>99%). Alternatively, Niwa and Saito (80)developed a complete feeder and animal-free monolayer system for iNK generation via a four-step differentiation. The method yielded high differentiation efficiency at both stages in serum-free conditions.

Table II: Summary of differentiation of iPSCs to NK cells via monolayer culture

hPSC source	Differentiation system	Hematopoietic differentiation stage (Stage 1) medium	Culture duration	Purity of hematopoietic progenitor	NK differentiation stage (Stage 2) medium	Culture duration	Purity of NK cells	Reference
hESC-H9	Stage 1: Monolayer culture of hESCs with S17 feeder cells Stage 2: Monolayer culture of CD34+ sorted HPCs with AFT024 feeder cells	RPMI 1640 (15% FBS) + 2 mM L-glutamine + 0.1 mM 2-ME + 1% MEM-nonessential amino acids + 1% P/S *HPCs (CD34+CD45+) are magnetically sorted before stage 2.*	14-17 days	CD34+: 9.7% (unsorted)	DMEM/F-12 (20% heat-inactivated hAB serum) + 5 ng/ml sodium selenite + 50 µM ethanolamine + 20 mg/L ascorbic acid + 25 µM 2-ME + 1% P/S + 10 ng/ml IL-15 + 20 ng/ml IL-7 + 20 ng/ml SCF + 10 ng/ml Flt-3L + 5 ng/ml IL-3 (first week only)	7-50 days	CD56+CD45+: 37.5% (at 28 days of differentiation)	(78)
hiPSC derived from PB	Stage 1: Monolayer culture of iPSCs with OP9 feeder cells Stage 2: Monolayer culture of iPSCs with OP9-DLL1 feeder cells	α-MEM (20% FBS)	12 days	CD34+: 18-19%	α-MEM (20% FBS) + 10 ng/mL SCF + 5 ng/mL Flt-3L + 5 ng/mL IL-7 and/or 10 ng/mL IL-15	Up to 35 days	CD56+CD45+: > 99%	(79)

CONTINUE

Table II: Summary of differentiation of iPSCs to NK cells via monolayer culture (CONT.)

hPSC source	Differentiation system	Hematopoietic differentiation stage (Stage 1) medium	Culture duration	Purity of hematopoietic progenitor	NK differentiation stage (Stage 2) medium	Culture duration	Purity of NK cells	Reference
hiPSCs (CB-A11 & 409B7) (Both derived from cord-blood mononuclear cells)	Complete feeder-free monolayer culture	Phase 1: *Primitive streak-like cell induction* Essential 8 + 2 μM CHIR-99021 + 80 ng/mL BMP4 + 80 ng/mL VEGF Phase 2: *Hemangioblast-like cell induction* Essential 6 + 2 μM SB431542 + 50 ng/mL SCF + 80 ng/mL VEGF Phase 3: *Hematopoietic progenitor induction* Stemline-II medium + 50 ng/mL SCF + 50 ng/mL Flt-3 Ligand	12 days Phase 1: Day 0-2 Phase 2: Day 2-4 Phase 3: Day 4-12	CD34+CD45+: A11: 92.47 ± 1.07% B7: 91.40 ± 4.29%	DMEM (20% hAB-serum) or Stemline-II + 50 ng/mL SCF + 50 ng/mL Flt-3L + 50 ng/mL IL-7 + 50 ng/mL IL-15	Up to 36 days	CD56+: 87.40 ± 0.61%	(98)

Abbreviations: CB – cord blood; DMEM/F12 – Dulbecco's modified Eagle medium/nutrient mixture F-12; FBS – fetal bovine serum; Flt-3L – FMS-like tyrosine kinase 3 ligand; hESC – human embryonic stem cell; hiPSC - human induced pluripotent stem cell; HPC – hematopoeitic progenitor cell; MEM – Minimum essential medium; NK- natural killer; PB – peripheral blood; SCF – stem cell factor; VEGF – vascular endothelial growth factor

Three-dimensional (3D) culture involving embryoid bodies (EBs) represents the main approach for generating iNKs (Table III) EBs provide a useful *in vitro* differentiation model as they recapitulate embryogenesis and demonstrate differentiation into three germ layers(81). Additionally, EBs provide access to early progenitor cells that are difficult to isolate(82). EBs have been demonstrated to generate haematopoietic lineages from hESCs with the addition of mesoderm-inducing factors including VEGF, BMP4, and cytokines including SCF, Flt3L and TPO(83, 84). The combination of the factors was sufficient to replace serum-derived factors in haematopoiesis, enabling feeder and serum-free haematopoietic development from hESCs(84). There are several methods to generate EBs from iPSCs, each encompassing specific advantages and weaknesses. These methods include hanging drop culture, spinner flask culture, and suspension culture in methylcellulose or low-adherent plates (85). The method of forming EBs influences their size and homogeneity, which in turn affects their survival and downstream differentiation (86, 87).

Table III: Summary of differentiation of iPSCs to NK cells via EB-based (3-D) system

hPSC source	Differentiation system	Hematopoietic differentiation stage (Stage 1) medium	Culture duration	Purity of hematopoietic progenitor	NK differentiation stage (Stage 2) medium	Culture duration	Purity of NK cells	Reference
hESC (H1 & H9) UCBiPS7, NHDFiPS, BJ1-iPS	Stage 1: Spin-EBs generated by seeding suspension iPSCs in 96-well ULA plate (3000 cells/well) and inducing forced aggregation via centrifugation (1500 rpm, 5 min) Stage 2: EBs cultured with EL08-1D2 feeder cells or in feeder free condition in uncoated plates	BPEL + 40 ng/mL SCF + 20 ng/mL BMP4+ 20 ng/mL VEGF	11 days	CD34+: hESCs: 55.9 ± 6.4% hiPSCs: 12.06 ± 5.40% CD45+: hESCs: 26.2 ± 6.6% hiPSCs: 3.20 ± 1.43%	IL-7 + IL-15 + SCF + Flt-3L + IL-3 (first week only)	28 days	CD56+: With feeder – 96.7% Feeder-free – 76.4%	(88)

CONTINUE

Table III: Summary of differentiation of iPSCs to NK cells via EB-based (3-D) system (CONT.)

hPSC source	Differentiation system	Hematopoietic differentiation stage (Stage 1) medium	Culture duration	Purity of hematopoietic progenitor	NK differentiation stage (Stage 2) medium	Culture duration	Purity of NK cells	Reference
hESC (H1 and H9), hiPSC (derived from UCB, CB, fibroblast)	Stage 1: Spin-EBs generated by seeding suspension iPSCs in 96-well ULA plate (3000 cells/well) and inducing forced aggregation via centrifugation (480 *g*, 8 ℃, 5 min) Stage 2: EBs cultured with EL08-1D2 feeder cells or in feeder free condition in uncoated plates	BPEL + 40 ng/mL SCF + 20 ng/mL BMP4+ 20 ng/mL VEGF	11 days	CD34+: 40-60% CD34+45+: 20-40%	56.6 % DMEM-high glucose + 28.3 % HAMS/F12 + 15 % heat-inactivated hAB serum + 2 mM L-glutamine +1 µM β-mercaptoethanol + 5 ng/mL sodium selenite + 50 uM ethanolamine + 20 mg/L ascorbic acid + 1 % P/S + 20 ng/mL SCF, 20 ng/mL IL-7 + 10 ng/mL IL-15 + 10 ng/mL Flt-3L + 5 ng/mL IL-3 (first week only)	28 days	Not mentioned	(99)
KOLF2 and hiPSC01(derived from fibroblast)	Stage 1: Spin-EBs generated by seeding suspension iPSCs in 96-well ULA plate (3000 cells/well) and inducing forced aggregation via centrifugation (220 *g*, 5 min) Stage 2: EBs cultured in uncoated plates	APEL + 40 ng/mL SCF + 20 ng/mL BMP4+ 20 ng/mL VEGF + 10 µM Y-27632	11 days	CD34: hiPSC01 – 60.40 ± 14.68% KOLF2 – 57.03 ± 13.61% CD45: hiPSC01 – 10.46 ± 2.57%, KOLF2 – 8.12 ± 3.50%	APEL 2 + 20 ng/mL SCF + 20 ng/mL IL-7 + 10 ng/mL IL-15 + 10 ng/mL Flt-3L+ 5 ng/mL IL-3 (first week only)	28 days	CD3–CD56+: hiPSC01 – 31.68 ± 8.90% KOLF2 – 17.06 ± 6.70%	(100)
hiPSC - MUSIi013-A (derived from UCB-NK cells)	Stage 1: Spin-EBs generated by seeding suspension iPSCs in 96-well ULA plate (5000 cells/well) and inducing forced aggregation via centrifugation (250 *g*, 5 minutes) Stage 2: EBs cultured in Matrigel-coated plates	APEL + 40 ng/mL SCF + 20 ng/mL BMP4+ 20 ng/mL VEGF + 10 µM Y-27632	6 days	CD34+: 70.5% (after 6 days incubation in NK media)	APEL 2 + 20 ng/mL SCF + 20 ng/mL IL-7 + 10 ng/mL IL-15 + 10 ng/mL Flt-3L+ 5 ng/mL IL-3 (first week only)	21-28 days	CD56+: 40.3%	(93)

CONTINUE

Table III: Summary of differentiation of iPSCs to NK cells via EB-based (3-D) system (CONT.)

hPSC source	Differentiation system	Hematopoietic differentiation stage (Stage 1) medium	Culture duration	Purity of hematopoietic progenitor	NK differentiation stage (Stage 2) medium	Culture duration	Purity of NK cells	Reference
hiPSC derived from PBMCs	Stage 1: Spin-EBs generated according to Zhu and Kaufman (92) protocol. Stage 2: EBs cultured in uncoated plates	APEL + 40 ng/mL SCF + 20 ng/mL BMP4+ 20 ng/mL VEGF + 10 µM Y-27632	6 days	Not mentioned	DMEM/F12 + 20% EliteGro-Adv + 1% P/S + 1% GlutaMAX +1 µM β-mercaptoethanol + 5 ng/mL sodium selenite + 50 µM ethanolamine + 20 mg/mL ascorbic acid + 20 ng/mL SCF + 20 ng/mL IL-7 + 10 ng/mL IL-15 + 10 ng/mL Flt-3L + 5 ng/mL IL-3 (first week only)	28-35 days	CD56+: 88.5%	(101)
QHJI-iPSC (Derived from blood)	Stage 1: EBs generated in low attachment plate by overnight incubation of suspension iPSCs in Stemfit AK03N + 10 µmol/L Y-27632. Stage 2: HPCs cultured on FcDLL4-coated plates to produce LPCs. LPCs are further expanded into NK/ILCs in co-culture of allogeneic PBMCs using phytohemagglutinin-P (PHA-P) scaffold.	Phase 1: *Mesodermal induction* EB medium (StemPro-34 + 2 mmol/L l-glutamine + 400 µmol/L monothioglycerol + 50 µg/mL ascorbic acid-2-phosphate + insulin-transferrin-selenium supplements) + 40 ng/mL hBMP-4 + 10 ng/mLFGF + 50 ng/mL VEGF Phase 2: *HPC induction* EB medium + 50 ng/mL hSCF + 20 ng/mL hFlt3L + 20 ng/mL hIL-3 + 30 ng/mL TPO	14 days Phase 1: Day 0-4 Phase 2: Day 4-14	CD34+43+: 69.2%	Phase 1: *Lymphocyte progenitor cell (LPC) induction* FcDLL4-coated plates + 10 ng/mL hFlt-3L + 5 ng/mL IL-7 + TPO Phase 2: *Expansion and differentiation of LPCs to NK/ILCs* FcDLL4-coated plates + IL-7 + IL-15	Phase 1: Up to 25 days Phase 2: Up to 15 days	CD56+: 98.5% (NK/ILCs at end of phase 2)	(95)

CONTINUE

Table III: Summary of differentiation of iPSCs to NK cells via EB-based (3-D) system (CONT.)

hPSC source	Differentiation system	Hematopoietic differentiation stage (Stage 1) medium	Culture duration	Purity of hematopoietic progenitor	NK differentiation stage (Stage 2) medium	Culture duration	Purity of NK cells	Reference
hiPSCs (derivation not mentioned)	Stage 1: EBs generated from hanging drops. Suspension iPSCs are seeded in DMEM/F12 + 10% serum replacement + 10 mM Y27633, 25 µL drops (4000 cells) are incubated for 3 days. Stage 2: EBs cultured in low attachment plate entirely in 3D system. Alternatively, LPCs obtained from 3D system are cultured in normal plate coated with STEMdiff lymphoid differentiation coating material.	Phase 1: *Mesoderm induction* STEMdiff Hematopoietic Kit-Medium A Phase 2: *Haemato-endothelial induction* STEMdiff Hematopoietic Kit-Medium B	10 days Phase 1: Day 0-3 Phase 2 Day 3-10	Not mentioned	Phase 1: *Lymphoid progenitor expansion* STEMdiff lymphoid expansion medium (contains 14 IU/mL IL-3, 4,500 IU/mL IL-15, 8,800 IU/mL IL-7, 26 IU/mL SCF, and 12 IU/mL Ftl3L) Phase 2 *NK maturation* STEMdiff NK cell differentiation medium (contains 4,500 IU/mL IL-15, 8,800 IU/mL IL-7, 26 IU/mL SCF, and 12 IU/mL Ftl3L)	28 days. Phase 1: 14 days Phase 2: 14 days	CD56+CD45+: 3D system – 14.3% 2D system – 32.3%	(102)
hiPSCs (derived from human normal dermal fibroblast)	Stage 1: EBs generated in spinner-flask bioreactor. Suspension iPSCs ($0.67\text{-}1.33 \times 10^6$) are cultured in NutriStem + Y27632 for 12h, forming EBs at 80-150 µm in diameter. Stage 2: HPCs differentiated in 3D differentiation system.	Phase 1: *Mesoderm and hemogenic endothelial induction* Nutristem + 25-50 ng/mL BMP4 + 25-50 ng/mL VEGF + 25-50 ng/mL bFGF + 3 µM CHIR99021 (Day 3-4) + 3 µM SB431542 (Day 4-5) Phase 2: *Hematopoietic differentiation and expansion* Nutristem + 10-25 ng/mL TPO + 10-25 ng/mL SCF + 10-25 ng/mL Flt3L + 2-10 ng/mL IL-3 + 2-10 ng/mL IL-6 + 0.75 µM SR1 + 2-10 ng/mL Oncostatin M (OSM) + 2 U/mL EPO (3 U/mL in expansion phase)	>13 days Phase 1: Day 0-6 (Hypoxia condition for Day 0-4) Phase 2: Differentiation – Day 6-11 Expansion – up to 40 days HPCs harvested around week 2.	Phase 1: CD34+: 15-30% CD43+: 7.5-20% Phase 2: CD34+CD45 in released HPCs: 34% (Day 14) Spheres: Up to 22% at Day 23	Basal media (10% FBS) + 10 ng/mL SCF + 5 ng/mL Flt-3L + 5 ng/mL IL-7 + 10 ng/mL IL-15 + 50 ng/mL sDLL- 1 + 10 ng/mL IL-6	21-50 days	CD56: 57% (at 21 days of differentiation)	(97, 103)

Abbreviations: 3D - three-dimensional; APEL – Albumin Polyvinylalcohol Essential Lipids; BPEL – Bovine Serum Albumin Polyvinylalcohol Essential Lipids; CB – cord blood; DMEM – Dulbecco's modified Eagle medium; EB – embryoid body; EPO – erythropoietin; FBS – fetal bovine serum; Flt-3L – FMS-like tyrosine kinase 3 ligand; FGF – fibroblast growth factor; HPC – hematopoeitic progenitor cell; LPC – lymphocyte precursor cell; NK- natural killer; PB – peripheral blood; PBMC – peripheral blood mononuclear cell; PHA-P – phytohemagglutinin-P; SCF – stem cell factor; TPO – thyroid peroxidase; UCB – umbilical cord blood; ULA – ultra low attachment; VEGF – vascular endothelial growth factor; hESC – human embryonic stem cell; hiPSC – human induced pluripotent stem cell

In 2013, the Kaufman group adapted a spin-EB approach to generate haematopoietic progenitors from iPSCs in feeder-free conditions (88, 89). The spin-EB protocol developed by Ng and colleagues detailed the formation of EBs in 96-well low-attachment plates by aggregating hiPSCs via centrifugation, allowing uniform generation of EBs which differentiates efficiently and synchronously (90, 91). Ng and colleagues also presented the formulation of the polyvinyl alcohol (PVA) inclusive APEL and BPEL media, which provided a highly reproducible and feeder-free platform for haematopoietic development from hiPSCs (91). An optimal EB cell density for hematopoietic derivation in an 11-day incubation was earlier developed by Ng and colleagues, whereby a minimal size of 500 cells/well was required for efficient generation of hematopoietic progenitors (90). An increase of cell density to 3000 cells/well increased hematopoietic yield but further increase in cell density did not improve hematopoietic yield (90). More recently, using higher cell densities was found to produce efficient hematopoietic yield in a shorter period. In the updated protocol from Kaufman group, the haematopoietic differentiation duration was shortened from 11 days to 6 days by increasing the cell density in each well to 8000 cells/well (92). Similarly, Klaihmon, Kang (93)also reported 6 days of haematopoietic differentiation duration with a cell density of 5000 cells/well.

Following haematopoietic differentiation, the EBs can be enzymatically digested for flow cytometry analysis to determine the CD34+ population. The Kaufman group reported in the updated protocol that the method can consistently generate more than 50% CD34+ population (92). However, a high CD34+ population appears to not be strictly required to generate mature NK cells. As low as 10% CD34 population in EBs was sufficient to generate mature NK cells from the Kaufman protocol. The CD34 population may be gradually released from the EBs following incubation in differentiation media (93). For initiating haematopoietic differentiation, EBs are seeded in cell culture plates that can be uncoated or coated with gelatine (92) or Matrigel (93) to aid with EB attachment.

While the development of spin-EB protocols successfully introduced reproducible iNK generation, the prohibitive cost of commercial ultra-low attachment (ULA) plates and specialized EB media remains a hurdle. In addition, patent barriers to the spin-EB method also present interest in pursuing alternative methods. A cost-effective alternative for obtaining ULA plates is by treating conventional U or V bottom 96-well plates with PVA or commercially available anti-adherence rinsing solution (94). One method which forgoes the need for spin-EBs was reported by Kaneko group, whereby EBs were generated by incubating suspension hiPSCs in a low-attachment plate overnight(95). Derived HPCs are then differentiated in Fc-DLL4-coated plates supplemented with T lineage cytokines and expanded in phytohemagglutinin-p (PHA) to obtain ILCs with NK functionality, referred to as NK/ILCs.

The fold expansion of NK cells from iPSC during differentiation is often insufficient, thereby requiring a further expansion system to generate a clinically relevant number of NK cells (14). Artificial presenting cells (APCs) expressing membrane-bound IL-21 (mbIL-21) are routinely used for NK expansion *in vitro*. The expansion of NK cells with mbIL-21 demonstrated a significant increase in proliferation ability with mature effector functionality (96). Additionally, the expansion system also sustained cell senescence, with improved telomere length, enabling prolonged expansion (96).

The use of bioreactors for haematopoietic differentiation and NK generation is of great interest for large-scale generation of clinical-grade NK cells. Despite this, there is a lack of reports on bioreactor systems for generating NK cells. Lu and Feng (97) patented a method for efficiently differentiating hiPSCs into NK cells in a serum-free bioreactor system96. In this process, suspension hiPSCs are expanded into 3D spheres in a spinner flask bioreactor, and subsequently induced into haematopoietic and NK lineages.

CHALLENGES AND PROSPECTS

Despite the advantages of iPSCs in generating NK cells, scalability of production remains the largest hurdle for large-scale adoption of NK cell therapy. Current methods can produce clinical-grade iNKs in limited quantities for clinical trials, but optimisation is required to enable the treatment of larger patient cohorts with multiple doses at a lower cost. Currently, clinical trials involving iNKs administer doses ranging from 106 to 108 cells per treatment (28, 30). However, improved NK production could enable higher doses exceeding 109 cells, which have been proven safe and effective with NK-92 cells (6).

The spin-EB approach developed by the Kaufman group can generate over 109 NK cells from a plate of spin-EBs after expansion with mbIL-21 in an 8-week process (92). However, the limited production of EBs poses a bottleneck for large-scale manufacturing. A potential solution is to use commercially available low-binding microwells, which can mass-produce thousands of homogeneous EBs from suspension iPSCs (80, 104). Additionally, rotary or stirred bioreactors can be employed for the mass production of homogeneous EBs (105). Despite these advances, the current EB-based system for iNK production remains labour-intensive, requiring the transfer of EBs and repeated media changes.

Further exploration of bioreactor platforms is essential for scaling up iNK production. The proprietary bioreactor platform developed by Lu and Feng can consistently

produce 1010 homogeneous and functional NK cells from a continuous 300 mL culture bioreactor over 45 days (97). This process eliminates the need for EB transfer and separate expansion systems involving APCs. Closed bioreactor systems, which also eliminate the need for repeated media changes(14), are being investigated as they may further reduce production costs by decreasing labour and minimising contamination risks. Previously, NK cells expanded using a closed bioreactor system demonstrated similar cytotoxic properties to those produced by conventional methods (106).

Advancements in automated cell processing systems, particularly those incorporating artificial intelligence (AI), promise significant improvements in iNK production. Automated processes can streamline the entire manufacturing workflow, from donor selection to automated manufacturing and quality control. Robust processes are necessary for selecting healthy iPSC lines for genetic manipulation, validating quality, and conducting clonal screening to establish genetically engineered iPSC primary cell lines. Fully automated systems can facilitate mass production of iNK cells from iPSCs, followed by automated functional validation and storage. Integrating automated processes throughout all phases will significantly enhance efficiency and reproducibility in iNK production while reducing the likelihood of human errors.

CONCLUSION

The use of NK cells as an allogeneic cell source for adoptive cell therapy offers the promise of readily available and cost-effective treatment. However, producing clinical-grade NK cells in large quantities remains a significant barrier to widespread adoption. HiPSCs present several advantages for NK cell generation, enabling the production of homogeneous and clinical-grade cells. Significant progress has been made in developing iPSC differentiation protocols for NK cells, particularly through embryoid body-based systems. Additionally, genetic engineering of iPSCs can further enhance cell viability and proliferation, improving manufacturing efficiency and cell function. The next step is to establish mass production systems for NK cells, focusing on automated bioreactor systems that can generate high-quality, homogeneous, and large quantities of clinical-grade cells while adhering to good manufacturing practices.

REFERENCES

1. Porter D, Frey N, Wood PA, Weng Y, Grupp SA. Grading of cytokine release syndrome associated with the CAR T cell therapy tisagenlecleucel. J Hematol Oncol 2018;11:35. doi: 10.1186/s13045-018-0571-y
2. Tang TCY, Xu N, Nordon R, Haber M, Micklethwaite K, Dolnikov A. Donor T cells for CAR T cell therapy. Biomark Res 2022;10:14. doi: 10.1186/s40364-022-00359-3
3. Sterner RC, Sterner RM. CAR-T cell therapy: current limitations and potential strategies. Blood Cancer J 2021;11:69. doi: 10.1038/s41408-021-00459-7
4. Paul S, Lal G. The molecular mechanism of natural killer cells function and its importance in cancer immunotherapy. Front Immunol 2017;8:1124. doi: 10.3389/fimmu.2017.01124
5. Jones AB, Rocco A, Lamb LS, Friedman GK, Hjelmeland AB. Regulation of NKG2D stress ligands and its relevance in cancer progression. Cancers (Basel) 2022;14. doi: 10.3390/cancers14092339
6. Tang X, Yang L, Li Z, Nalin AP, Dai H, Xu T, et al. First-in-man clinical trial of CAR NK-92 cells: Safety test of CD33-CAR NK-92 cells in patients with relapsed and refractory acute myeloid leukemia. American Journal of Cancer Research 2018;8:1083. doi:
7. Liu E, Marin D, Banerjee P, Macapinlac HA, Thompson P, Basar R, et al. Use of CAR-transduced natural killer cells in CD19-positive lymphoid tumors. N Engl J Med 2020;382:545-53. doi: 10.1056/NEJMoa1910607
8. Lin H, Yang X, Ye S, Huang L, Mu W. Antigen escape in CAR-T cell therapy: Mechanisms and overcoming strategies. Biomed Pharmacother 2024;178:117252. doi: 10.1016/j.biopha.2024.117252
9. Maalej KM, Merhi M, Inchakalody VP, Mestiri S, Alam M, Maccalli C, et al. CAR-cell therapy in the era of solid tumor treatment: Current challenges and emerging therapeutic advances. Molecular Cancer 2023;22:20. doi: 10.1186/s12943-023-01723-z
10. Zhu H, Blum RH, Bernareggi D, Ask EH, Wu Z, Hoel HJ, et al. Metabolic reprograming via deletion of CISH in human iPSC-derived NK cells promotes *in vivo* persistence and enhances anti-tumor activity. Cell Stem Cell 2020;27:224-37 e6. doi: 10.1016/j.stem.2020.05.008
11. Peled T, Brachya G, Persi N, Lador C, Olesinski E, Landau E, et al. Enhanced *in vivo* persistence and proliferation of NK cells expanded in culture with the small molecule nicotinamide: Development of a clinical-applicable method for NK expansion. Blood 2017;130:657. doi: https://doi.org/10.1182/blood.V130.Suppl_1.657.657
12. Biasco L, Izotova N, Rivat C, Ghorashian S, Richardson R, Guvenel A, et al. Clonal expansion of T memory stem cells determines early anti-leukemic responses and long-term CAR T cell persistence in patients. Nat Cancer 2021;2:629-42. doi: 10.1038/s43018-021-00207-7
13. Pan K, Farrukh H, Chittepu V, Xu H, Pan CX, Zhu Z. CAR race to cancer immunotherapy: from CAR T, CAR NK to CAR macrophage therapy. J Exp Clin Cancer Res 2022;41:119. doi: 10.1186/s13046-022-02327-z

14. Fang F, Xie S, Chen M, Li Y, Yue J, Ma J, et al. Advances in NK cell production. Cell Mol Immunol 2022;19:460-81. doi: 10.1038/s41423-021-00808-3
15. Solana R, Campos C, Pera A, Tarazona R. Shaping of NK cell subsets by aging. Curr Opin Immunol 2014;29:56-61. doi: 10.1016/j.coi.2014.04.002
16. Gounder SS, Abdullah BJJ, Radzuanb N, Zain F, Sait NBM, Chua C, et al. Effect of aging on NK cell population and their proliferation at ex vivo culture condition. Analytical Cellular Pathology 2018;2018:7871814. doi: 10.1155/2018/7871814
17. Damele L, Spaggiari GM, Parodi M, Mingari MC, Vitale M, Vitale C. Cord blood-derived natural killer cell exploitation in immunotherapy protocols: More than a promise? Cancers (Basel) 2022;14. doi: 10.3390/cancers14184439
18. Shimasaki N, Jain A, Campana D. NK cells for cancer immunotherapy. Nat Rev Drug Discov 2020;19:200-18. doi: 10.1038/s41573-019-0052-1
19. Goldenson BH, Zhu H, Wang YM, Heragu N, Bernareggi D, Ruiz-Cisneros A, et al. Umbilical cord blood and iPSC-derived natural killer cells demonstrate key differences in cytotoxic activity and KIR profiles. Front Immunol 2020;11:561553. doi: 10.3389/fimmu.2020.561553
20. Klingemann H, Boissel L, Toneguzzo F. Natural killer cells for immunotherapy–advantages of the NK-92 cell line over blood NK cells. Front Immunol 2016;7:91. doi: 10.3389/fimmu.2016.00091
21. Tonn T, Schwabe D, Klingemann HG, Becker S, Esser R, Koehl U, et al. Treatment of patients with advanced cancer with the natural killer cell line NK-92. Cytotherapy 2013;15:1563-70. doi: 10.1016/j.jcyt.2013.06.017
22. Navarrete-Galvan L, Guglielmo M, Cruz Amaya J, Smith-Gagen J, Lombardi VC, Merica R, et al. Optimizing NK-92 serial killers: Gamma irradiation, CD95/Fas-ligation, and NK or LAK attack limit cytotoxic efficacy. Journal of Translational Medicine 2022;20:151. doi: 10.1186/s12967-022-03350-6
23. Walcher L, Kistenmacher A-K, Sommer C, Buhlen S, Ziemann C, Dehmel S, et al. Low energy electron irradiation is a potent alternative to gamma irradiation for the inactivation of (CAR-)NK-92 cells in ATMP manufacturing. Front Immunol 2021;12. doi: 10.3389/fimmu.2021.684052
24. Takahashi K, Yamanaka S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell 2006;126:663-76. doi: 10.1016/j.cell.2006.07.024
25. Karagiannis P, Kim S-I. iPSC-Derived Natural Killer Cells for Cancer Immunotherapy. Molecules and Cells 2021;44:541-8. doi: https://doi.org/10.14348/molcells.2021.0078
26. Yamanaka S. Pluripotent stem cell-based cell therapy—promise and challenges. Cell Stem Cell 2020;27:523-31. doi: 10.1016/j.stem.2020.09.014
27. Haridhasapavalan KK, Borgohain MP, Dey C, Saha B, Narayan G, Kumar S, et al. An insight into non-integrative gene delivery approaches to generate transgene-free induced pluripotent stem cells. Gene 2019;686:146-59. doi: 10.1016/j.gene.2018.11.069
28. Bachanova V, Ghobadi A, Patel K, Park JH, Flinn IW, Shah P, et al. Safety and efficacy of FT596, a first-in-class, multi-antigen targeted, off-the-shelf, ipsc-derived CD19 CAR NK cell therapy in relapsed/refractory B-cell lymphoma. Blood 2021;138:823. doi: https://doi.org/10.1182/blood-2021-151185
29. Ramachandran I, Rothman S, Clausi M, McFadden K, Salantes B, Jih G, et al. Multiple doses of CNTY-101, an iPSC-derived allogeneic CD19 targeting CAR-NK product, are safe and result in tumor microenvironment changes associated with response: A case study. Blood 2023;142:1654. doi: https://doi.org/10.1182/blood-2023-182313
30. Harano K, Kaneko S, Nakatsura T, Yuda J, Fuse N, Sato A, et al. Abstract 5185: First in human trial of off-the shelf iPS derived anti-GPC3 NK cells for recurrent ovarian clear cell carcinoma with peritoneal dissemination. Cancer Research 2022;82:5185-. doi: 10.1158/1538-7445.Am2022-5185
31. Baghbaderani BA, Tian X, Neo BH, Burkall A, Dimezzo T, Sierra G, et al. cGMP-manufactured human induced pluripotent stem cells are available for pre-clinical and clinical applications. Stem Cell Reports 2015;5:647-59. doi: 10.1016/j.stemcr.2015.08.015
32. Poetsch MS, Strano A, Guan K. Human induced pluripotent stem cells: From cell origin, genomic stability, and epigenetic memory to translational medicine. Stem Cells 2022;40:546-55. doi: 10.1093/stmcls/sxac020
33. Kim K, Doi A, Wen B, Ng K, Zhao R, Cahan P, et al. Epigenetic memory in induced pluripotent stem cells. Nature 2010;467:285-90. doi: 10.1038/nature09342
34. Arnheim N, Cortopassi G. Deleterious mitochondrial DNA mutations accumulate in aging human tissues. Mutat Res 1992;275:157-67. doi: 10.1016/0921-8734(92)90020-p
35. Ono T, Uehara Y, Saito Y, Ikehata H. Mutation theory of aging, assessed in transgenic mice and knockout mice. Mech Ageing Dev 2002;123:1543-52. doi: 10.1016/s0047-6374(02)00090-8
36. Ema H, Morita Y, Suda T. Heterogeneity and hierarchy of hematopoietic stem cells. Exp Hematol 2014;42:74-82 e2. doi: 10.1016/j.exphem.2013.11.004
37. Zhang Y, Gao S, Xia J, Liu F. Hematopoietic hierarchy - An updated roadmap. Trends Cell Biol 2018;28:976-86. doi: 10.1016/j.tcb.2018.06.001
38. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool

by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity 2006;25:977-88. doi: 10.1016/j.immuni.2006.10.016
39. Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain haematopoietic stem cells. Nature 2012;481:457-62. doi: 10.1038/nature10783
40. Rybtsov S, Batsivari A, Bilotkach K, Paruzina D, Senserrich J, Nerushev O, et al. Tracing the origin of the HSC hierarchy reveals an SCF-dependent, IL-3-independent CD43(-) embryonic precursor. Stem Cell Reports 2014;3:489-501. doi: 10.1016/j.stemcr.2014.07.009
41. Crane GM, Jeffery E, Morrison SJ. Adult haematopoietic stem cell niches. Nat Rev Immunol 2017;17:573-90. doi: 10.1038/nri.2017.53
42. Scoville SD, Freud AG, Caligiuri MA. Modeling human natural killer cell development in the era of innate lymphoid cells. Front Immunol 2017;8:360. doi: 10.3389/fimmu.2017.00360
43. Yu J, Freud AG, Caligiuri MA. Location and cellular stages of natural killer cell development. Trends Immunol 2013;34:573-82. doi: 10.1016/j.it.2013.07.005
44. Freud AG, Caligiuri MA. Human natural killer cell development. Immunol Rev 2006;214:56-72. doi: 10.1111/j.1600-065X.2006.00451.x
45. Freud AG, Yokohama A, Becknell B, Lee MT, Mao HC, Ferketich AK, et al. Evidence for discrete stages of human natural killer cell differentiation *in vivo*. J Exp Med 2006;203:1033-43. doi: 10.1084/jem.20052507
46. Schippel N, Sharma S. Dynamics of human hematopoietic stem and progenitor cell differentiation to the erythroid lineage. Exp Hematol 2023;123:1-17. doi: 10.1016/j.exphem.2023.05.001
47. Gilfillan S, Ho EL, Cella M, Yokoyama WM, Colonna M. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. Nat Immunol 2002;3:1150-5. doi: 10.1038/ni857
48. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol 2001;22:633-40. doi: 10.1016/S1471-4906(01)02060-9
49. Lanier L, Le AM, Civin C, Loken M, Phillips J. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. Journal of Immunology 1986;136:4480-6. doi: 10.4049/jimmunol.136.12.4480
50. Abel AM, Yang C, Thakar MS, Malarkannan S. Natural killer cells: Development, maturation, and clinical utilization. Front Immunol 2018;9:1869. doi: 10.3389/fimmu.2018.01869
51. Romagnani C, Juelke K, Falco M, Morandi B, D'Agostino A, Costa R, et al. CD56^{bright}CD16− killer Ig-like receptor− NK cells display longer telomeres and acquire features of CD56^{dim} NK cells upon activation. J Immunol 2007;178:4947-55. doi: 10.4049/jimmunol.178.8.4947
52. Bjorkstrom NK, Ljunggren HG, Michaelsson J. Emerging insights into natural killer cells in human peripheral tissues. Nat Rev Immunol 2016;16:310-20. doi: 10.1038/nri.2016.34
53. Lopez-Verges S, Milush JM, Pandey S, York VA, Arakawa-Hoyt J, Pircher H, et al. CD57 defines a functionally distinct population of mature NK cells in the human CD56^{dim} CD16+ NK-cell subset. Blood 2010;116:3865-74. doi: 10.1182/blood-2010-04-282301
54. Beziat V, Descours B, Parizot C, Debre P, Vieillard V. NK cell terminal differentiation: Correlated stepwise decrease of NKG2A and acquisition of KIRs. PLoS One 2010;5:e11966. doi: 10.1371/journal.pone.0011966
55. Paust S, Blish CA, Reeves RK. Redefining memory: Building the case for adaptive NK cells. J Virol 2017;91. doi: 10.1128/JVI.00169-17
56. Wu Y, Tian Z, Wei H. Developmental and functional control of natural killer cells by cytokines. Front Immunol 2017;8:930. doi: 10.3389/fimmu.2017.00930
57. Grzywacz B, Kataria N, Kataria N, Blazar BR, Miller JS, Verneris MR. Natural killer-cell differentiation by myeloid progenitors. Blood 2011;117:3548-58. doi: 10.1182/blood-2010-04-281394
58. Meazza R, Azzarone B, Orengo AM, Ferrini S. Role of common-gamma chain cytokines in NK cell development and function: Perspectives for immunotherapy. J Biomed Biotechnol 2011;2011:861920. doi: 10.1155/2011/861920
59. Ring AM, Lin JX, Feng D, Mitra S, Rickert M, Bowman GR, et al. Mechanistic and structural insight into the functional dichotomy between IL-2 and IL-15. Nat Immunol 2012;13:1187-95. doi: 10.1038/ni.2449
60. Waldmann TA. The biology of interleukin-2 and interleukin-15: Implications for cancer therapy and vaccine design. Nat Rev Immunol 2006;6:595-601. doi: 10.1038/nri1901
61. Kundig TM, Schorle H, Bachmann MF, Hengartner H, Zinkernagel RM, Horak I. Immune responses in interleukin-2-deficient mice. Science 1993;262:1059-61. doi: 10.1126/science.8235625
62. Suzuki H, Duncan GS, Takimoto H, Mak TW. Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor beta chain. J Exp Med 1997;185:499-505. doi: 10.1084/jem.185.3.499
63. Lodolce JP, Boone DL, Chai S, Swain RE, Dassopoulos T, Trettin S, et al. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. Immunity 1998;9:669-76. doi: 10.1016/s1074-7613(00)80664-0
64. Carson WE, Fehniger TA, Haldar S, Eckhert K, Lindemann MJ, Lai CF, et al. A potential role for

interleukin-15 in the regulation of human natural killer cell survival. J Clin Invest 1997;99:937-43. doi: 10.1172/JCI119258
65. Cooper MA, Bush JE, Fehniger TA, VanDeusen JB, Waite RE, Liu Y, et al. *In vivo* evidence for a dependence on interleukin 15 for survival of natural killer cells. Blood 2002;100:3633-8. doi: 10.1182/blood-2001-12-0293
66. Vosshenrich CA, Garcia-Ojeda ME, Samson-Villeger SI, Pasqualetto V, Enault L, Richard-Le Goff O, et al. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. Nat Immunol 2006;7:1217-24. doi: 10.1038/ni1395
67. Michaud A, Dardari R, Charrier E, Cordeiro P, Herblot S, Duval M. IL-7 enhances survival of human CD56^{bright} NK cells. J Immunother 2010;33:382-90. doi: 10.1097/CJI.0b013e3181cd872d
68. Cella M, Otero K, Colonna M. Expansion of human NK-22 cells with IL-7, IL-2, and IL-1beta reveals intrinsic functional plasticity. Proc Natl Acad Sci U S A 2010;107:10961-6. doi: 10.1073/pnas.1005641107
69. Roda JM, Joshi T, Butchar JP, McAlees JW, Lehman A, Tridandapani S, et al. The activation of natural killer cell effector functions by cetuximab-coated, epidermal growth factor receptor positive tumor cells is enhanced by cytokines. Clin Cancer Res 2007;13:6419-28. doi: 10.1158/1078-0432.CCR-07-0865
70. Skak K, Frederiksen KS, Lundsgaard D. Interleukin-21 activates human natural killer cells and modulates their surface receptor expression. Immunology 2008;123:575-83. doi: 10.1111/j.1365-2567.2007.02730.x
71. Brady J, Hayakawa Y, Smyth MJ, Nutt SL. IL-21 induces the functional maturation of murine NK cells. J Immunol 2004;172:2048-58. doi: 10.4049/jimmunol.172.4.2048
72. Sivori S, Cantoni C, Parolini S, Marcenaro E, Conte R, Moretta L, et al. IL-21 induces both rapid maturation of human CD34+ cell precursors towards NK cells and acquisition of surface killer Ig-like receptors. Eur J Immunol 2003;33:3439-47. doi: 10.1002/eji.200324533
73. Lyman SD, Jacobsen SEW. c-kit ligand and Flt3 ligand: Stem/progenitor cell factors with overlapping yet distinct activities. Blood 1998;91:1101-34. doi: 10.1182/blood.V91.4.1101
74. Wodnar-Filipowicz A. Flt3 ligand: role in control of hematopoietic and immune functions of the bone marrow. News Physiol Sci 2003;18:247-51. doi: 10.1152/nips.01452.2003
75. Shibuya A, Nagayoshi K, Nakamura K, Nakauchi H. Lymphokine requirement for the generation of natural killer cells from CD34+ hematopoietic progenitor cells. Blood 1995;85:3538-46. doi: 10.1182/blood.V85.12.3538.bloodjournal85123538
76. Luevano M, Madrigal A, Saudemont A. Generation of natural killer cells from hematopoietic stem cells *in vitro* for immunotherapy. Cell Mol Immunol 2012;9:310-20. doi: 10.1038/cmi.2012.17
77. Zeng J, Tang SY, Toh LL, Wang S. Generation of “Off-the-Shelf” Natural Killer Cells from Peripheral Blood Cell-Derived Induced Pluripotent Stem Cells. Stem Cell Reports 2017;9:1796-812. doi: 10.1016/j.stemcr.2017.10.020
78. Woll PS, Martin CH, Miller JS, Kaufman DS. Human embryonic stem cell-derived NK cells acquire functional receptors and cytolytic activity. J Immunol 2005;175:5095-103. doi: 10.4049/jimmunol.175.8.5095
79. Zeng J, Tang SY, Toh LL, Wang S. Generation of "off-the-shelf" natural killer cells from peripheral blood cell-derived induced pluripotent stem cells. Stem Cell Reports 2017;9:1796-812. doi: 10.1016/j.stemcr.2017.10.020
80. Niwa A, Saito MK. Induction of human natural killer cells under defined conditions by seamless transition from maintenance culture of pluripotent stem cells. Methods Mol Biol 2022;2463:47-52. doi: 10.1007/978-1-0716-2160-8_4
81. Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, et al. Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. Molecular Medicine 2000;6:88-95. doi: 10.1007/BF03401776
82. Trounson A. The production and directed differentiation of human embryonic stem cells. Endocrine Reviews 2006;27:208-19. doi: 10.1210/er.2005-0016
83. Chadwick K, Wang L, Li L, Menendez P, Murdoch B, Rouleau A, et al. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. Blood 2003;102:906-15. doi: 10.1182/blood-2003-03-0832
84. Tian X, Morris JK, Linehan JL, Kaufman DS. Cytokine requirements differ for stroma and embryoid body-mediated hematopoiesis from human embryonic stem cells. Exp Hematol 2004;32:1000-9. doi: 10.1016/j.exphem.2004.06.013
85. Kurosawa H. Methods for inducing embryoid body formation: *In vitro* differentiation system of embryonic stem cells. J Biosci Bioeng 2007;103:389-98. doi: 10.1263/jbb.103.389
86. Peerani R, Rao BM, Bauwens C, Yin T, Wood GA, Nagy A, et al. Niche-mediated control of human embryonic stem cell self-renewal and differentiation. EMBO Journal 2007;26:4744-55. doi: 10.1038/sj.emboj.7601896
87. Van Winkle AP, Gates ID, Kallos MS. Mass transfer limitations in embryoid bodies during human embryonic stem cell differentiation. Cells Tissues Organs 2012;196:34-47. doi: 10.1159/000330691
88. Knorr DA, Ni Z, Hermanson D, Hexum MK,

Bendzick L, Cooper LJ, et al. Clinical-scale derivation of natural killer cells from human pluripotent stem cells for cancer therapy. Sci Transl Med 2013;2:274-83. doi: 10.5966/sctm.2012-0084

89. Bock AM, Knorr D, Kaufman DS. Development, expansion, and *in vivo* monitoring of human NK cells from human embryonic stem cells (hESCs) and and induced pluripotent stem cells (iPSCs). J Vis Exp 2013:e50337. doi: 10.3791/50337
90. Ng ES, Davis RP, Azzola L, Stanley EG, Elefanty AG. Forced aggregation of defined numbers of human embryonic stem cells into embryoid bodies fosters robust, reproducible hematopoietic differentiation. Blood 2005;106:1601-3. doi: 10.1182/blood-2005-03-0987
91. Ng ES, Davis R, Stanley EG, Elefanty AG. A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. Nat Protoc 2008;3:768-76. doi: 10.1038/nprot.2008.42
92. Zhu H, Kaufman DS. An improved method to produce clinical-scale natural killer cells from human pluripotent stem cells. Methods Mol Biol 2019;2048:107-19. doi: 10.1007/978-1-4939-9728-2_12
93. Klaihmon P, Kang X, Issaragrisil S, Luanpitpong S. Generation and functional characterization of anti-CD19 chimeric antigen receptor-natural killer cells from human induced pluripotent stem cells. Int J Mol Sci 2023;24. doi: 10.3390/ijms241310508
94. Choy Buentello D, Koch LS, Trujillo-de Santiago G, Alvarez MM, Broersen K. Use of standard U-bottom and V-bottom well plates to generate neuroepithelial embryoid bodies. PLoS One 2022;17:e0262062. doi: 10.1371/journal.pone.0262062
95. Ueda T, Kumagai A, Iriguchi S, Yasui Y, Miyasaka T, Nakagoshi K, et al. Non-clinical efficacy, safety and stable clinical cell processing of induced pluripotent stem cell-derived anti-glypican-3 chimeric antigen receptor-expressing natural killer/innate lymphoid cells. Cancer Sci 2020;111:1478-90. doi: 10.1111/cas.14374
96. Denman CJ, Senyukov VV, Somanchi SS, Phatarpekar PV, Kopp LM, Johnson JL, et al. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. PLoS One 2012;7:e30264. doi: 10.1371/journal.pone.0030264
97. Lu SJ, Feng Q. CAR-NK cells from engineered pluripotent stem cells: Off-the-shelf therapeutics for all patients. Stem Cells Transl Med 2021;10 Suppl 2:S10-S7. doi: 10.1002/sctm.21-0135
98. Matsubara H, Niwa A, Nakahata T, Saito MK. Induction of human pluripotent stem cell-derived natural killer cells for immunotherapy under chemically defined conditions. Biochem Biophys Res Commun 2019;515:1-8. doi: 10.1016/j.bbrc.2019.03.085
99. Hermanson DL, Ni Z, Kaufman DS. Human pluripotent stem cells as a renewable source of natural killer cells. In: Cheng T, editor. Hematopoietic Differentiation of Human Pluripotent Stem Cells. Dordrecht: Springer Netherlands; 2015. p. 69-79.
100. Lupo KB, Moon JI, Chambers AM, Matosevic S. Differentiation of natural killer cells from induced pluripotent stem cells under defined, serum- and feeder-free conditions. Cytotherapy 2021;23:939-52. doi: 10.1016/j.jcyt.2021.05.001
101. Meng F, Zhang S, Xie J, Zhou Y, Wu Q, Lu B, et al. Leveraging CD16 fusion receptors to remodel the immune response for enhancing anti-tumor immunotherapy in iPSC-derived NK cells. J Hematol Oncol 2023;16:62. doi: 10.1186/s13045-023-01455-z
102. Ching PY, Wang C, Hang S, Liu P, Sugimura R. Generation of natural killer cells from human expanded potential stem cells. J Vis Exp 2023. doi: 10.3791/64608
103. Feng Q, Zhang M-Y, Lu S-J, inventorsMethods and systems for manufacturing hematopoietic lineage cells patent WO/2020/086889. 2021 2020.
104. Antonchuk J. Formation of embryoid bodies from human pluripotent stem cells using AggreWell™ plates. In: Helgason CD, Miller CL, editors. Basic Cell Culture Protocols. Totowa, NJ: Humana Press; 2013. p. 523-33.
105. Pettinato G, Wen X, Zhang N. Engineering strategies for the formation of embryoid bodies from human pluripotent stem cells. Stem Cells Dev 2015;24:1595-609. doi: 10.1089/scd.2014.0427
106. Granzin M, Soltenborn S, Мьller S, Kollet J, Berg M, Cerwenka A, et al. Fully automated expansion and activation of clinical-grade natural killer cells for adoptive immunotherapy. doi: