Derivation of Lymphocytes from Human induced Pluripotent Stem Cells

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Dedication

This thesis is dedicated to my parents, Mr. Li-lian Ma and Mrs. Ai-min Zhang and my sister Ms. Ping Ma and my brother-in-law Mr. Pei-zheng Fu. Thank you for your support and encouragement throughout my academic endeavors.

Abstract

Human pluripotent stem cells have the potential to produce essentially unlimited numbers of mature and functional blood lineage populations to study human hematopoiesis. Particularly, human induced pluripotent stem cells (hiPSCs) have the advantage to provide a source of autologous transplantable blood cell populations suitable for treatment of patient specific hematological diseases. This research aims to derive human lymphocytes from hiPSCs. There are three projects: The overall generation of human lymphocytes (B cell, T cell and NK cell) from hiPSCs is explored in Project I. In Project II and III, based on the derivation of NK cells, two human immunodeficiency disease models, both caused by specific somatic gene mutation, are established using human pluripotent stem cells. The ultimate goal of this research is to use hiPSCs to study the normal development of human lymphocytes in vitro, as well as model human immunodeficiency diseases by combining with gene therapy methods, thus providing a novel approach for immunotherapy.

The **hypothesis** is that human lymphocytes can be derived from hiPSCs and this will enable the establishment of in vitro models to study human immunodeficiency diseases.

Specific aims:

1. To generate human lymphocytes from hiPSCs in vitro;
2. To establish two human immunodeficiency disease models (X-SCID and WAS) through in vitro derivation of lymphocytes from hESCs/hiPSCs.

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Introduction

The overall goal of this research project is to use human induced pluripotent stem cells (hiPSCs) to establish an in vitro model to compare normal lymphocyte development to diseases with defective lymphocyte development. It is divided into three projects. The first general project is using hiPSC lines to study the normal development of human lymphocytes (B cell, T cell and natural killer cell) in vitro by co-culturing with different murine stromal layers. The Project I provides us a comprehensive characterization of hiPSCs derived natural killer (NK) cell development in vitro, but with a severe lack of B and T cell development. Based on Project I, we conduct two individual projects to use human pluripotent stem cells as a tool to study immunodeficiency diseases caused by specific gene mutations required for lymphocyte development. Project II aims to use the common γ chain (γ_c) gene targeted human embryonic stem cells (hESCs) to establish an in vitro lymphocyte development model to study X-linked severe combined immunodeficiency (X-SCID) disease. The Project II shows that wild type γ_c hESCs can generate NK cells, while the γ_c knocked out hESCs are deficient in NK cell development in vitro. Project III is establishing a model to study Wiskott-Aldrich syndrome (WAS), which is another X-linked immunodeficiency disease caused by the mutation of WAS gene. WAS patient derived iPSCs contain the mutated WAS gene and lack of NK cell development. Once they are corrected by gene manipulation in vitro, they are able to make normal NK cells. Thus, Project II and Project III establish two in vitro models to study NK cell development diseases.

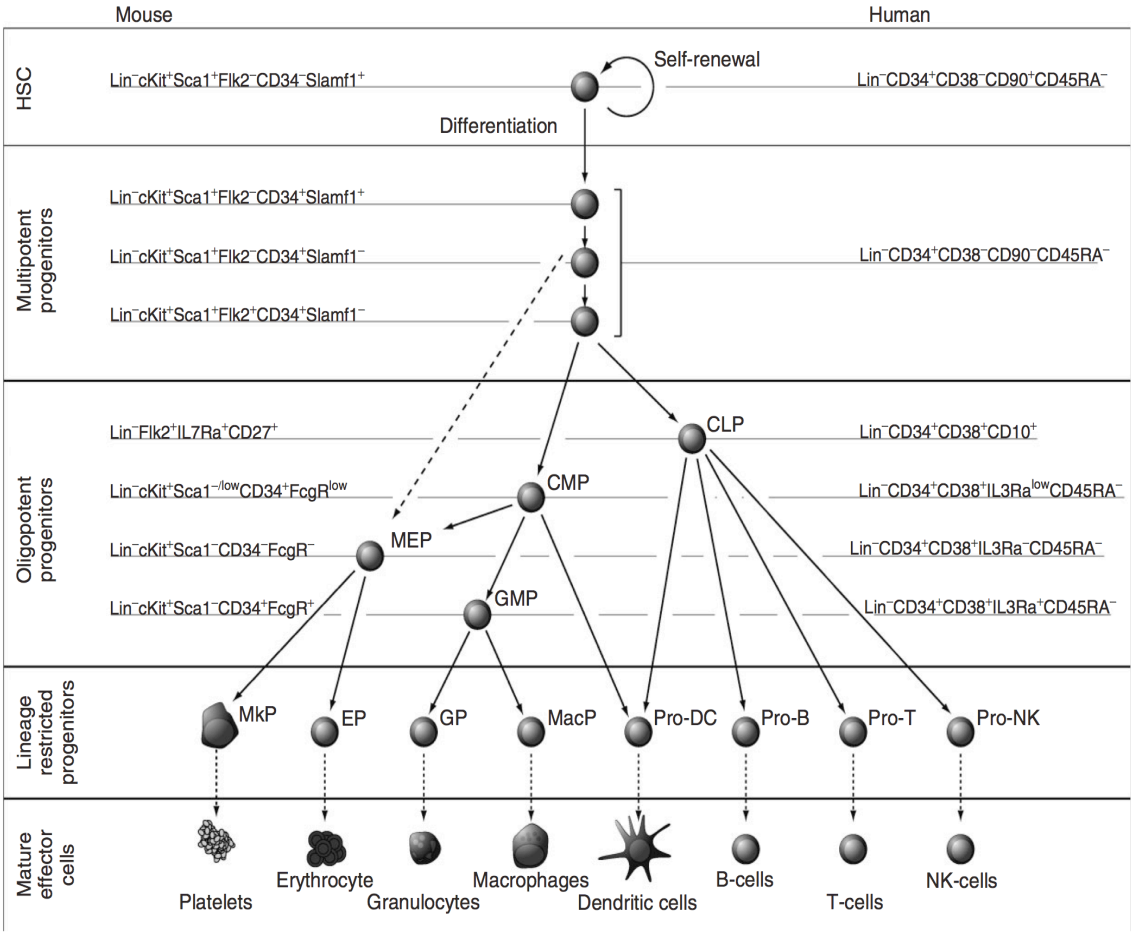
Human Hematopoiesis and Lymphocyte Development

Conducted by Till and McCulloch in the early 1960s, the concept of "stem cell" was first proposed by experimental validation through the pioneering studies of the blood system regeneration in vivo. After transplanting limited number of syngeneic bone marrow (BM) cells into irradiated recipient mice for ten days, secondary passage of cellular colonies that formed in the spleens of recipient mice was observed. Thus the analysis of these colonies implied that a very small subpopulation of the donor BM cells probably

possessed two remarkable attributes: one is to generate multiple types of myeloerythroid cells, and the other is to self-replicate [1-4].

Since then, hematopoietic stem cells (HSCs) have been defined by the ability to give rise to two daughter cells by the way of asymmetric cell division. One daughter cell maintains the HSC characteristics, indicating the self-renewal of the HSCs. The other daughter cell differentiates into all types of mature blood cells. Hematopoiesis is commonly viewed as a hierarchical model (Figure 1, [5]). First, HSCs give rise to the multipotent progenitors (MPPs), which no longer possess self-renewal ability yet keeping full-lineage differentiation potential [6, 7]. Then MPPs further downstream differentiate into two important oligopotent progenitors: common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). The CLPs mainly generate B cells, T cells and NK cells [8-10]. The CMPs give rise to mature blood cells, such as granulocytes, monocytes, erythrocytes, megakaryocytes [11].

Figure 1: Hierarchical structure model of mouse and human hematopoietic development. This figure is taken from Seita, J. and I.L. Weissman, *Hematopoietic stem cell: self-renewal versus differentiation.* Wiley Interdiscip Rev Syst Biol Med, 2010. 2(6): p. 640-53. [5]
The HSCs undergo asymmetric cell division, which is defined as the cell that has both the self-renewal capacity and the potential to give rise to all hematopoietic cell types. The HSCs give rise to the MPPs, which no longer possess self-renewal ability yet keeping full-lineage differentiation potential. Then MPPs further downstream differentiate into two important oligopotent progenitors: CLP and CMP. Through lineage restricted progenitors, the CLPs mainly generate B cells, T cells and NK cells. The CMPs give rise to mature blood cells, such as granulocytes, monocytes, erythrocytes, megakaryocytes. The cell surface phenotype of each population is shown for the mouse and human systems. HSC, hematopoietic stem cell; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DC, dendritic cell; EP, erythrocyte progenitor; GMP, granulocyte/macrophage progenitor; GP, granulocyte progenitor; MacP, macrophage progenitor; MEP, megakaryocyte/erythrocyte progenitor; MkP, megakaryocyte progenitor; NK, natural killer; Lin, lineage markers.



Development of NK cells

The progression of gene regulation in NK cell development mostly has been done in mice, but humans share many similarities with mice. Regardless of a variety of transcription factors involved in development, over-expression of some of them, most notably the inhibitors of DNA binding (ID) proteins (ID2 and ID3), delays B cell and T cell development by driving NK cell development [12, 13]. E proteins have been shown to play a central role in the transcriptional regulatory network in the development of B and T lineages [14, 15]. Since ID proteins are known to be the major antagonist for E proteins by forming dimers and make E proteins unable to bind DNA, ID proteins negatively regulate B lymphopoiesis in both mice and humans [14, 16]. ID proteins are supposed to inhibit E proteins' NK cell suppressing activity. By exploring ID gene profiling patterns, two groups have found that high expression levels of ID genes could be detected in hESCs derived hematopoietic progenitor cells, but not in umbilical cord blood (UCB) isolated CD34+ cells, indicating differential ID gene expression profiles between hESC- and UCB-derived hematopoietic progenitors [17, 18].

Development of B cells

The use of conditional knockout mice has greatly advanced our understanding of the roles of important transcription factors during mouse B cell development. However, such an approach is unavailable thus far in a human setting. Early B cell development is critically coordinated and dependent on five notable transcription factors, PU.1, Ikaros, E2A, EBF and PAX5. Among these, EBF and PAX5 work exclusively within the B cell fate and are considered as the key transcription regulators for B cell development, while the others are also involved in other hematopoietic lineages [19]. It has been shown that enforced expression of EBF1 in IL-7R$\alpha^{-/-}$ bone marrow mice potently down-regulates Id2 and Id3 mRNA expression and restores B cell differentiation in vivo [20]. EBF1 can restore the PU.1$^{-/-}$ and Ikaros$^{-/-}$ multipotent progenitors to differentiate into a pro-B cell state [21, 22]. Moreover, in the absence of E2A and PAX5, EBF1 is still able to promote B lymphopoiesis [23, 24]. Although EBF1 has recently been found to have an important role not only in specification but also in commitment of the B cell lineage, commitment

to the B cell lineage has been commonly attributed to a single transcription factor, PAX5. This is because only PAX5 deficiency can change the fate of cells committed to the B cell lineage [25-28]. Thus, EBF1 and PAX5 act in a positive feedback loop and determine the specification and commitment of the B cell identity.

Development of T Cells

Based on gain and loss of function approaches, the Notch1 receptor has been shown to be essential and sufficient in T cell lineage commitment [29-32]. A clear consensus is that Notch1 receptor regulates the earliest stages of T cell commitment by providing a directive signal that promotes differentiation of hematopoietic progenitors into the T cell lineage, while inhibiting B cell differentiation [33]. Notch signaling is initiated through ligand-receptor interactions, leading to proteolytic cleavage of the receptor, a process that liberates the cytoplasmic domain of Notch (intracellular notch domain, ICN). ICN translocates to the nucleus and heterodimerizes with the transcription factor CBF-1 in humans, converting it from a repressor into an activator, thus activating downstream target genes responsive for T cell specification. Therefore, the murine bone marrow stromal cell line OP9, engineered with delta like ligand-1 (DL1) or delta like ligand-4 (DL4) to generate OP9-DL1 and OP9-DL4 cells, promotes in vitro human T cell development by providing continuous Notch engagement [34].

NK Cells and Immunodeficiency Diseases

NK cells are large granular lymphocytes belonging to the innate immune system. They play a critical role in killing infected cells or cancer cells without prior sensitization. Functionally, NK cells are an important source of immune-regulatory cytokines, especially interferon (IFN)-γ, tumor-necrosis factors (TNFs), granulocyte macrophage colony-stimulating factor (GM-CSF) or interleukin-10. These secreted cytokines interact with other immune cells to trigger the adaptive immune response. NK cells have the ability to directly kill target cells through the exocytosis of cytoplasmic granules containing perforin and granzyme. Also they can kill target cells via death receptors like TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL). In addition, NK

cells can mediate antibody-dependent cellular cytotoxicity (ADCC) via the membrane receptor FcγRIII (CD16), which binds to the Fc portion of IgG antibody [35]. Due to these attributes, NK cells are a very attractive cell population for immunotherapy. Ruggeri et. al. have developed a cancer therapy by applying NK cells. NK cell-mediated alloreactivity could also eliminate relapse, graft rejection, and protect against graft-versus-host-disease (GVHD) for patients with acute myelogenous leukemia (AML) [36]. While several different approaches toward T cell based adoptive cell transfer (ACT) has been studied and used in clinic, NK cell therapy is rapidly progressing and widely applied in allogenic hematopoietic stem cell transplantation [37].

X-linked severe combined immunodeficiency disease (X-SCID) is a profound immunodeficiency disease that accounts for approximately half of the cases of SCID. It's the most common form of SCID. It is characterized by an absence of T cells, B cells and NK cells [38]. In 1993, it was found that mutations in the interleukin-2 receptor gamma (IL-2RG, CD132, γ_c) gene caused X-SCID disease [39]. The common γ chain is shared by a family of cytokines, including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. The IL-2 and IL-15 are heterotrimeric and also share another subunit: IL-2/15 receptor β (known as IL-2RB, CD122). The IL-2RB and IL-2RG involves signaling pathways of Janus tyrosine-kinase 1 (JAK1), JAK3 and signal transducer and activator of transcription 5 (STAT5). The interaction of IL-2RB and IL-2RG mediated phosphorylation of JAK1 and JAK3, leading to the dissociation of STAT5 and subsequent dimerization of STAT5. Then the phosphorylated STAT5 dimers translocate to the nucleus and promote the transcription of target genes. Although many other cytokines can also activate JAK1, the γ_c-dependent cytokines are the only ones that activate JAK3. JAK3 deficient patients had a T-NK-B+ SCID phenotype [40, 41]. In most cases, SCID can be cured by bone marrow transplantation [42]. In addition, the development of gene therapy provides new strategies for treatment of X-SCID [43, 44].

Wiskott-Aldrich syndrome (WAS) is an X-linked primary immunodeficiency disease characterized by thrombocytopenia, recurrent infections and increased autoimmunity

[45]. The disease is caused by mutations in the WAS gene which encodes for WAS protein (WASp). WASp is exclusively expressed in hematopoietic cells and required for proper lymphocyte and platelet development and function. WASp is critical for immune synapse formation and the polarization of effector T cells through its effects on the actin cytoskeleton. By activating the Arp2/3 complex to initiate actin polymerization, WASp helps to transduce signals to the cell cytoskeletal framework. Compared to the minor role of WASp in early haematopoietic cell development, differentiated haematopoietic cells, especially lymphocytes in WASp-deficient hosts have a significant survival disadvantage in humans and mice [45]. There is a lower number of T cells in WAS patients than in normal individuals, probably due to abnormal thymopoiesis [46]. It is also clear that WASp deficiency mediates a selective depletion of mature B cells by affecting B cell homeostasis with a greater effect on some B cell subpopulations than others [47, 48]. It is found that in the absence of WASp, NK cell function is abnormal. NK cell cytotoxicity is defective as a result of impaired immune synapse formation between an NK cell and its target cell, impaired perforin localization at the immune synapse and defective signaling downstream of CD16 engagement [49, 50].

Human Pluripotent Stem Cells

Human pluripotent stem cells include both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), and they are capable of self-renewal and differentiation into all the three germ layers: endoderm, mesoderm and ectoderm. In 1981, the first pluripotent stem cell line, mouse embryonic stem cell (mESC) lines were established from mouse blastocysts [51, 52]. Thus mESCs could provide an initial population of pluripotent stem cells as a tool to study the development of terminal differentiated cells. Due to the differences between mouse and human, hESCs have been more difficult to culture in vitro. But hESCs can provide a better model to benefit human regeneration medicine. The hESCs were not successfully derived until 1998 by James Thomson [53]. Since then, hESCs became the first human pluripotent stem cell line that enabled translational and clinical research around the world. A breakthrough occurred in 2006 when Takahashi and Yamanaka were able to convert murine fibroblasts into a

pluripotent state by retroviral expression of a set of four genes (Oct4, Sox2, Klf4 and c-Myc). The new derived cells were named induced pluripotent stem cells (iPSCs), which were similar to ESC [54]. The following year Yamanaka's group and Thomson's group described the derivation of human iPSCs using terminally differentiated human fibroblasts [55, 56]. In the study of human pluripotency, hESCs still remain the gold standard, from which its results can benefit the future application of iPSCs. The field of hiPSCs is growing rapidly, because they are more useful in the study of disease modeling and enable the potential of patient specific autologous cell transplantation as a future clinical therapy [57].

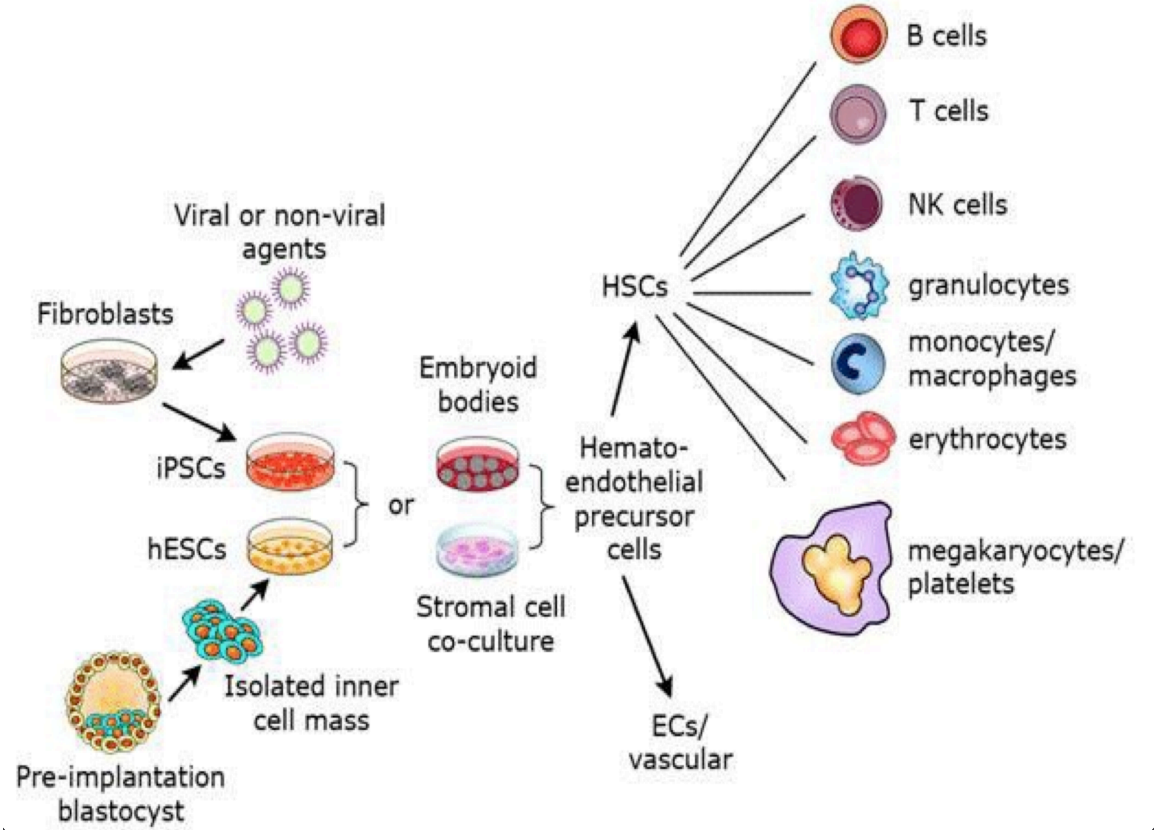
Human iPSC-Based Gene and Cell Therapy

When combined with gene editing methods, iPSC technology enables the treatment of diseases with somatic genetic mutations by using genetically corrected iPSCs for autologous transplantation. First, the patient's somatic cells containing the genetic mutations can be transformed into iPSCs ectopic expression of transcription factors in vitro. Then the mutated genes can be corrected by using gene specific editing techniques, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPRs) [58]. Finally the gene corrected hiPSCs can be differentiated into the appropriate progenitor cells or mature cells for autologous transplantation to the patient. The first "proof of concept" study was performed in a humanized mouse model with sickle cell disease by Jaenisch and Townes groups in 2007 [59]. Mouse iPSCs was derived from the tip-tail fibroblasts of a humanized knock-in mouse model with sickle cell anemia. Then the miPSCs had the mutated sickle hemoglobin allele corrected using homologous recombination and differentiated into hematopoietic progenitor cells. When transplanted back in the mouse models, the gene corrected miPSCs derived hematopoietic progenitor cells completely restored normal. Thus, human pluripotent stem cells, especially iPSCs, when combined with gene correcting techniques, would provide an unlimited cellular source for curing genetic diseases through autologous transplantation therapy.

Human Pluripotent Stem Cells Derived Hematopoiesis

Shortly after the derivation of hESCs, in 2001, Kaufman et.al demonstrated the first hematopoietic differentiation of hESCs using an in vitro co-culture model [60]. Since then, several different groups have been trying to optimize the hematopoietic differentiation conditions by using different methods such as embryoid body formation or stromal cell co-culture techniques [61-63]. After the reprogramming of human somatic cells into pluripotency, these hESCs derived hematopoietic conditions have been also applied to the differentiation of hiPSCs [64-66]. These hESCs/iPSCs derived hematopoietic progenitors can produce both myeloid and lymphoid cells in vitro (Figure 2, [67]), but the generation of hematopoietic stem cells with long-term and multilineage engraftment potential still remains a significant challenge [67, 68].

Figure 2: Derivation of hematopoietic and related cell lineages from human pluripotent stem cells. This figure is taken from Kaufman, D.S., *Toward clinical therapies using hematopoietic cells derived from human pluripotent stem cells.* Blood, 2009. 114(17): p. 3513-23. [67]
Human pluripotent stem cells, including hESCs and hiPSCs, both can serve an initial population of cells to investigate blood cell development. Here the figure described two main approaches to induce hESCs/hiPSCs derived hematopoietic development in vitro: embryoid body formation and stromal cell co-culture. Essentially all mature hematopoietic cell populations (lymphoid and myeloid lineages) have been derived from hESCs and hiPSCs from in vitro or in vivo studies.



NK Cells Derived from Human Pluripotent Stem Cells

In 2005, for the first time, by using hESCs, our lab could generate NK cells with functional mature markers, including KIRs, natural cytotoxic receptors and CD16. Also, these NK cells could lyse malignant cells through both direct cell mediated cytotoxicity and antibody dependent cellular cytotoxicity [69]. Then in 2009, hESCs generated NK cells were successfully tested for in vivo anti-tumor activity using a mouse model of human leukemia [70]. Furthermore, using both hESCs and hiPSCs, our lab developed a combination technique of two-stage NK culture system and expansion with membrane-bond interleukin 21-expressing artificial antigen presenting cells to promote the large-scale amplification of NK cells into clinical translational research [71].

B Cells Derived from Human Pluripotent Stem Cells

In vitro B cell generation from hESCs and iPSCs is severely restricted. Several groups has reported the possibility of generation of CD19+ cells from hESCs/iPSCs both in vitro and in vivo, but most of them has no functional data, such as antibody production, to fully support successful B cell development. Zambidis et al obtained hematopoietic cells derived from hESCs on OP9 stromal cells, then differentiated them into B cells and NK cells. Only a few weakly staining CD19+ cells (~1%) could be observed, which expressed low levels of RAG and scant surrogate light chains (VpreB, λ5) [72]. Vodyanik et al used sorted hESCs derived CD34+ cells and plated them on MS5 stromal cells for 3 weeks. Then CD19+CD45+ B cells could be detected albeit at very low frequency (~1%) [73]. By only replacing hESCs with hiPSCs, Carpenter et al were able to show that one of their hiPS cell line (c18) derived from normal human dermal fibroblasts could generate CD45+CD10+CD19+ B cells (~8%). The positive RT-PCR results for transcripts Pax5, IL-7αR, λ-like, and VpreB receptor indicated a pre-B cell identity [74]. Unfortunately, no further characterization of these cells was reported from either of these studies. However, Amabile et.al. co-injected OP9 murine bone marrow stromal cells with hiPSCs into immunodeficient NOD/SCID/IL2Rγc-/- (NSG) mice, and showed that they generated teratomas containing hematopoietic cells, including myeloid, lymphoid and erythroid cells. They also found that when co-injecting OP9 cells that

produce Wnt3a or express the Notch ligand Delta-like 1 (DL1), more CD19+ B cells and CD3+ T cells could be derived. In addition, when NSG mouse engrafted with these hiPSC teratoma derived hematopoietic cells (CD34+CD45+), human B cells producing IgG antibody and T cells making cytokines could be derived [75].

T Cells Derived from Human Pluripotent Stem Cells

T cell generation from human pluripotent stem cells first showed feasibility from in vivo experiments. Galic et al. used OP9 stromal cells to differentiate GFP-expressing hESCs into CD34+ and CD133+ cells. These cells were injected into human thymic tissues engrafted into immunodeficient mice (SCID-hu mouse model). Though at low frequency (~1%) of engraftment, T cell progenitors were generated with typical phenotype of CD4, CD8, CD1a and CD7 [76]. Subsequently the same group showed that similar T cell derivation could be achieved using an EB-mediated method in the SCID-hu mouse model [77]. Although in vivo experiments were useful for T cell derivation, more attention was attracted to human pluripotent stem cell derived T cells purely from in vitro cell culture system in view of the translational medicine. Timmermans et al. have shown that after differentiation of hESCs on OP9 stromal cells, selected “hematopoietic zones” enriched of CD34+/CD43+ cells were transferred onto OP9-DL1 stromal cells for T cell derivation. For the first time they provided an in vitro condition critical for the identification of T cell potential [78]. Two studies have shown that human antigen-specific cytotoxic T cell derived iPSCs (T-iPSCs) could produce T cell progenitors using OP9-DL1 co-culture. These T cell progenitors not only could be stimulated into CD8+ T cell with a memory for their original tumor or virus antigen, but also have elongated telomeres and greater proliferative capacity, indicating that T-iPSCs could be re-differentiated into “rejuvenated” T cells [79, 80]. Recently, using peripheral T lymphocytes as the initial cell source, by combining iPSC and chimeric antigen receptor (CAR) technologies, the T-iPSC derived CD19 CAR expressing T cells, displayed the phenotype and tumor-killing function resembling of innate γδ T cells. CAR-T-iPSC derived hematopoietic cells could be generated from feeder free conditions and then induced to T lymphoid commitment through the OP9-DL1 co-culture [81]. These T-

iPSCs could be potentially more accessible to differentiate into a T cell phenotype, because of the cell surface marker memorization. Not only restricted to T-iPSCs, T lymphoid progenitors could be derived from both hESCs and hiPSCs and used to mark the definitive hematopoiesis in the human pluripotent stem cell differentiation cultures [82]. And for the first time, αβ and γδ T lymphocytes with a broad TCR repertoire was successfully produced from human iPSCs in vitro [83]. Although T cells could be derived from hESCs/iPSCs by different groups, the efficiency and reproducibility of these experiments is a significant problem. It still remains unclear the differences between hESCs and iPSCs lines and the different hematopoietic differentiation culture conditions that may effect the development of T cells in the in vitro system. Less time consuming and more stable and translational methods to derive T cells need to be explored.

Thus, gaining a better understanding for derivation of human lymphocytes from hESCs/iPSCs can enable novel in vitro development models and potentially promote new therapeutic strategies against immunodeficiency, infections or cancer. NK cells with antitumor and antiviral capacity can be routinely derived from hESCs and iPSCs either with the use of murine stromal cell layers or in a feeder free conditions [71]. However, difficulties in the derivation of B cells and T cells from human pluripotent stem cells remains the main hindrance to promote the use of hESCs/iPSCs as a source to establish B cell and T cell in vitro developmental models and develop appropriate immunotherapies.

Methods

Maintenance of undifferentiated human iPSCs

Undifferentiated human iPS cells (UCBiP7) for use in hematopoietic differentiation were grown in 6-well plates that were coated with a monolayer of irradiated mouse embryonic fibroblasts (MEF) at a high density (180,000 cells/well). Each plate was stored in incubators at 37°C with 5.0% CO_2. Human iPS cells were passed on to fresh MEF plates every 6-7 days depending on the confluency and amount of colonies that showed signs of differentiation (non-linear borders, 3-dimensional growth, discoloration). If it was estimated that more than 10% of the colonies were undergoing differentiation, glass picking sticks would be used to remove the unwanted colonies. Wells were fed daily with 2.5mL of culture media containing 15% knockout serum replacement, 1% non-essential amino acids (NEAA), 1% L-glutamine, 0.5% pen/strep, 0.1mM β-mercaptoethanol, and 8ng/mL basic fibroblast growth factor (bFGF). Passaging the undifferentiated cells was done by incubating each well in 1mL of 1mg/mL collagenase IV in DMEM-F12 media for 5 minutes. Cells were mechanically scraped off with a glass pipette re-suspended in ES media, and centrifuged for 5 minutes at 1500 RPM. For undifferentiated cells, the supernatant was aspirated and the cells were split into 6 well plates coated with new MEFs with the culture media at appropriate ratios.

Hematopoietic differentiation of human iPSCs

Our hematopoietic differentiation method was adapted from a protocol [84]. Human iPSCs were first adapted by TrypLE Select (Invitrogen) and maintained on low density MEFs (90,000 cells/well) for a minimum of 10 passages. Then these TrypLE adapted hiPSCs were induced into embryonic bodies (EB) through the "spin-EB" approach. To generate TypLE adapted iPSCs, cultures around 60-70% confluency were dissociated and filtered through a 70-μm sterile filter. Only pure cultures of iPSCs lacking any signs of differentiation were used. Cells were passaged 1:1 on low density MEFs in regular hESC media until cellular proliferation allowed passing at more dilute ratios, typically occurring around passage 10. To set up TrypLE passaged hESCs into Stage I spin EBs,

adapted cells around 70% confluency were dissociated with TrypLE and filtered through a 70 micron filter to remove any clumps. Cells were then counted and placed at a concentration of 3000 cells per well (100 µl volume) of a round-bottom 96-well plate in BPEL ((Bovine Serum Albumin (BSA) Polyvinylalchohol Essential Lipids) media containing stem cell factor (SCF, 40 ng/ml, PeproTech, Rocky Hill NJ), vascular endothelial growth factor (VEGF, 20 ng/ml, R&D Systems, Minneapolis), and bone morphogenic protein 4 (BMP4, 20 ng/ml, PeproTech). BPEL media was made in 200 mL volumes and contained Iscove's Modified Dulbecco's Medium (IMDM, 86 mL, Invitrogen), F12 Nutrient Mixture with Glutamx I (86 mL, Invitrogen), 10% deionized Bovine Serum Albumin (BSA, 5 mL, Sigma), 5% Polyvinyl alcohol (10 mL, Sigma), Linoleic acid (20uL of 1 mg/mL solution, Sigma), Linolenic acid (20uL of 1 mg/mL solution, Sigma), Synthecol 500X solution (Sigma), α-monothioglyceral (Sigma), Protein-free hybridoma mix II (Invitrogen), ascorbic acid (5 mg/mL, Sigma), Glutamax I (Invitrogen), Insulin-transferrin-selenium 100x solution (Invitrogen), Penicillin/Streptomycin (P/S, Invitrogen). The outer wells of the plate were filled with sterile water to prevent any evaporation of the media. Plates were then spin aggregated at 1,500 RPMs for 5 minutes at room temperature and placed undisturbed in a 37°C incubator with 5% CO_2. Typical spin-EBs consists of a solid embryonic body in the center and non-adherent cells at the margin were formed after 11 or 12 days of incubation. Non-adherent cells could be collected after mechanically suspending the whole spin-EBs and filtered by a 70-µm sterile filter.

Magnetic Separation for CD34+ cells from umbilical cord blood (UCB) samples

Healthy UCB donating samples were kindly provided from University of Colorado Cord Blood Bank. UCB samples were transferred from collection bags to T150 culture flask (BD Falcon) using luer lock 60 mL syringe. Initially 1mL heparin (Sigma) is added to the T150 flask before starting. Cord blood units are not mixed. Each donor is processed separately. Based on the original volume of UCB per bag, dilute the sample 1:1 with IMDM medium (Thermo Scientific). Prepare appropriate number of tubes with 20 mL Ficoll-Paque Plus (GE Healthcare). Slowly layer 30 mL of diluted UCB on top of the

Ficoll, and spin the tubes for 30 min, 1,500 RPM (room temperature, with no brake). After spinning, carefully aspirate most of the medium from the top of each tube and try not to disturb the interphase, including any remaining medium left on top of the layer of Ficoll. Take the buffy coat layer carefully and pool the buffy coats from all the Ficoll tubes. Centrifuge the buffy coats at 1,700 RPM for 10 minutes at room temperature. After centrifugation, aspirate off the supernatant and the pellet should be red. Wash cells with IMDM, combine all the pellets into one. Centrifuge at 1,700 RPM for 10 minutes at room temperature. After centrifugation, the supernatant was aspirated. Add ice-cold lysis solution ammonium chloride to the pellets, vortex a little and keep on ice for 5 minutes. Alternatively, the pellets could be suspended with water for 30 seconds on ice. Add 10 mL IMDM to the tube. Centrifuge at 1,700 RPM for 10 minutes at room temperature. The pellet should be white. If the pellet is still red, lysis process should be repeated. The UCB samples are then sorted for CD34+ hematopoietic progenitor cells using CD34 Microbead kit (Miltenyi Biotec). First, the CD34+ UCB cells were magnetically labeled with CD34 Microbeads. Then, the cell suspension is loaded onto a MACS column, which is placed in the magnetic field of a MACS separator. The magnetically labeled CD34+ cells are retained within the column. The unlabeled cells run through, this fraction is thus depleted of CD34+ cells. After removing the column from the magnetic field, the magnetically retained CD34+ cells can be eluted as the positively selected cell fraction. Thus, UCB CD34+ cells could be magnetically sorted from the whole UCB samples and used as a positive control for real long-term hematopoietic stem cells for the experiments.

In vitro generation of NK cells

Hematopoietic progenitor cells were transferred to 24-well plates with a confluent monolayer of irradiated stromal cells (EL08-1D2, OP9, OP9-DL1, OP9-DL4) in the NK medium designed to maximize NK cell growth as described previously [71]. Briefly, cells were co-cultured in 2:1 mixture of Dulbecco modified Eagle medium/Ham's F12 (DMEM/Ham's F-12, Cellgro/Mediatech) basal media supplemented with 20% heat-inactivated human AB serum (Nabi), 5 ng/ml sodium selenite (Sigma), 50 μM ethanolamine (MP Biomedicals), 20 mg/L ascorbic acid (Sigma), 25 μM 2-ME, 2mM L-

glutamine, 1% P/S (Invitrogen), 10 ng/ml Interleukin-15 (IL-15, PeproTech), 5 ng/ml Interleukin-3 (IL-3, PeproTech), 20 ng/ml Interleukin-7 (IL-7, PeproTech), 20 ng/ml SCF (PeproTech), and 10 ng/ml FMS-like tyrosine kinase 3 ligand (Flt3L, PeproTech). Medium containing fresh cytokines was changed twice a week with the exception of IL-3 which was only included for the first week of co-culture. Stromal cells were replaced with fresh cells once a week. Wells were harvested at appropriate time points during NK cell co-culture, counted for viable cells, and assayed for phenotypic and functional analysis.

In vitro generation of B cells

Hematopoietic progenitors were plated onto 24-well plates with a confluent monolayer of non-irradiated MS-5 stromal cells in the B cell medium as previously described [85]. The B cell medium was MS5 medium supplemented with recombinant human SCF to a concentration of 100 ng/mL each of SCF and granulocyte colony-stimulating factor (G-CSF, Fairview Pharmacy, Minneapolis, MN, USA) to a concentration of 10 ng/ml. Feed the cells twice per week, each time by removing half of the old medium and replacing it with fresh medium. Stromal cells were replaced with fresh cells once a week. Then the cells were collected at selected time points for flow cytometric analysis.

In vitro generation of T cells

Hematopoietic progenitors were plated onto 24-well plates with a confluent monolayer of non-irradiated OP9-DL1 or OP9-DL4 stromal cells in the T cell medium as previously described [17, 31]. The T cell medium was OP9 medium supplemented with recombinant human cytokines Flt3L (5 ng/mL), IL-7 (5 ng/mL) and SCF (10 ng/ml) (Peprotech). The fresh medium containing cytokines were changed every two days and the cells were transferred onto fresh stromal cells every four days. Co-cultures were disaggregated by vigorous pipetting and passaged through a 70-μm filter to reduce stromal cell line aggregates and eliminate contaminating with stromal cells.

Maintenance of stromal cells

Different murine stromal cells were maintained in cell culture to support human lymphocyte development. Murine bone marrow stromal cells MS-5 were used to support the growth of progenitor B cells [85]. MS5 cells were maintained in MS5 media which was defined as RPMI-1640 (Invitrogen) medium containing 10% standard fetal bovine serum (FBS, ATLANTA BIO, Premium), 1% L-glutamine (Invitrogen), 1% NEAA (Invitrogen), 1% P/S (Invitrogen). EL08-1D2 stromal cells supported the differentiation of NK cell precursors, NK cell commitment, and proliferation [86]. EL08-1D2 cells were maintained in EL08-1D2 medium containing 50% M5300 (StemCell Technologies), 40% α-minimal essential medium (α-MEM, Invitrogen), 8% standard FBS (ATLANTA BIO, Premium), 1% Glutamax I (Invitrogen), 1% P/S (Invitrogen) and 0.1% mM β-mercaptoethanol (Sigma). OP9 stromal cells were ordered from ATCC (CRL-2749). The cells were established from newborn op/op mouse calvaria, and do not produce functional macrophage colony-stimulating factor (M-CSF) due to an osteopetrotic mutation in the gene encoding M-CSF [87]. OP9 cells were cultured in OP9 medium, which was composed of 79% of α-MEM medium (Invitrogen), without ribonucleosides and dexyribonucleosides but with 2 mM L-glutamine, 20% of characterized FBS (ATLANTA BIO, Optima), 1% of P/S (Invitrogen) and 1.5 g/L sodium bicarbonate (Sigma). OP9-DL1 and OP9-DL4 stromal cells were made and kindly provided by Juan Carlos Zúñiga-Pflücker lab from Sunnybrook Research Institute (SRI). OP9-DL1 and OP9-DL4 cells were made from OP9 cells but ectopically express the Notch ligands: Delta-like 1 (DL1) and Delta-like 4 (DL4), respectively [30, 88]. They were both maintained in OP9 medium. When passaging the OP9, OP9-DL1 and OP9-DL4 cells, the cell density is very important for them. They should not overgrow, very large cells and adipocytes tend to appear after overgrowth. Make sure to do the subculture just before confluence.

Phenotype analysis by flow cytometry

Cells were prepared in buffer (PBS containing 1% sodium azide and 2% standard FBS) and were labeled with a combination of fluorochrome-conjugated monoclonal antibodies specific for cell surface antigens. They were all mouse anti-human monoclonal antibodies unless indicated. Approximately 200,000 cells were used for one sample, and incubated

with antibodies at 4 °C for 20 minutes. Following incubation, cells were washed with buffer and the supernatant was aspirated following centrifugation at 1500 RPM for 5 minutes to remove any excess unbound antibody. Then samples were analyzed using BD LSRII (BD Biosciences) flow cytometers. List mode files were analyzed by FlowJo software version 8.87 and version 10 (Tree Star). Antibodies, which were used according to the manufacturers' recommendations, were from BD Biosciences unless otherwise indicated. Live cells were distinguished from dead cells with LIVE/DEAD fixable dead cell stain kit (Invitrogen, L10120), CYTOX blue dead cell stain (Invitrogen) and fixable viability dye eFluor 450 (eBiosciences).

Antibodies used for hematopoietic progenitor cell phenotype analysis were: CD34 (APC-clone 581; PE-clone 581; FITC-clone 581; PE-Cy7-clone 581); CD43 (APC-clone 1G10; PE-clone 1G10); CD45 (APC-clone HI30; PE-clone HI30; FITC-clone HI30; APC-H7-clone 2D1; Alexa Fluor 700-clone HI30; PE-CF594-clone HI30; PE-Cy7-clone HI30; PerCP-Cy 5.5- clone HI30). Antibodies used for NK cell phenotype analysis were: CD56 (APC-clone B159; PE-Cy7-clone B159); CD117 (PerCP-Cy5.5-clone YB5.B8); CD16 (Alexa Fluor 700-clone 3G8); CD94 (APC-clone HP-3D9); PE-CF594-clone RPA-2.10); CD7 (Alexa Fluor 700-clone M-T701); CD2 (FITC-clone RPA-2.10; PE-CF594-clone RPA-2.10); CD161 (FITC-clone DX12); CD122 (APC-TU27, BioLegend); CD132 (PE-clone TUGh4, eBioscience); CD11a (PE-clone HI111); NKp44 (CD336, PE-clone p44-8.1); NKp46 (CD335, PE-clone 9E2/Nkp46); NKG2D (CD314, PE-clone 1D11); KIR (CD158a,h-PE, CD158a-PE, CD158i-PE, CD158e1/e2-PE, CD158b1/b2-PE, Beckman Coulter.); NKG2A (CD159a, PE, Beckman Coulter); NKG2C (CD159c, R&D Systems); Fas Ligand (CD95L, PE-NOK-1, eBioscience); TRAIL (CD253, PE-RIK-2); CD14 (APC-H7-clone MφP9; PE-MφP9); CD3 (FITC-clone UCHT1). Antibodies used for B cell phenotype analysis were: CD10 (APC-clone HI10a, BioLegend); CD19 (PE-clone HIB19; PE-Cy7-clone HIB19); CD20 (Alexa Fluor 700-clone 2H7); CD21 (PE-Cy7-clone B-ly4). Antibodies used for T cell phenotype analysis were: CD4 (PE-CF594-clone L200); CD8 (APC-clone RPA-T8); CD5 (PerCP-Cy5.5-clone L17F12); CD7 (Alexa Fluor 700-clone M-T701); CD1a (PE-clone HI149).

In vitro cytotoxicity assay

This assay is used to assess the killing capabilities of cells. Target cells are labeled with radioactive chromium, which is taken up into the cells. The labeled cells are then incubated with the effector cells in a four-hour assay. If the effector cells kill the target cells, they will burst, and release chromium. So in this experiment, tumor targets (K562 cells) were incubated with ^{51}Chromium for 2 hours at 37°C, washed three times and co-cultured with effectors (NK cells) at indicated effector to target (E: T) ratios. After 4 hours, cells were harvested and analyzed by the gamma counter machine
Specific ^{51}Cr lysis was calculated using the equation: % specific lysis =
100 × (test release - spontaneous release) / (maximal release - spontaneous release) [18].

Analysis of NK cell degranulation

We assessed NK cell activation by monitoring the CD107a up-regulation of the stimulated NK cells [89]. NK cells were incubated with target cells at an E: T ratio of 1:5, 1:2, 1:1 and 1:0 at 37 °C and 5% CO_2 for 6 h. Fluorochrome-conjugated CD107a mAb (PE-H4A3, BD Biosciences) or the corresponding IgG1 isotype control was added at the initiation of the assay. After 1 h of incubation, GolgiStop (BD Biosciences) was added to each well and incubated for another 5 hours. Surface staining was done by incubating the cells with CD56 mAb (APC-clone B159, BD Biosciences) for 20 mins on ice. The cells were washed and analyzed by flow cytometry.

Artificial antigen presenting (aAPC) cell expansion

To stimulate and promote NK cell maturation and expansion, NK cells were placed in culture with Clone 9.mbIL-21 aAPCs [90]. For the first week of stimulation, aAPCs were irradiated with 10,000 rads and added to NK cells at a ratio of 2:1 (Day 0). The following stimulations with aAPCs (every 7 days) were at a 1:1 ratio. Cultures were fed twice a week. The medium was containing 88% of RPMI-1640 (Invitrogen), 10% of standard FBS (ATLANTA BIO, Premium), 1% P/S (Invitrogen), 1% L-glutamine (Invitrogen) and

50 U/mL interleukin-2 (IL-2, PeproTech). Total cells were maintained at 250,000 cells/mL for optimal expansion.

Results

Project I:

The goal of the research is to use human pluripotent stem cells to study the development of human lymphocytes in vitro. It includes three projects. The first project is using human iPSC lines to study the general in vitro derivation of human lymphocytes (NK cells, B cells and T cells) by co-culturing with different murine stromal layers. The results of Project I provide a comprehensive characterization of hiPSCs derived NK cell development in vitro, but with a severe lack of B cell and T cell development. Based on Project I, we establish two immunodeficiency disease models by using human pluripotent stem cells.

Human iPSCs Can Generate Hematopoietic Progenitor Cells

Different sources of human iPSCs have been shown to produce hematopoietic progenitor cells (HPCs) in appropriate hematopoietic differentiation conditions. Other labs and our lab have conducted different protocols to induce efficient and consistent hematopoietic differentiation from iPSCs [64-66, 71]. iCell-HPCs are one of the "iCell products" developed by Cellular Dynamics International (CDI) to study human hematopoietic development in vitro. iCell-HPCs are derived from human iPSCs through CDI's proprietary derivation and purification protocols. Our analysis demonstrates more than 99% of the iCell-HPCs express the hemato-endothelial cell surface glycoprotein CD34, as well as 95% of cells express CD34+/CD43+ and 78% of cells express CD34+/CD45+ (Figure 3A). A homogenous population of the iCell-HPCs can be seen in Figure 3B. UiPS (UCBiPS7) is a human iPSC line derived and characterized in our lab [71]. We used a novel "spin-EB" method to induce the hematopoietic differentiation. Briefly, 3,000 undifferentiated UiPS cells were aggregated in 96-well plate through mechanical spinning. Through 12 days of induction in the BPEL medium with cytokines of SCF, VEGF and BMP4, a typical morphology of embryonic body (EB) was formed (Figure 3B). Particularly, there was a circle of non-adherent cells emerging from the center of the

EB. These non-adherent cells were characterized as the UiPS-HPCs, as shown by the flow cytometry data (Figure 3A). UiPS-HPCs are enriched with 84% CD34 cells, 68% CD34+/CD43+ cells and 82% CD34+/CD45+ cells (Figure 3A). Including the whole EBs, UiPS-EBs have an expression of CD34+ cells at 70%, CD34+/CD43+ cells at 58% and CD34+CD45+ cells at 62% (Figure 3A). Compared to non-adherent UiPS-HPCs, UiPS-EBs have almost 10% reduced expression of CD34+ cells. UCB CD34+ cells (UCB) were magnetic sorted for CD34 surface antigen from human umbilical cord blood cells using a CD34 Micro-Bead kit. The sorted UCB cells are 94% CD34+, 85% CD34+/CD43+ and CD34+/CD45+ (Figure 3A). UCB CD34+ cells served as a positive control for HPCs.

Based on the data of Figure 3A, these four sources of HPCs have 70% to 99% of CD34 expression, each providing an ideal initial cell source for further blood lineage development. Especially, iCell-HPCs and UiPS-HPCs with 99% and 84% of CD34 expression, representing a pure homogenous population to study the human iPS derived hematopoietic development in vitro.

Human iPSCs Derived HPCs Can Be Differentiated into NK Cells

Although previous studies of human iPSCs were able to generate NK cells with different types of stromal layers or in feeder free condition, a comprehensive phenotype analysis for the overall NK cell surface antigen has been rarely shown [71]. In order to show the NK cell in vitro development from hiPSC derived HPCs as well as how different stromal layers can affect the NK cell differentiation, multiple stromal cell populations (EL08-1D2, OP9, OP9-DL1 and OP9-DL4) were used to support the NK differentiation co-cultures. The defined NK differentiation media (NKDM) was supplemented with cytokines of IL-3, IL-7, IL-15, SCF and Flt-3L. IL-3 was only used for the first week of co-culture. In order to only gate the NK cells from the whole cell culture dish, CD14+ monocytes (may also express CD56) are excluded from the flow cytometric analysis. Then CD45+/CD56+ NK cells are gated only from CD14- populations. Two subsets of CD56+ cells can be further distinguished as immature NK cells (imNK:

CD56+/CD117+/CD94-) and mature NK cells (mNK: CD56+/CD117-/CD94+) [91]. At two weeks, only 1.21% of CD14-/CD45+/CD56+ cells can be found from iCell-HPCs derived NK cells (iCell-NK) in OP9-DL1 and OP9-DL4 condition, while no CD56+ cells can be seen in EL08-1D2 and OP9 condition (Figure 4). For UiPS-HPCs derived NK cells (UiPS-NK), only a small amount of CD14-/CD45+/CD56+ cells can be found with 8.29% in EL08-1D2 condition and 5.55% in OP9 condition (Figure 4A, B). Almost double the amount of NK cells with the same phenotype are observed with OP9-DL1 (16.4%) and OP9-DL4 (19.2%) (Figure 4C, D). The ratio of UiPS-iNK cells to UiPS-mNK cells is different for each condition; more than 2:1 is found in EL08-1D2 condition, 1:1 in OP9 condition, and 1:2 in OP9-DL1 and OP9-DL4 conditions (Figure 4). UiPS-EB derived NK cells (EB-NK) have only 2.73%~5.2% of CD14-/CD45+/CD56+ cells within the four stromal co-culture conditions (Figure 4). NK cells derived from UCB CD34+ cells (UCB-NK) set up a good example of early NK cell derivation. Within the first two weeks, the CD14-/CD45+/CD56+ cell population is 13.2% in EL08-1D2 condition, 4.82% in OP9 condition, 18% in OP9-DL1 condition and 14.4% in OP9-DL4 condition (Figure 4). Both immature and mature UCB-NK cells can be seen in each condition, which we typically observed before. To be noticed, among the four stromal conditions, the CD14- population of iCell-NK, EB-NK and UCB-NK is ranging from 76.6% to 92.7%, while CD14- population of UiPS-NK is limited as 43.2% in EL08-1D2 and 27.7% in OP9 condition, indicating 56.8% and 72.3% of CD14+ monocyte populations are occupied in these two stromal conditions. However, in OP9-DL1 and OP9-DL4 conditions, UiPS-NK has a majority of 73.1% and 79.1% of the cells are CD14-. As early as two weeks NK co-culture, UiPS-HPCs, compared to its low derivation of CD14-/CD45+/CD56+ NK cells in EL08-1D2 and OP9 conditions (8.29% and 5.55%), in OP9-DL1 and OP9-DL4 conditions, there is more than double percentage of NK cells derived (16.4% and 19.2%).

Mature NK Cells with Typical NK Receptors Are Identified

In order to show a complete NK derivation from all the conditions, a full panel of NK cell surface antigens assessed by flow cytometry at four weeks is provided. NK cell

maturation is accompanied with coordinated acquisition of inhibitory and activating receptors [91]. The inhibitor receptors (KIRs, CD161 and NKG2A) and activating receptors (NKp44, NKp46, NKG2D, NKG2C and CD16) are tested here. At four weeks, all the monocytes are gone from each condition, 99% of the remaining cells are CD14-/CD45+ (Figure 5.1). Based on CD45 and CD56 expression, up to 99% of CD45+ cells are also CD56+, implying that all cells become NK cells (Figure 5.1), with the exception of iCell-NK in OP9 and OP9-DL4 conditions. The CD45+/CD56+ population of iCell-NK in OP9 and OP9-DL4 conditions is approximately 53% for both conditions (Figure 5.1B, D). EB-NK in EL08-1D2 and OP9-DL4 conditions, shows more than 95% of the CD45+/CD56+ NK cells are CD117-/CD94+/-, indicating a transition of immature NK cells into mature NK cells during 2~4 weeks of NK differentiation (Figure 5.1). Based on the CD45+/CD56+ population, the CD117+/CD94- versus CD117-/CD94+ population of EB-NK in EL08-1D2 and OP9-DL4 condition is 79.7% versus 13.3% and 25.3% versus 65.3% (Figure 5.1A). Interestingly, two sub-populations of CD45+/CD56+ are clearly distinguishable. Through further analysis, the CD45+low/CD56+low population represents the CD117+/CD94- population, while CD45+high/CD56+high population represents the CD117-/CD94+ population. A full panel of mature NK inhibitory and activating receptors are detected by flow cytometry, but due to varied cell proliferation capability as shown in Figure 6.1, not all the derived NK cells in different conditions are covered. UCB-NK cells, which serve as a positive control for deriving NK cells, consistently show typical and high expression of NK cell markers. In EL08-1D2 condition, the expression of inhibitory and activating receptors on UCB-NK is a little bit higher (3~10%) than on iCell-NK and UiPS-NK cells, while the iCell-NK and UiPS-NK have similar levels of these receptors (Figure 5.2A). In OP9 condition, UiPS-NK and UCB-NK have similar expression of NK receptors except for NKG2A. NKG2A binds to CD94 expression and CD94/NKG2A expression of UiPS-NK cells and UCB-NK cells is 44.9%~46.7% and 73.0%~79.4%, indicating there is 28.1%~32.7% more mature NK cells in UCB-NK (Figure 5.2B). In OP9-DL4 condition, EB-NK and UCB-NK cells share similar expression pattern of different NK cell markers (Figure 5.2C). Based on the phenotype of NK cells, the best stromal cell population for the support of NK cells is the

OP9-DL1 stromal cells. All the NK populations (iCell-NK, UiPS-NK, EB-NK and UCB-NK) share a typical and similar phenotype of mature NK cell markers, with CD56+ cells ranging from 95.6%~99.3%. Based on the CD56 expression, the inhibitory receptors, KIRs is 24.1~37.1%, CD161 is 90.8%~93.9% and NKG2A is 78.0%~88.3%; for the activating receptors, NKp44 is 96.9%~98.8%, NKp46 is 90.5%~97.4%, NKG2D is 71.0~76.1%, NKG2C is 9.42%~13.7% and CD16 is 17.6%~31.4% (Figure 5.2C). Therefore, iCell-HPCs and UiPS-HPCs can be differentiated into mature NK cells with typical inhibitory and activating receptors but with varied ability on different stromal cells, with OP9-DL1 condition showing the most comprehensive expression of NK cell markers from all the NK populations.

Expansion Potential of hiPSCs Derived NK Cells

To better understand the proliferation ability of the NK cells, the total living cells were counted in each individual NK condition at a time course of four weeks (Figure 6.1, 6.2). The starting cell number of the initial HPC sources is: UCB 10,000, iCell-HPCs 50,000, UiPS-HPCs 50,000 and UiPS-EBs 45,000 (calculated from 1EB contains 5670 cells). In Figure 6.1, by four weeks' co-culture, 10,000 UCB-NK could reach to 10^9 cells, representing a 10^6 fold increase in the cell number, and has the best proliferation capability among all the NK cell populations. In OP9 and OP9-DL4 conditions, all the other NK cell populations have only a two fold increase in cell number (Figure 6.1B, D). However, in EL08-1D2 and OP9-DL1 conditions, except for EB-NK in EL08-1D2 condition, all the other NK cell populations have at least 20 fold expansion by four weeks (Figure 6.1A, C). Derived from Figure 6.1, Figure 6.2 is based on the same HPCs derived NK cell populations, trying to compare the different stromal co-culture conditions according to the NK cell expansion potential. Therefore, in the OP9-DL1 condition, both iCell-NK and UiPS-NK experience 20 fold increase in proliferation from 50,000 cells expanded to 1,000,000 cells.

Function of hiPSCs Derived NK Cells

The cytotoxic function of the derived NK cells could be tested in a ^{51}Chromium release assay. The target cells are K562 cells, an established immortalized myelogenous leukemia cell line. Due to the varied expansion potential of the NK cells shown in Figure 6.1, only from the OP9-DL1 condition, enough NK cells could be collected and used for cytotoxicity assay at three E:T ratios (10:01; 5:01; 2.5:01). In EL08-1D2 condition, although missing EB-NK, we could also collect enough cells from the other NK populations to set up the cytotoxicity. In OP9-DL1 condition, although they share pretty similar mature NK phenotype in Figure 5.2C, iCell-NK has an almost 20% decreased killing ability (60%~30%) compared to the other NK populations (80%~50%). The UiPS-NK and EB-NK has a similar and even better lysis ability compared to UCB-NK (80%~50%) (Figure 7A). In EL08-1D2 condition, iCell-NK and UiPS-NK have similar killing ability (60%~30%), but UCB-NK has a better killing (Figure 7A). Derived from Figure 7A, Figure 7B showed the killing ability between two condition derived NK cells. Therefore, derived from EL08-1D2 and OP9-DL1, although varied, both iCell-NK and UiPS-NK obtained normal cytotoxicity (Figure 7B).

Limited Derivation of B cells from hiPSCs

Derivation of B cells from hiPSCs has been tried by different groups but was not successful [74]. Previously, our group has used hESCs derived HPCs for B cell co-culture, UCB CD34+ cells could robustly become CD45+/CD19+ B cells, while sorted hESCs derived CD34+CD45+ could not survive in the same condition [17]. The iCell-HPCs from the CDI company and UiPS-HPCs derived from Day 12 spin-EB protocol were used for B cell co-culture. UCB CD34+ cells were chosen as a positive control for generating B cells. The murine bone marrow stromal cells MS5 were used as stromal cells to support human B cell development. The B cell co-culture medium was MS5 medium supplemented with G-CSF and SCF cytokines [85]. On day 0, iCell-HPCs and UiPS-HPCs were plated with 100,000 cells, UCB CD34+ cells were plated with 10,000 cells in each well of a 24-well plate. As early as two weeks, under microscope, we can observe that iCell-HPCs and UiPS-HPCs are almost diminished on the stromal cells (Figure 8B), while UCB CD34+ cells proliferate very well (Figure 8A). At day 14, UCB

CD34+ cells can generate 13.1% of CD45+/CD10+ cells, 6.45% of CD10+/CD19+ cells and 5.25% of CD19+/CD20+ cells (Figure 8A). At day 28, there are 67.2% of CD45+/CD10+ cells, 59.8% of CD10+/CD19+ cells and 42.3% of CD19+/CD20+ cells (Figure 8A). So these UCB cells are making CD45+/CD10+/CD19+/CD20+ progenitor B cells (pro-B). However, the iCell-HPCs and UiPS-HPCs cannot produce any B cells. (Figure 8B). Thus, derivation of B cells from hiPSCs is severely restricted in the B cell co-culture condition.

Limited Derivation of T cells from hiPSCs

Derivation of T cells from hESCs and hiPSCs has been successfully shown before. Several groups have published papers on how to generate T cells from hiPSCs [79-83]. However, depending on different sources of HPCs, the stromal cells used as well as other factors in the T cell condition, the reproducibility of these experiments has been a big issue. Our group tried to use hESCs derived CD34+/CD45+ cells to set up T cell co-culture, as compared to UCB CD34+ cells, the results turned out that UCB CD34+ cells could make T cell progenitors but hESCs derived CD34+/CD45+ sorted cells couldn't [17]. Here, we used iCell-HPCs from the CDI company and our UiPS-HPCs derived from D12 spin-EB protocol as two sources of iPSC derived HPCs to test their T cell differentiation ability. UCB CD34+ cells, still served as a positive control for making T cells [31]. The murine stromal cell line OP9 was transduced with delta ligand 1 to generate OP9-DL1 stromal cells [30]. The T cell co-culture medium was OP9 medium supplemented with SCF, IL-7 and Flt3-L cytokines. The early T cell markers are CD1a, CD5 and CD7. During the T cell development, double positive (DP) stage of T cells is marked as CD4+/CD8+. On day 0, iCell-HPCs and UiPS-HPCs were plated with 100,000 cells, and UCB CD34+ cells were plated with 10,000 cells in each well of a 24-well plate. As early as two weeks, it is observed that both iCell-HPCs and UiPS-HPCs cannot survive on the stromal cells (Figure 9B). UCB CD34+ cells proliferate very well (Figure 9A). Flow cytometric analysis of iCell-HPCs and UiPS-HPCs cannot demonstrate a real population of T cells (Figure 9B). However, by 28 days, 12.3% expresses CD1a+/CD7+, 68.3% expresses CD5+/CD7+, 4.84% expresses CD4+/CD8+ as well as 5.9% expresses

CD3+/CD56-; by 59 days of culture, UCB CD34+ cells have an increased expression of CD1a+/CD7+ at 69.2%, CD5+/CD7+ at 90.9%, CD4+/CD8+ at 20% and CD3+/CD56- at 59.1% (Figure 9A). Therefore, there is a severe lack of in vitro T cell development from hiPSCs derived HPCs.

Project II:

Project II aims to use IL-2RG gene targeted human ESCs to establish an in vitro lymphocyte development model to study X-SCID disease. David Russell's lab group from University of Washington knocked out IL-2RG gene from hESCs by adeno-associated virus (AAV) mediated gene targeting. Both knock out clones (clone 1 and clone 7) and wild type clone (clone 9) were able to be induced into hematopoietic progenitors through EB formation. Day 13 EBs were shipped to our lab for further derivation. Here, in Project II, collaborating with Russell's lab, day 13 EBs from all clones were co-cultured with murine fetal live stromal cells EL08-1D2 toward NK cell development. The results indicated that the wild type clone could generate immature NK cells, and upon stimulation with artificial antigen presenting cells, they could also become functional mature NK cells. However, although knock out clones gave rise to a few monocytes and neutrophils, there's no NK cells induced.

Human Pluripotent Stem Cells Can Generate Hematopoietic Progenitor Cells

Human pluripotent stem cells, including hESCs and hiPSCs, can both be differentiated into hematopoietic progenitor cells (HPCs) through different approaches [64-66]. By collaborating with David Russell's group, they used AAV to target the IL-2RG gene from hESCs (H1 cell line) and generated three isogenic clones: Clone 1 (C1) and Clone 7 (C7) were knock out clones, and Clone 9 (C9) was a wild type clone. By adapting a hematopoietic differentiation protocol, both knock out and wild typ hESC clones were formed into EBs [63]. Then the day 13 EBs were shipped to us overnight in sterile conical tubes at 4 °C. Flow cytometric analysis shows that knock out clone 1 (KO-C1) and knock out clone 7 (KO-C7) and wild type clone 9 (WT-C9) express 34.4±2.9%,

29±4.5% and 19.6±6.3% CD34+/CD45+ cells (Figure 10A). Applying the same "spin-EB" differentiation protocol as described in Project I, UiPS (UCBiPS7) cells can be routinely differentiated into HPCs. On Day 12 of spin-EB differentiation, non-adherent cells were collected and analyzed by flow cytometry showing that 33% of the cells express CD34 and 30% of the cells express CD34+/CD45+ (Figure 10B). UCB sorted CD34+ cells served as a control for HPCs. More than 97% of the UCB cells express CD34 (Figure 10B). Therefore, both hESCs and hiPSCs can be routinely differentiated into HPC populations (KO-C1 HPCs, KO-C7 HPCs, WT-C9 HPCs) through different methods, regardless of genetic background.

Derivation of NK Cells Reveals Differential Expression of IL-2RG

Both hESCs and hiPSCs can be differentiated into NK cells by co-culturing with different stromal cells or feeder free condition [69, 71]. EL08-1D2 is a murine fetal liver derived stromal cell lines used to support human NK cell development in vitro [86]. To induce all these HPC populations into NK cells, defined NK differentiation medium supplemented with cytokines of IL-3, IL-7, IL-15, SCF and Flt-3L was used. After two weeks of co-culture, HPCs derived cells in the dish were analyzed by flow cytometry. Particularly, the KO-C1 HPCs and KO-C7 HPCs derived cells express 13% and 10% of CD56+ cells, but none of them express IL-2RG (CD132), that is 0% of CD56+/CD132+ cells (Figure 11Aa, Ba). For WT-C9 HPCs, UiPS-HPCs and UCB derived cells at two weeks, the expression of CD56+/CD132+ cells is 22%, 29% and 45% respectively (Figure 11Ca, Da, Ea). Thus, the KO-C1 and KO-C7 cells has no CD56+/CD132+ expression, indicating these two IL-2RG knock out cell clones cannot generate NK cells without IL-2RG gene expression. Although they each has 13% and 10% of CD56+ cells, when excluded from CD14+ cells (monocyte), they express 0% of CD14-CD56+ cells, which are characterized as real NK cell populations (Figure 11Ab, Bb). To further characterize the NK cell development stages, NK cells are first excluded from CD14+ cells, CD56+/CD161+/- population is defined as the real NK population, then based on LFA-1 (CD11a) expression, mature NK cells (mNK) and immature NK (imNK) are distinguished [92]. This characterization of mNK and iNK cells is correlated to what had

been found before: mNK cells are CD117-/CD94+/CD16+, imNK cells are CD117+/CD94-/CD16- [91]. Thus, mNK cells are defined as CD14-/CD56+/CD161+/-/LFA-1+/CD117-/CD94+/CD16+, and imNK cells are defined as CD14-/CD56+/CD161+/-/LFA-1-/CD117+/-/CD94-/CD16-. Specifically, in WT-C9-NK cells, gated on CD14- cells, CD56+/CD161+/- NK cells are 26%, and of that population 46% of the cells express LFA-1 while 50% of the cells lack of LFA-1 expression (Figure 11Cb). So mNK and imNK cells are mixed in half and half. In UiPS-NK cells, gated on CD14- cells, CD56+/CD161+/- NK cells are 37%, for this NK population, and 97% of the cells express LFA-1, while 3% of the cells has no LFA-1 expression (Figure 11Db). Most of the NK cells become mNK cells. In UCB-NK cells, gated on CD14- cells, CD56+/CD161+/- NK cells is 40%, among these NK cells, 35% of the cells express LFA-1, while 64% of the cells has no LFA-1 expression (Figure 11Eb). So UCB-NK cells are mixed with mNK cells (64%) and imNK cells (35%). Therefore, as early as two weeks co-culture, KO-C1 and KO-C7 HPCs do not make any NK cells and has no CD132 expression, while WT-C9 HPCs have undergone CD132 expression as they begin to generate 22.3% of CD56+/CD132+ NK cells. UiPS-HPCs and UCB serve as good examples to make NK cells.

Homogenous NK Cell Populations Display Different Stages of NK Cell Development

To further understand how IL-2RG knock out and wild type clones undergone differentiation in NK condition, we did flow cytometry to analyze the cells at a longer time point (4 weeks). During four weeks co-culture, KO-C1 and KO-C7 cells underwent an initial cell proliferation in the first two weeks, then after two weeks, cells began to diminish and by four weeks only a few cells survived. At four weeks, different cell surface markers were analyzed to identify the cell types of the remaining KO-C1 and KO-C7 cells. After four weeks culture, KO-C1 and KO-C7 derived cells are not NK cells (CD56), B cells (CD19), T cells (CD3, CD1a) or dendritic cells (CD11c, CD123), but they are characterized as 27% and 32% of CD45+CD14+ cells (monocyte) as well as 65% and 61% of CD45+CD15+ cells (neutrophil) (Figure 12A,B). As for WT-C9 HPCs derived cells, more than 99% of the cells become CD14-/CD56+ NK cells, with 88% of

cells expressing CD132 and CD122 (Figure 12C). Both UiPS-HPCs and UCB generated 92% and 99% of CD14-CD56+ NK cells. UiPS-NK cells express 42% and 83% of CD132 and CD122 (Figure 12Da). UCB-NK cells express 77% and 95% of CD132 and CD122 (Figure 12Ea). Interestingly, among the three homogenous derived NK cell populations, they are mixed with mNK cells and imNK cells at different ratios. For WT-C9-NK cells, 97% of the NK cells didn't express LFA-1 and are represented as CD117+/CD94-/CD16- immature NK cells (Figure 12Cb), while 94% of the UiPS-NK cells express LFA-1 and are CD117-/CD94+/CD16+ mature NK cells (Figure 12Db). As for UCB-NK cells, 71% of the NK cells express LFA-1 while 27% of the cells has no LFA-1 expression, representing a much more mixed population of mature and immature NK cells (Figure 12Eb). Based on CD45+/CD56+/CD3- NK populations, selected important NK inhibitory (KIRs, NKG2A and CD161) and activating receptors (NKp44, NKp46, NKG2D, NKG2C and CD16) as well as apoptosis ligands (FasL and TRAIL) were analyzed by flow cytometry. In WT-C9-NK cells, we found very low expression of inhibitory receptors (0% of KIRs, 3% of NKG2A), activating receptors (34% of NKp44, 3% of NKp46, 6% of NKG2D, 0% of NKG2C and 1% of CD16) and apoptosis ligands (0% of FasL and TRAIL) (Figure 12Cc). However, in UCB-NK cells, we found relative high expression of inhibitory receptors (11% of KIRs, 67% of NKG2A), activating receptors (90% of NKp44, 64% of NKp46, 61% of NKG2D, 7% of NKG2C and 5% of CD16) and apoptosis ligands (0% of FasL and 4% of TRAIL) (Figure 12Ec). Therefore, by four weeks NK co-culture, KO-C1 and KO-C7 HPCs are greatly diminished and only a few monocytes and neutrophils survived, but WT-C9 HPCs are successfully differentiated into a homogenous NK cell population and 97% are immature NK cells. Homogenous NK cell populations mixed with more mature and less immature NK cells are also derived in NK co-culture of UiPS-HPCs and UCB.

Expansion Potential of hESCs Derived NK Cells

The proliferation potential of the HPCs in the NK derivation condition reflects their NK differentiation capability. To determine the expansion ability of the different clones of hESCs derived HPCs, for each individual cell line, equal amount of cells were seeded

onto each well of a 24-well plate coated with EL08-1D2 stromal cells. That is, on day 0, KO-C1 HPCs were plated at 33,000 cells per well, KO-C7 HPCs were plated at 55,000 cells per well, WT-C9 HPCs were plated at 83,000 cells per well and UCB CD34+ cells were plated at 10,000 cells (Figure 13A). Within two weeks of co-culture, all the starting cell populations are experiencing a trend of cells expanding, at two weeks, the total cells of Clone 1-KO and Clone 7-KO reach the maximum of total cells (Figure 13A). However, after two weeks' co-culture, Clone 1-KO and Clone 7-KO cells begin to diminish and finally remain 10^5 cells by four weeks. Clone 9-WT and UCB cells keep expanding after two weeks, then reach more than 10^7 cells per well at four weeks, which are indicated as CD14-/CD56+ NK cells (Figure 13A). Based on percentage of CD14-/CD56+ and CD56+/CD132+ analyzed every week, Clone 9-WT and UCB are experiencing a growing population of CD14-/CD56+ NK cells and CD56+/CD132+ NK cells, while the Clone 1-KO and Clone 7-KO has no expression of CD56 throughout four weeks co-culture (Figure 13B). By four weeks, 100% of the Clone 9-WT and UCB derived cells become NK cells, among them, 88% and 77% of the NK cells express CD132 (Figure 13B). Therefore, throughout four weeks NK co-culture, WT-C9 HPCs and UCB are sufficiently differentiated into NK cells with a robust expansion potential, while KO-C1 and KO-C7 HPCs cannot produce NK cells.

Maturation and Function of hESCs Derived Wild Type Clone 9 NK Cells

Considering for translational research, hESCs and iPSCs derived NK cells should have normal cytotoxicity function that could kill tumor cells. Based on the four weeks phenotype for wild type Clone 9 NK (WT-C9-NK) cells, 97% of them are still immature NK cells (CD14-/CD56+/LFA-1-/CD117+/CD94-) (Figure 12Cb). UCB-NK cells have 71% mature NK cells with 27% of immature NK cells (Figure 12Eb). Initially we used these four weeks WT-C9-NK and UCB-NK cells as effectors for ^{51}Chromium release assay to test the cytotoxicity function of NK cells. Peripheral blood NK cells are considered as normal functional mature NK cells, serving as a positive effector for the assay [71, 89, 93]. The tumor cell line K562 cells are chosen as the targets for the assay. The effector to target ratio is 10:01, 5:01 and 2.5:01. The assay shows that the percentage

of the targets killed by different ratio of WT-C9-NK cells is all below 10%, indicating the killing ability of these immature WT-C9-NK cells is under 10% (Figure 15). But the killing ability of the PB-NK and UCB-NK is between 70%~40%, significantly better than WT-C9-NK (Figure 15). In order to make W-C9-NK cells become mature NK cells, these immature NK cells were stimulated with artificial antigen presenting cells (aAPCs) with membrane bond IL-21 expression. These aAPCs has been proved to be able to expand PB-NK cells while maintaining telomere length and in vitro activity [90]. For the first week of stimulation, aAPCs were irradiated with 10,000 RADs and added to both WT-C9-NK and UCB-NK cells at a ratio of 2:1 (Day 0). In the second week, cells were stimulated with aAPCs at a 1:1 ratio. Cultures were fed twice a week. After two weeks stimulation, we performed another ^{51}Chromium release assay to test the cytotoxicity of stimulated NK cells. Stimulated WT-C9-NK, UCB-NK and PB-NK were effectors against the target K562 cells at a ratio of 10:01, 5:01 and 2.5:01. The assay demonstrates that stimulated WT-C9-NK has increased killing ability and almost the same to stimulated UCB-NK, between 60%~50% (Figure 15). PB-NK cells with a relative better killing ability between 80%~60% still serve as a positive control for the experiment (Figure 15).

The stimulated WT-C9-NK and UCB-NK cells used for the cytotoxicity assay are also analyzed by flow cytometry on the same day. WT-C9-NK and UCB-NK cells maintain high expression of CD132 and CD122 at 87%, 76% and 88%, 96% (Figure 14A). CD2 is a NK cell adhesion and co-stimulatory molecule expressed on the surface of NK cells. Interestingly, CD2 expression experienced a significant reduction after NK stimulation, that is WT-C9-NK and UCB-NK cells' CD2 expression decreased from 50% to 11.2% and from 8.54% to 1.23% respectively (Figure 12Ca, Ea; Figure 14A). After stimulation, the WT-C9-NK cells, the population of immature NK cells (CD14-/CD56+/LFA-1-) decreases from 97.3% to 72.3%, while mature NK cells (CD14-/CD56+/LFA-1+) increase from 2.21% to 24% (Figure 12Cb, Figure 14Ba). For UCB-NK cells, the population of immature NK cells decreases from 71.3% to 5.53% and the mature NK cells increase from 21.4% to 93.4% (Figure 12Eb, Figure 14). The increased percentage

of mature NK cells and decreased percentage of immature NK cells indicate that both WT-C9-NK and UCB-NK are becoming mature during the stimulation of aAPCs. Specifically, for WT-C9-NK cells, the expression of mature NK markers are increased after stimulation, the inhibitory receptors of KIRs from 0% to 16%, NKG2A from 3% to 72%, activating receptors of NKp44 from 34% to 58%, NKp46 from 3% to 91%, NKG2D from 6% to 43.7%, NKG2C from 0% to 27%, CD16 from 1% to 30%, and apoptosis ligands of TRAIL from 0% to 30% (Figure 12Cc; Figure 14Bc). Therefore, after stimulation with aAPCs, WT-C9-NK cells become mature NK cells with increased expression NK function markers and improved cytotoxicity.

Project III:

Wiskott-Aldrich syndrome (WAS) is another X-linked immunodeficiency disease, which is caused by the mutation of WAS gene. Gene modification of human patient iPSCs could potentially provide a method to do autologous transplantation for the disease. As a "proof of concept" experiment, Brian Davis' lab from University of Texas Health Science Center used zinc-finger nuclease (ZFN) to mediate genome editing to correct the WAS gene from WAS patient derived iPSCs. WAS patient iPSCs and gene corrected iPSCs could both be differentiated into hematopoietic progenitor cells. When they were co-cultured with OP9-DL1 or OP9-DL4 cells toward NK differentiation, WAS gene corrected iPSCs could generate NK cells with normal function, WAS patient derived iPSCs without gene correction had NK cell development in vitro.

Human WAS Patient Derived iPSCs Can Generate Hematopoietic Progenitor Cells

To study the lymphocyte development of the patient derived iPSCs, these iPSCs should be first derived into hematopoietic progenitor cells (HPCs). By adapting the original "spin-EB" protocol, Brian Davis' lab group applied OP9 stromal cells to better improve the hemato-endothelial cell surface antigen expression level on HPCs. First, on day 0, 3,000 undifferentiated human iPS cells (cWAS and WAS) and ESCs (H9 cell line) were aggregated in 96-well plate through mechanical spinning. Day 0 to day 4 was an early

hematopoietic differentiation. Then on day 4, EBs were moved onto irradiated confluent OP9 stromal cells to grow for another 8~10 days for a later hematopoietic differentiation. The media used through 12 days' of induction was commercially purchased BPEL basal media with added cytokines of SCF, VEGF and BMP4 on the day of use. Then on day 12~14, total cells were stained for CD34 and CD43, HPCs were sorted by a fluorescence activated cell sorting (FACS) machine. On day 14, sorted WAS-iPSCs derived HPCs (WAS-HPCs), WAS gene corrected WAS-iPSCs derived HPCs (cWAS-HPCs) and hESCs (H9 cell line) derived HPCs (H9-HPCs) were shipped to us overnight in sterile tubes at 4 °C. Among them, H9-HPCs serve as a hESCs control compared to hiPSCs. Our flow cytometric analysis demonstrates that all the cWAS-HPCs, WAS-HPCs and H9-HPCs express more than 99% of CD34+/CD43+ cells, representing a homogenous population of HPCs (Figure 16). UCB sorted CD34+ cells have 80% of CD34+/CD43+ expression, and serve as a positive control for the derivation of NK cells from HPCs (Figure 16). Therefore, all these HPC populations (cWAS-HPCs, WAS-HPCs, H9-HPCs and UCB) provide ideal hematopoietic progenitor cell sources for the following lymphocyte differentiation, with 99% of CD34+/CD43+ expression.

Derivation of NK Cells from Different Hematopoietic Progenitor Cell Populations

In an effort to induce these ideal sources of hematopoietic progenitor cells to become NK cells, they were separately seeded onto irradiated confluent OP9-DL1 and OP9-DL4 stromal cells in NK differentiation media. The defined NK differentiation media was supplemented with cytokines of IL-3, IL-7, IL-15, SCF and Flt-3L. CD33 is a myeloid progenitor marker, but is also expressed on some activated T cells and NK cells [94]. Considered as a prethymic lymphocyte marker for T cells and NK cells, CD7 is also found on mature T cells and NK cells [95]. Excluded from CD14 positive monocytes, NK cells are characterized as CD14-/CD56+ cells. In less than two weeks, we found these HPCs made an obvious difference in the NK differentiation conditions. For cWAS-HPCs in OP9-DL1 condition, 66% of the cells are CD14-/CD56+ NK cells, 31% of these cells express CD56+/CD33+ and 53% of the cells express CD56+/CD7+. Gated on CD56+/CD14- population, 52% of the NK cells are CD56+/CD117-/CD94+ mature NK

cells (Figure 17A). In OP9-DL4 condition, similar results are found, 45% of the cells expressed CD14-/CD56+, with 24% express CD56+/CD33+, 42% express CD56+/CD7+, and 14% of the NK cells are mature NK cells (Figure 17B). The NK co-culture of H9-HPCs and UCB, in OP9-DL1 condition, 48% and 33% of cells express CD14-/CD56+, 33% and 28% of cells express CD56+/CD33+, 37% and 22% of cells express CD56+/CD7+ as well as 70% and 30% of the CD14-/CD56+ NK cells share a mature phenotype (Figure 17A). In OP9-DL4 condition, 64% and 70% of cells express CD14-/CD56+, 33% and 61% of cells express CD56+/CD33+, 54% and 64% of cells express CD56+/CD7+ as well as 64% and 70% of the CD14-/CD56+ NK cells had a mature phenotype (Figure 17B). Specifically, the WAS-HPCs in OP9-DL1 co-culture, CD14-/CD56+ cells can only be found at 5%, with 2% of the cells expressing CD56+/CD33+ and CD56+/CD7+ (Figure 17A). While in OP9-DL4 condition, no CD56+ cells are expressed (Figure 17B). Therefore, cWAS-NK, H9-NK and UCB-NK cells are successfully characterized in both NK conditions as early as 13 days of co-culture, while WAS-NK derivation is severely limited in these conditions. Thus, it indicates that WAS gene corrected iPSCs restores the development of NK cells in vitro.

Function of Gene Corrected cWAS-NK Cells In Vitro

To better understand whether the gene corrected cWAS-NK cells regained the normal function of NK cells, they were tested in a CD107a assay. The CD107a is a marker for NK cell degranulation, it reflects the NK cells direct exocytosis to the target cells by releasing perforin and granzymes. So CD107a assay has been used as a measurement for NK cell killing ability [89]. By co-culturing cWAS-NK cells for 34 days, 83% and 86% of the cells in OP9-DL1 and OP9-DL4 conditions express CD56, and 75% and 72% of the NK cells in OP9-DL1 and OP9-DL4 conditions are CD117-/CD94+ mature NK cells (Figure 18). Based on CD56 expression, some NK inhibitory and activating receptors are further characterized for cWAS-NK cells. Flow cytometric analysis demonstrates that the KIR surface expression was 38% in OP9-DL1 and 13% in OP9-DL4, and activating receptors of NKp44 and NKG2D express 79% and 93% in OP9-DL1 condition, 67% and 57% in OP9-DL4 condition (Figure 18). Surprisingly, two other major NK activating

receptors of CD16 and NKp46 are rarely expressed in the two conditions (Figure 18A). In the CD107a assay, flow cytometric analysis shows that from an E: T ratio of 1:5, 1:2, 1:1 and 1:0, OP9-DL1 co-cultured cWAS-NK cells express CD107a at 61%, 40%, 25% and 4%, and OP9-DL4 co-cultured cWAS-NK cells express CD107a at 56%, 39%, 31% and 6% (Figure 18B). Therefore, both OP9-DL1 and OP9-DL4 co-cultured cWAS-NK cells have significantly increased expression of CD107a upon increased dose of target stimulation, implying that cWAS-NK cells obtain the normal killing ability of NK cells.

Figure 3: Different sources of hiPSCs can generate HPCs. A. Phenotypic analysis for common HPC surface antigens' (CD34, CD43, CD45) expression from multiple sources of HPCs. **B.** Morphology of iCell-HPCs and Day 12 UiPS-EBs. iCell-HPCs: human iPSCs derived HPCs generated by CDI's proprietary protocols; UiPS-HPCs: human iPSCs (UCBiPS7) derived HPCs separated as non-adherent cells from Day 12 Spin-EB protocol; UiPS-EBs: human iPSCs (UCBiPS7) derived embryonic bodies from Day 12 Spin-EB protocol; UCB: human umbilical cord blood sorted CD34+ cells. HPC: hematopoietic progenitor cell.

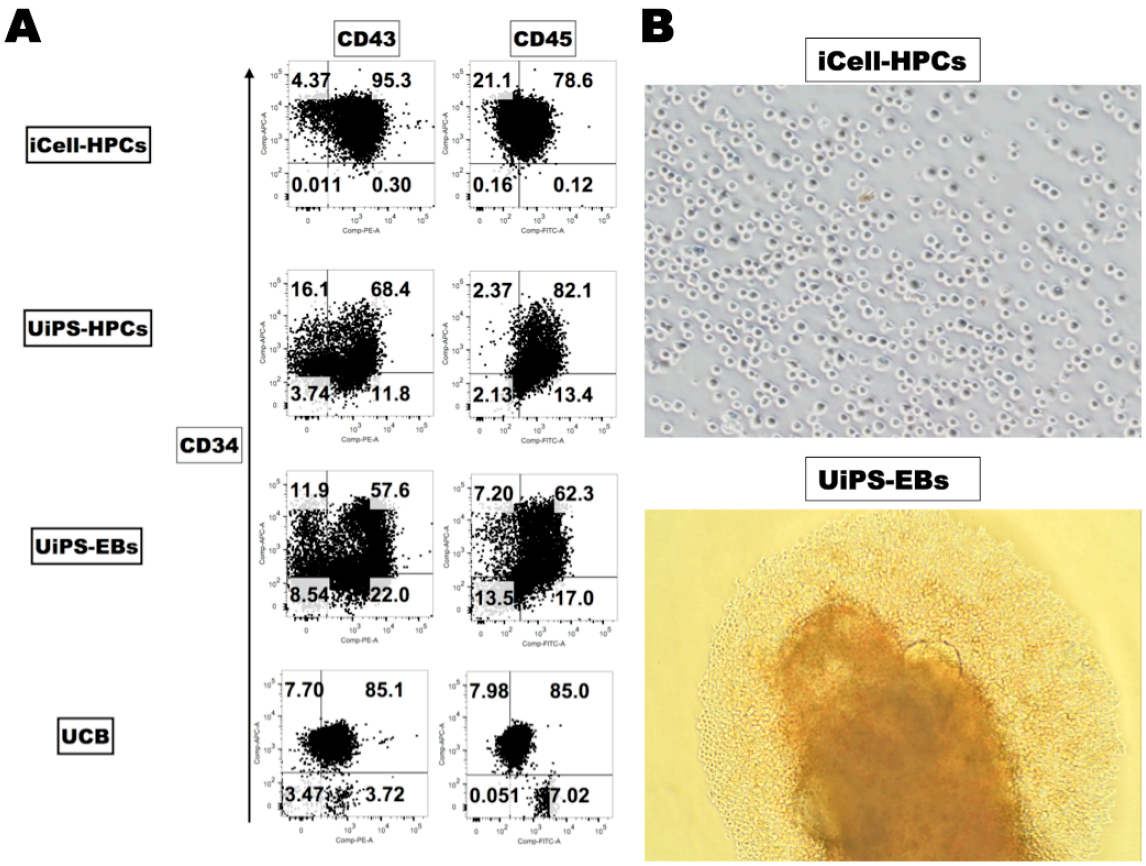
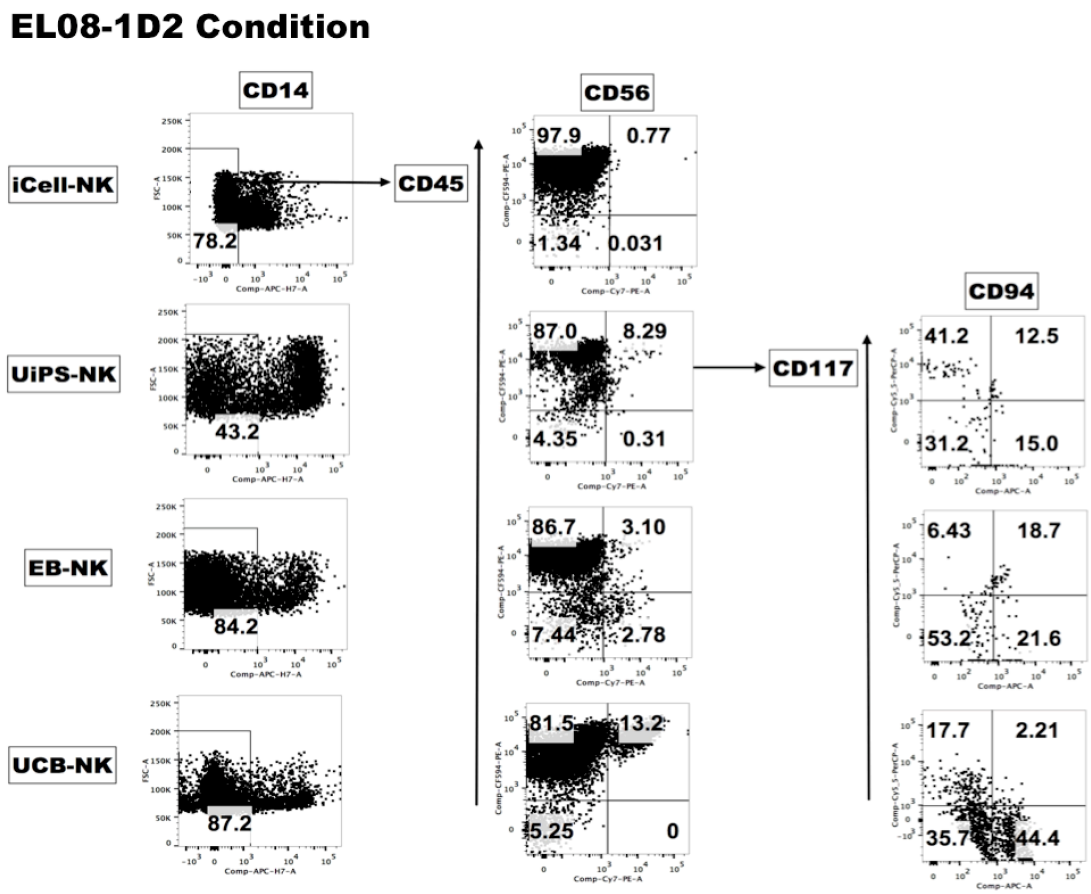
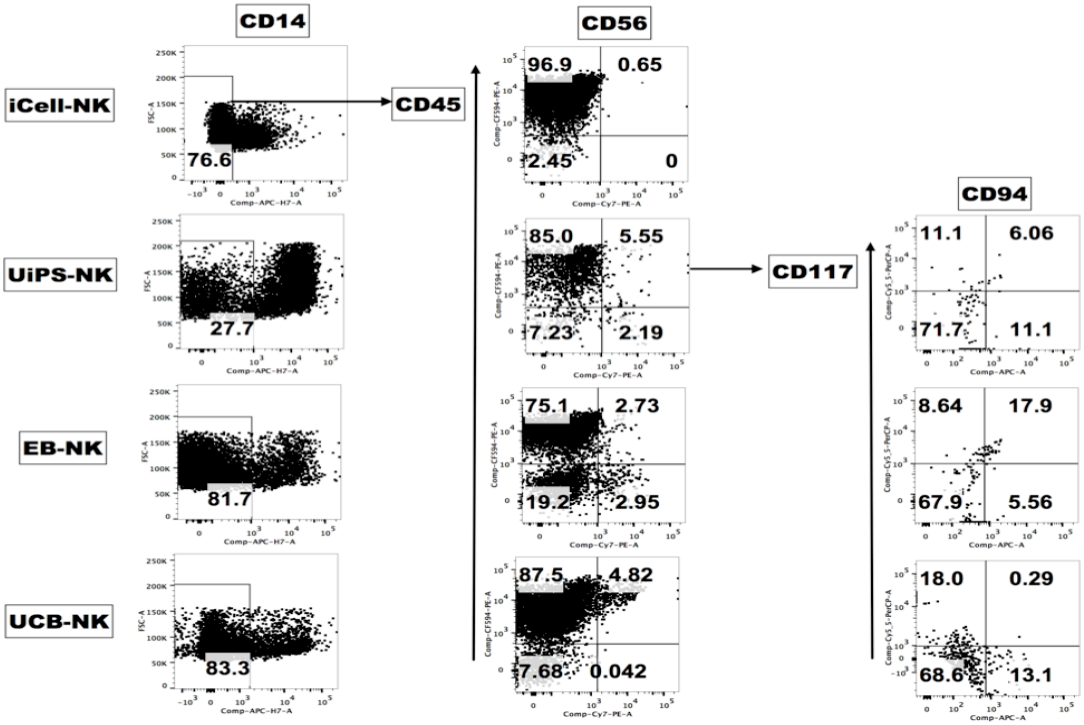


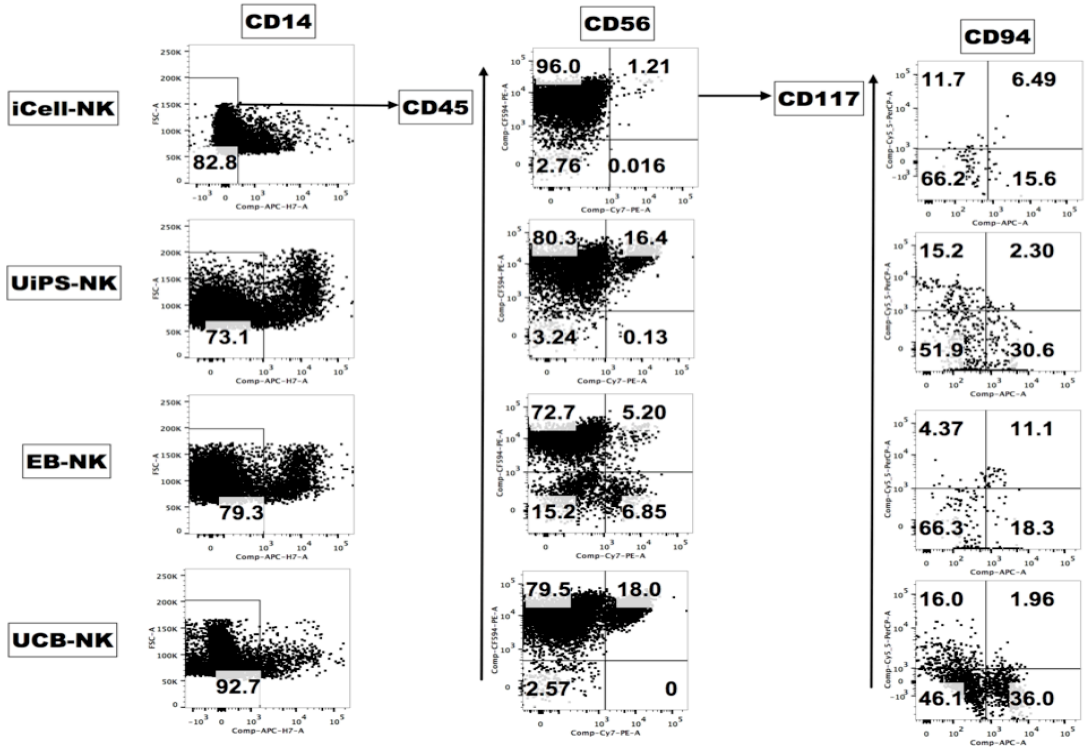
Figure 4: Derivation of NK cells in two weeks. Flow cytometric analysis of common NK cell markers (CD56, CD117, CD94) is shown here. Under the same NK differentiation media, different stromal cells are used to support the NK cell derivation: **A.** EL08-1D2 Condition; **B.** OP9 Condition; **C.** OP9-DL1 Condition; **D.** OP9-DL4 Condition. iCell-NK: iCell-HPCs derived NK cells; UiPS-NK: UiPS-HPCs derived NK cells; EB-NK: UiPS-EBs derived NK cells; UCB-NK: UCB derived NK cells



B **OP9 Condition**



C **OP9-DL1 Condition**



D **OP9-DL4 Condition**

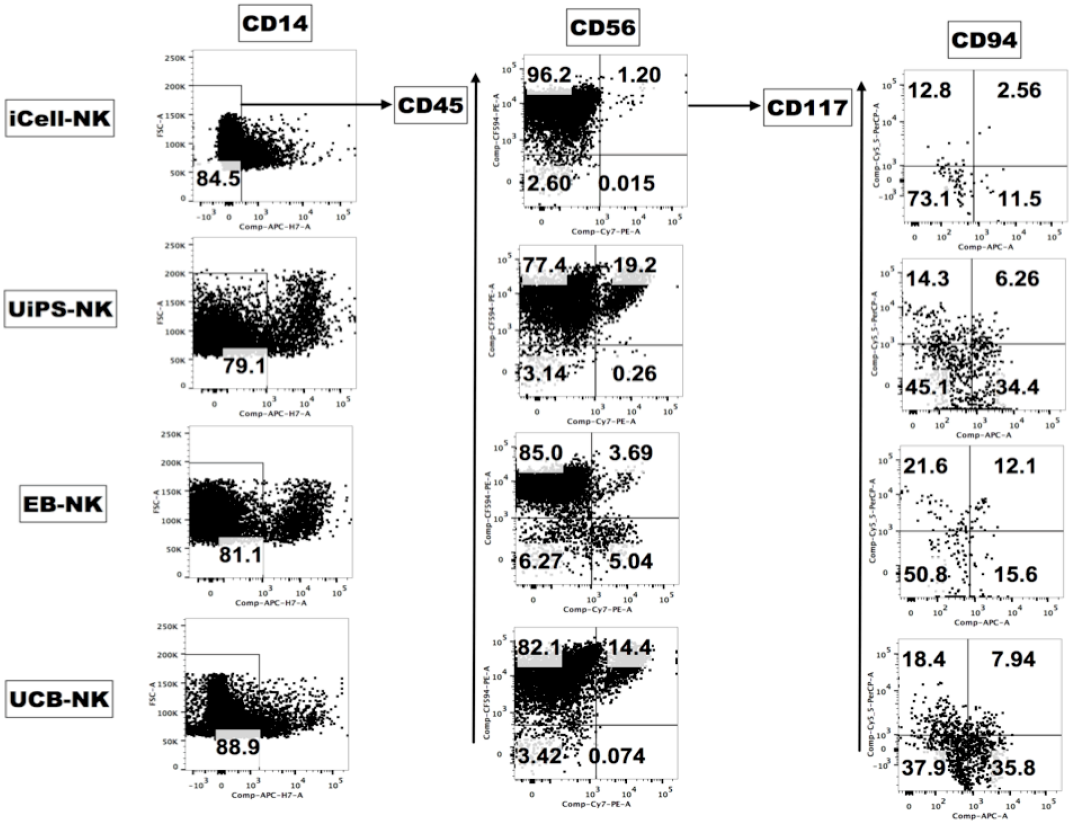
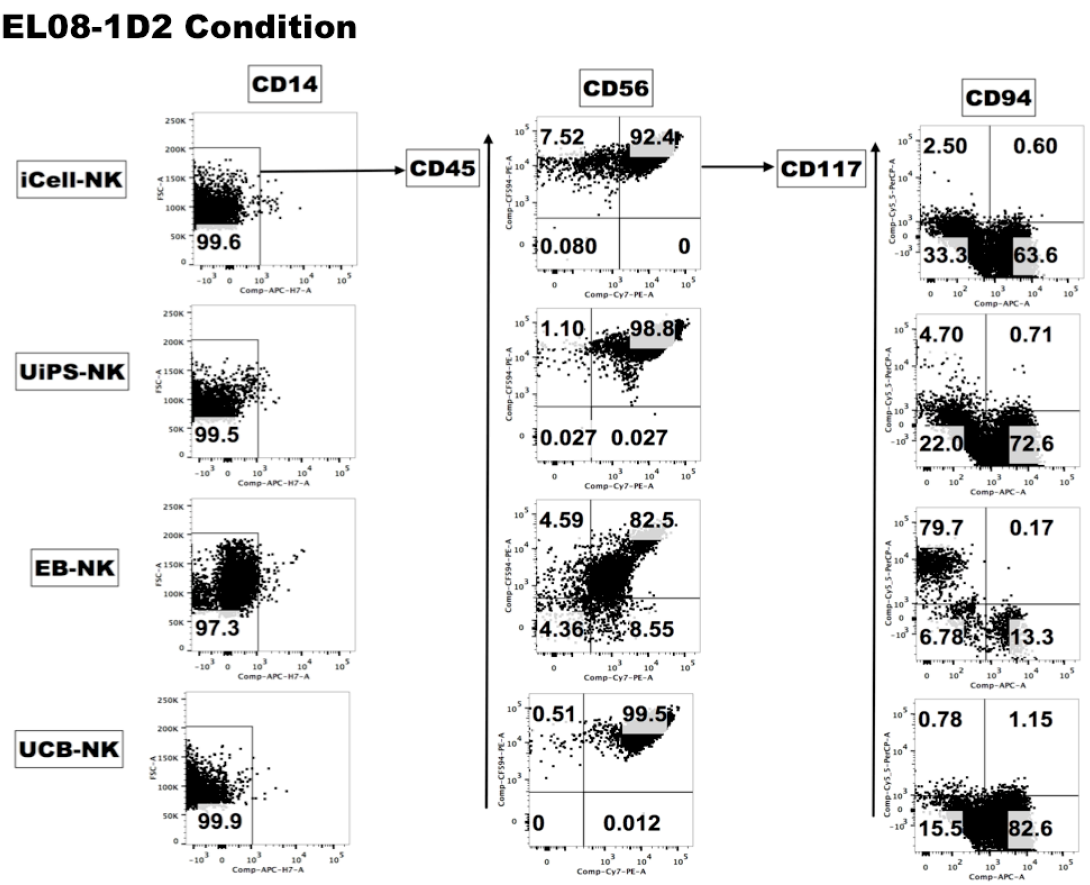
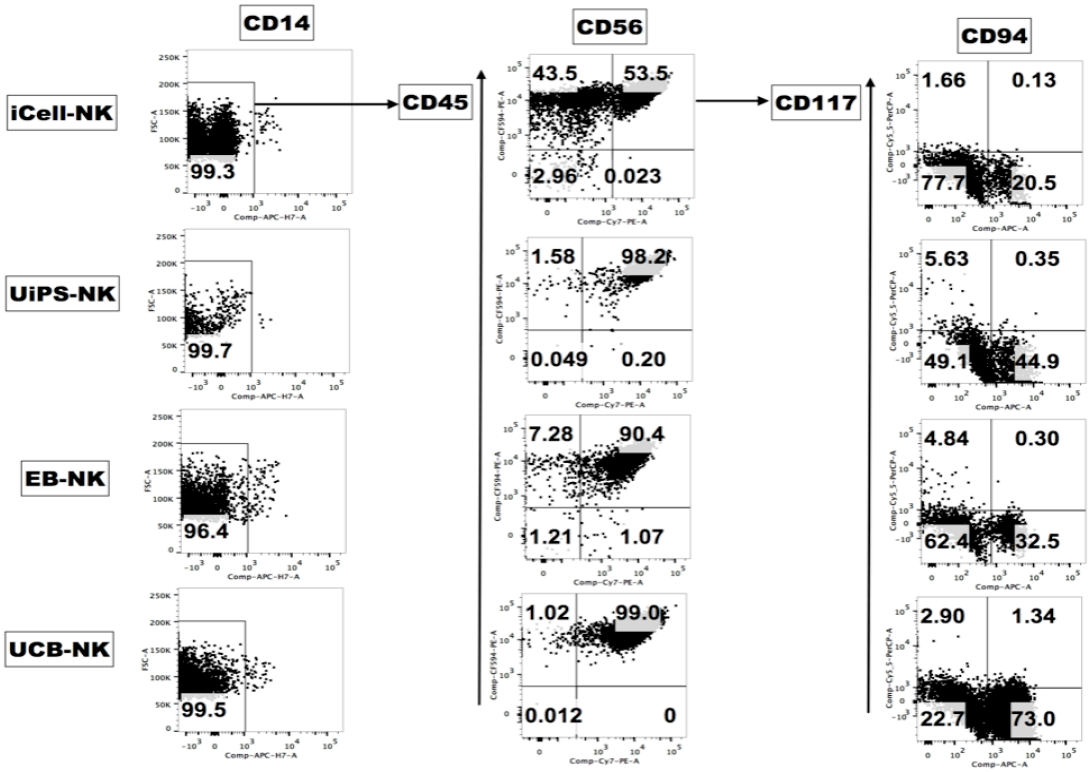


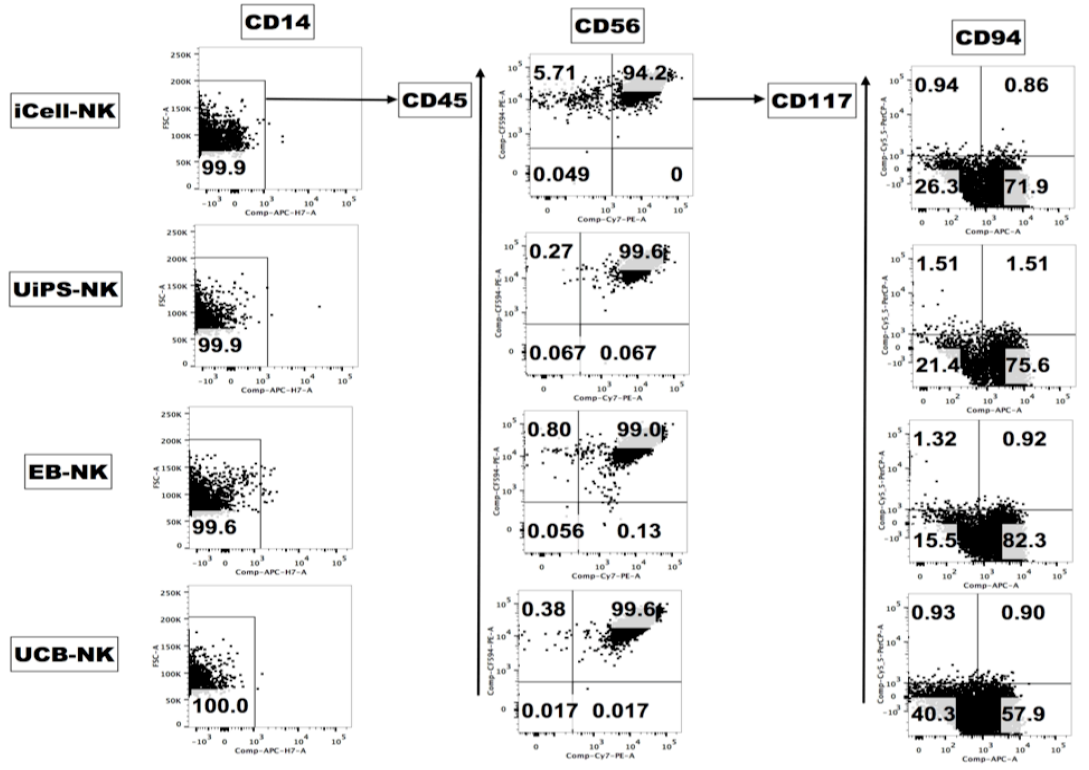
Figure 5.1: Derivation of NK cells in four weeks. Flow cytometric analysis of common NK cell markers (CD56, CD117, CD94) is shown here. Under the same NK differentiation media, multiple stromal cells are used to support the NK derivation: **A.** EL08-1D2 Condition; **B.** OP9 Condition; **C.** OP9-DL1 Condition; **D.** OP9-DL4 Condition.



B **OP9 Condition**



C **OP9-DL1 Condition**



D **OP9-DL4 Condition**

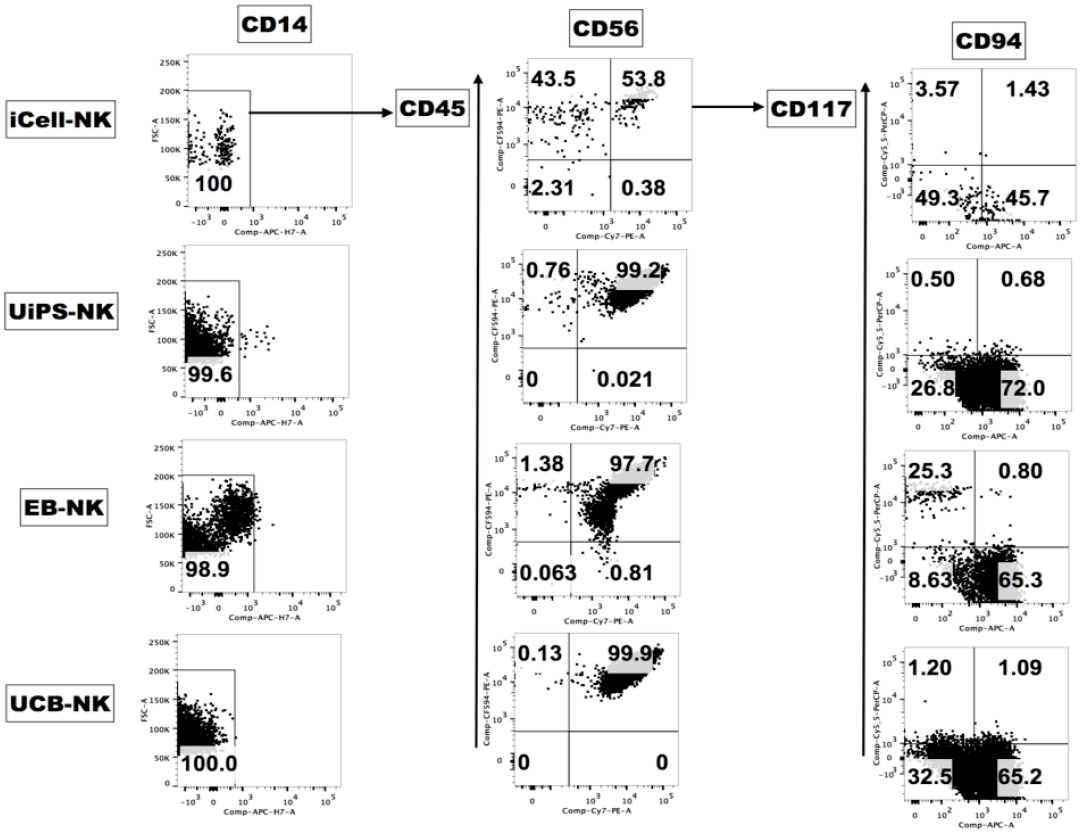
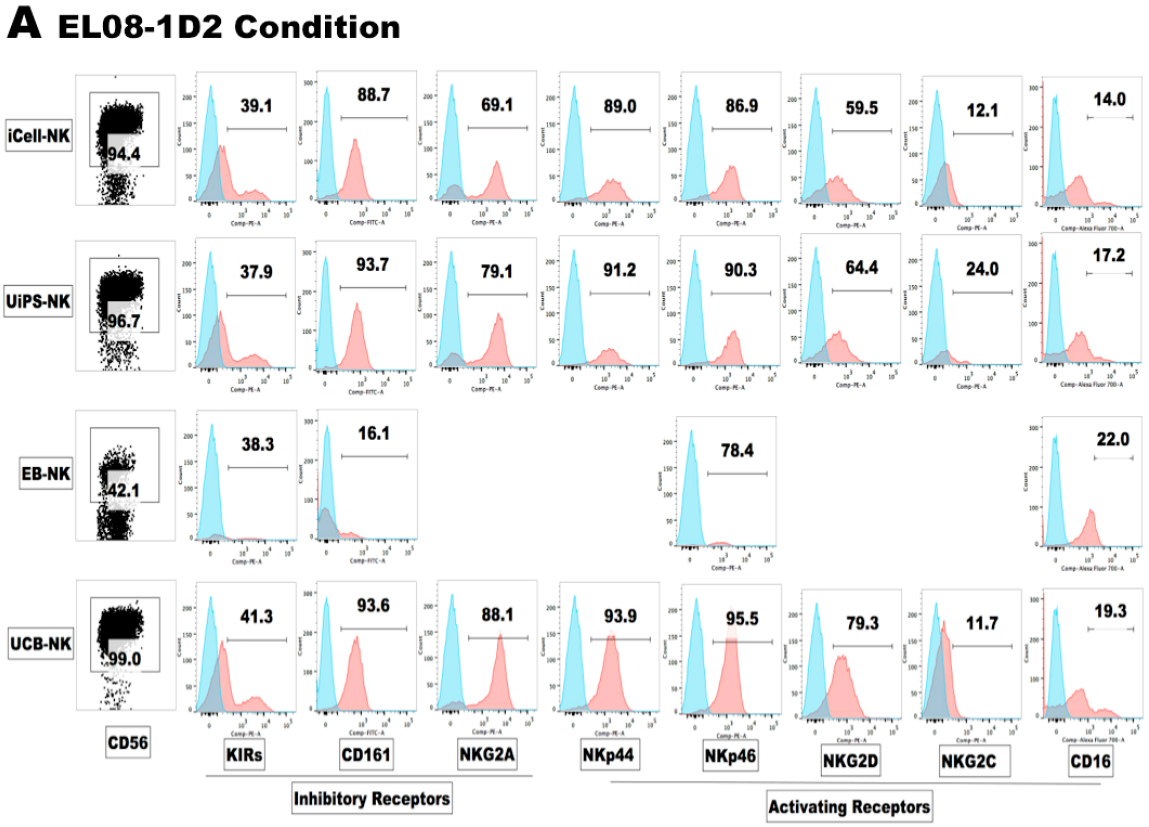
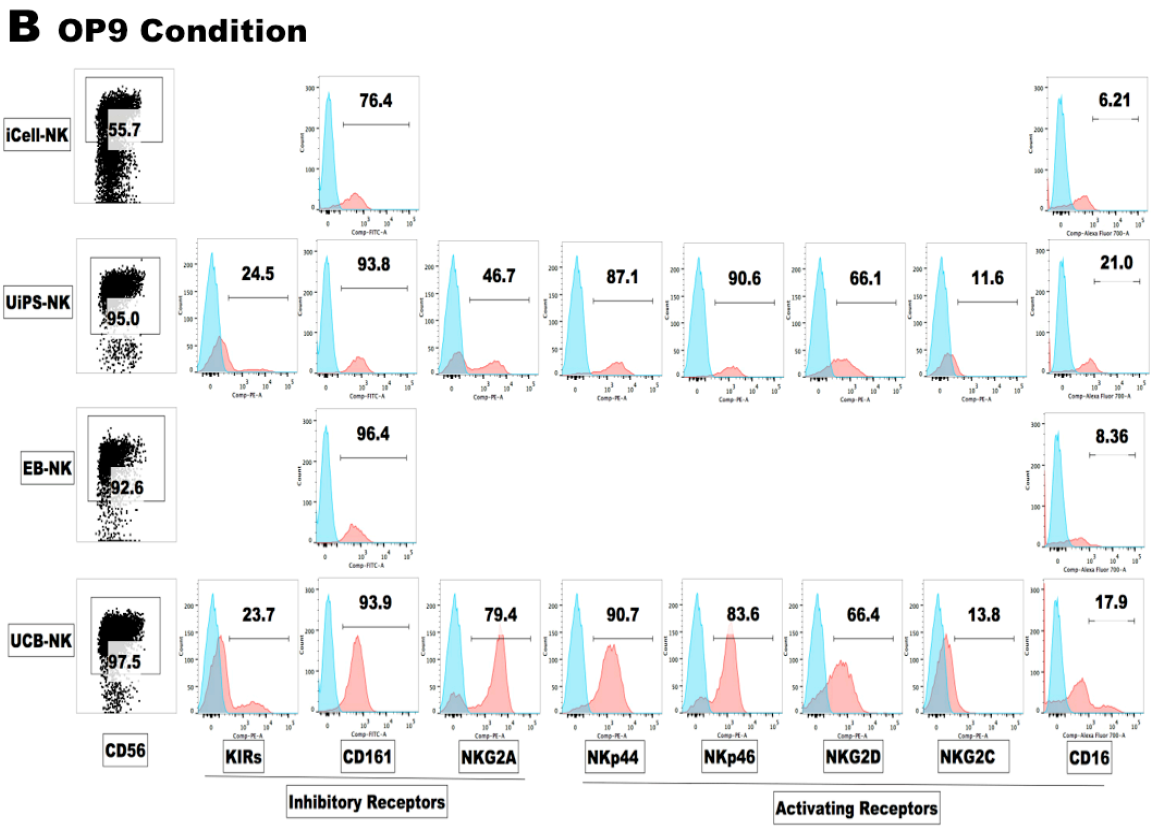


Figure 5.2: Characterization of mature NK cells in four weeks. Inhibitory NK cell receptors are listed as KIRs, CD161 and NKG2A. Activating NK cell receptors are listed as NKp44, NKp46, NKG2D, NKG2C and CD16. Under the same NK differentiation media, multiple stromal cells are used to support the NK derivation: **A.** EL08-1D2 Condition; **B.** OP9 Condition; **C.** OP9-DL1 Condition; **D.** OP9-DL4 Condition.




B OP9 Condition
iCell-NK
55.7
76.4
6.21
UiPS-NK
95.0
24.5
93.8
46.7
87.1
90.6
66.1
11.6
21.0
EB-NK
92.6
96.4
8.36
UCB-NK
97.5
23.7
93.9
79.4
90.7
83.6
66.4
13.8
17.9
CD56
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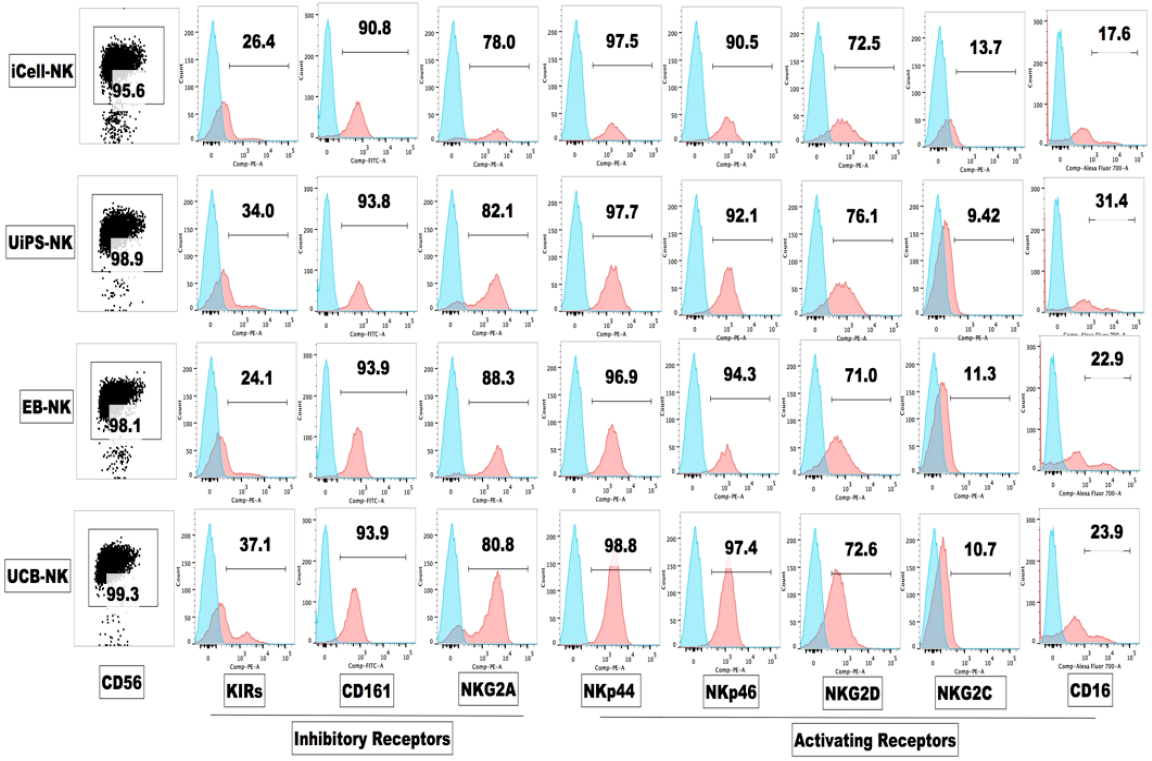
NKp44
NKp46
NKG2D
NKG2C
CD16
Inhibitory Receptors
Activating Receptors


C OP9-DL1 Condition
iCell-NK
95.6
26.4
90.8
78.0
97.5
90.5
72.5
13.7
17.6
UiPS-NK
98.9
34.0
93.8
82.1
97.7
92.1
76.1
9.42
31.4
EB-NK
98.1
24.1
93.9
88.3
96.9
94.3
71.0
11.3
22.9
UCB-NK
99.3
37.1
93.9
80.8
98.8
97.4
72.6
10.7
23.9
CD56
KIRs
CD161
NKG2A
NKp44
NKp46
NKG2D
NKG2C
CD16
Inhibitory Receptors
Activating Receptors

D OP9-DL4 Condition

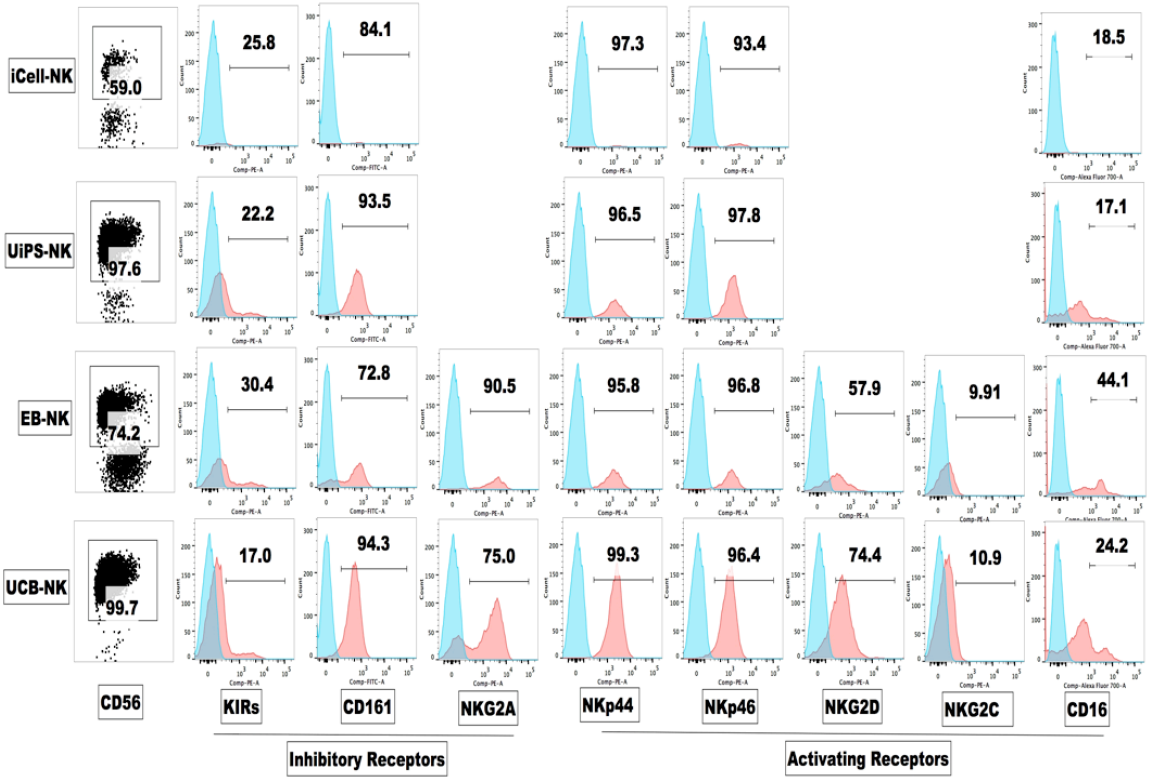


Figure 6.1: Expansion potential of hiPSCs derived NK cells in four weeks. Within one condition, different sources of NK cells are compared and counted at multiple time points (day 0, day 7, day 14, day21 and 28). **A.** EL08-1D2 Condition; **B.** OP9 Condition; **C.** OP9-DL1 Condition; **D.** OP9-DL4 Condition.

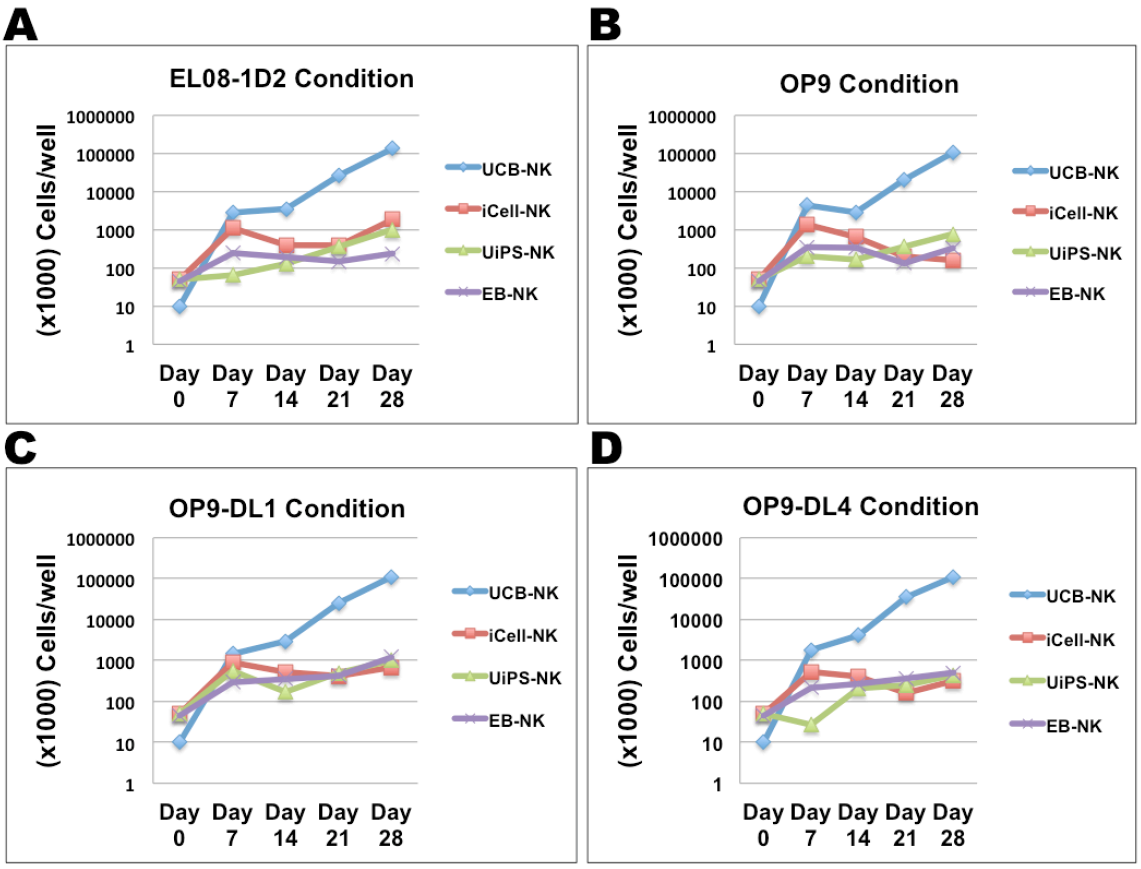


Figure 6.2: Comparison of NK cell expansion potential among different stromal cell co-culture conditions. Within one source of NK cells, different stromal cells are compared to promote NK cells and total cell number is counted at multiple time points (day 0, day 7, day 14, day21 and 28). **A**. iCell-NK; **B.** UiPS-NK; **C.** EB-NK; **D.** UCB-NK.

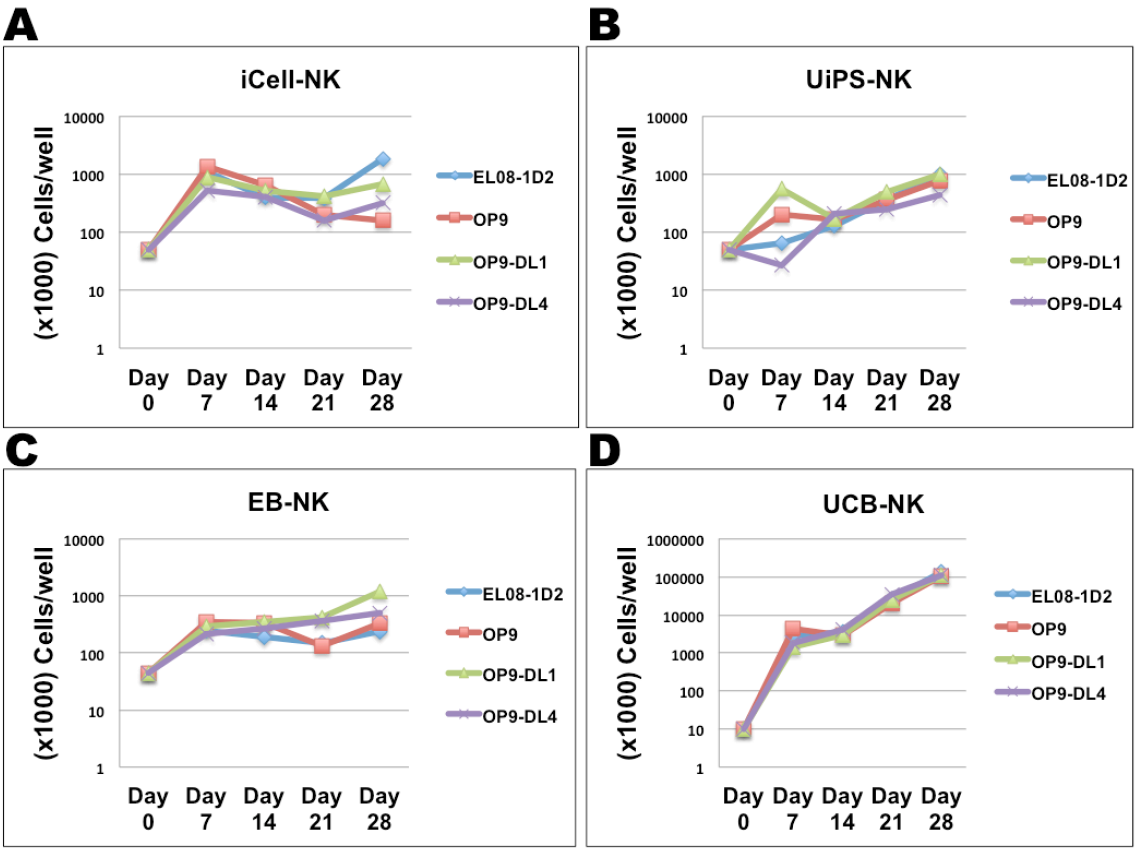


Figure 7: The cytotoxicity of hiPSCs derived NK cells in four weeks. There are two conditions: EL08-1D2 condition and OP9-DL1 condition. **A.** The ^{51}Chromium release assay against K562 cells using multiple NK cells derived from EL08-1D2 condition and OP9-DL1 condition. **B.** The comparison of derived NK cells' ^{51}Chromium release assay between EL08-1D2 condition and OP9-DL1 condition.

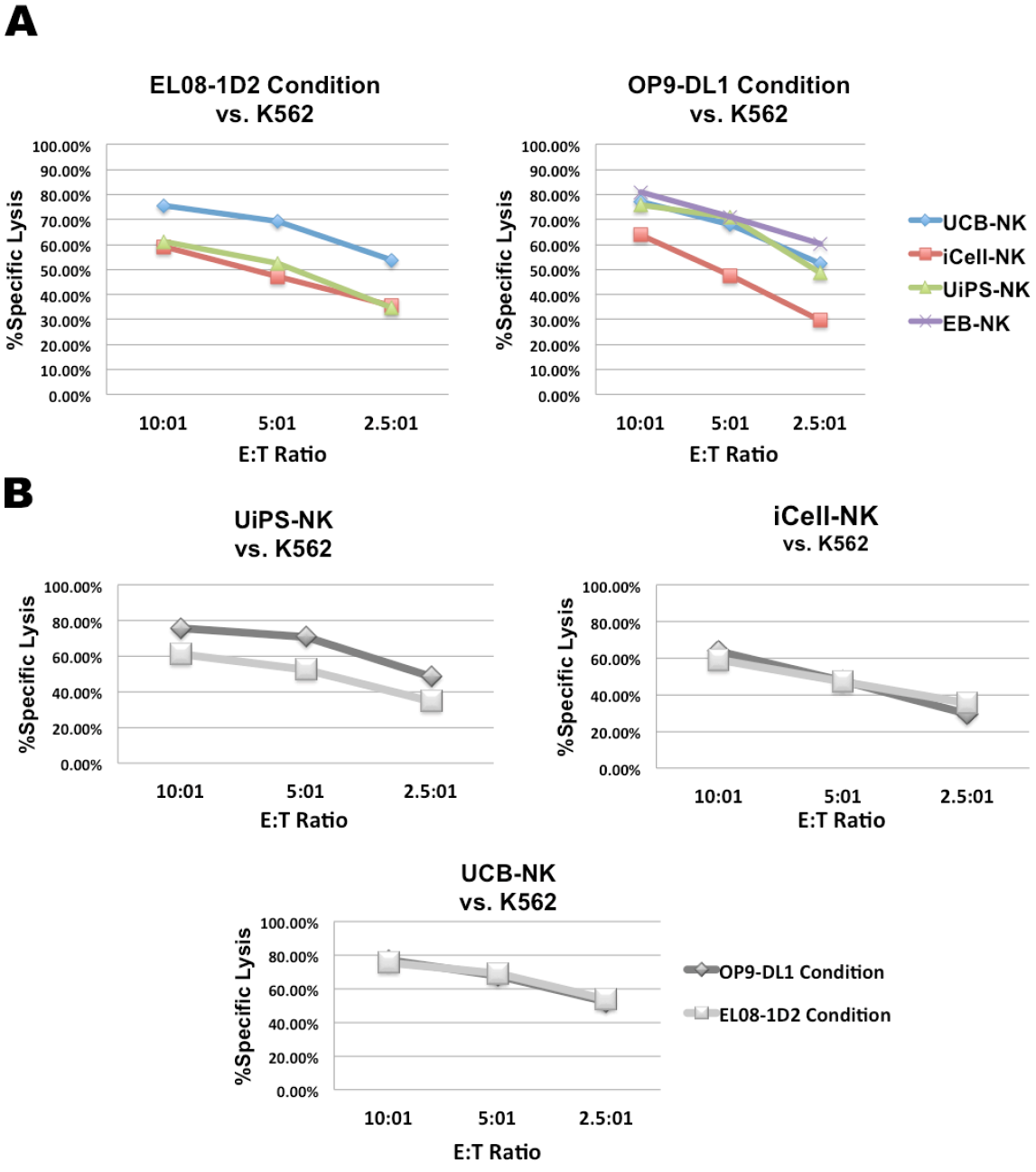
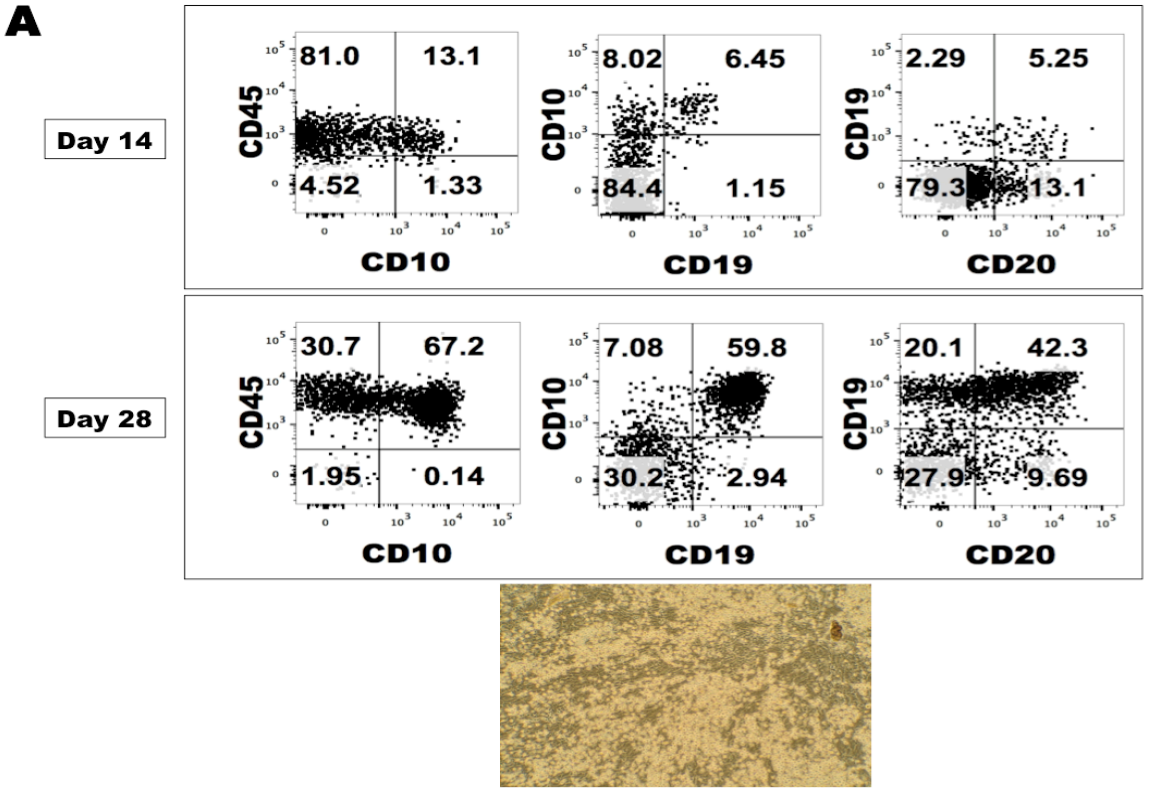


Figure 8: Derivation of B cells from hiPSCs. A. UCB cells were co-cultured in B cell condition for 28 days. Flow cytometric analysis for B cell marker is shown at Day 14 and Day 28. The morphology of the cells was shown at Day 14. **B**. iCell-HPCs and UiPS-HPCs were co-cultured in B cell condition for two weeks.



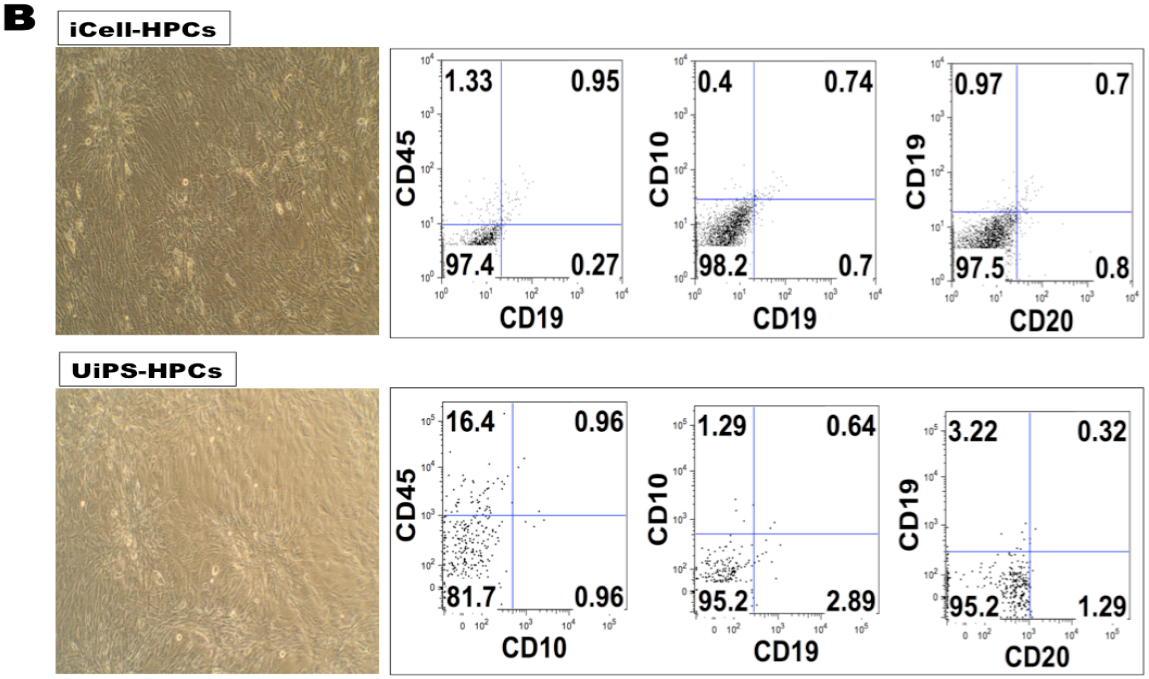


Figure 9: Derivation of T cells from hiPSCs. A. UCB cells were co-cultured in T cell condition for 59 days. Flow cytometric analysis for T cell marker is shown at Day 28 and Day 59. The morphology of the cells was shown at Day 28. **B.** iCell-HPCs and UiPS-HPCs were co-cultured in T cell condition for two weeks.

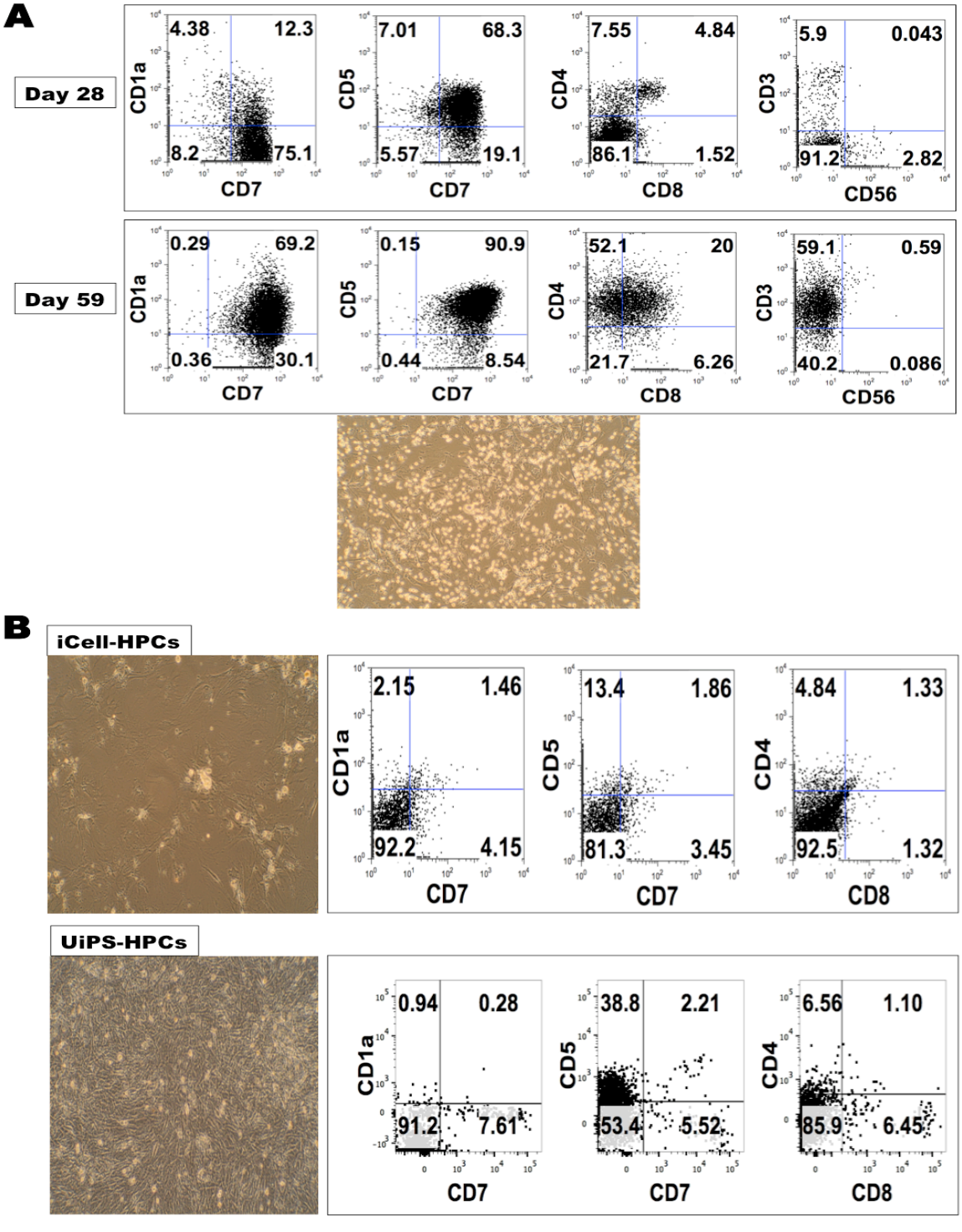


Figure 10: Human ESC clones (IL-2RG WT/KO) can generate HPCs. A. CD34 and CD45 expression of HPCs. IL-2RG knock out hESCs (H1 line) clone 1 derived HPCs (KO-C1 HPCs), IL-2RG knock out hESCs (H1 line) clone 7 derived HPCs (KO-C1 HPCs) and wild type hESCs (H1 line) clone 9 derived HPCs (WT-C9 HPCs) were shipped to us as day 13 EBs. Data are represented as mean +/- SEM. **B.** CD34, CD43 and CD45 expression of UiPS-HPCs and UCB. UiPS-HPCs: human iPSCs (UCBiPS7) derived HPCs separated as non-adherent cells from Day 12 Spin-EB protocol; UCB: human umbilical cord blood sorted CD34+ cells.

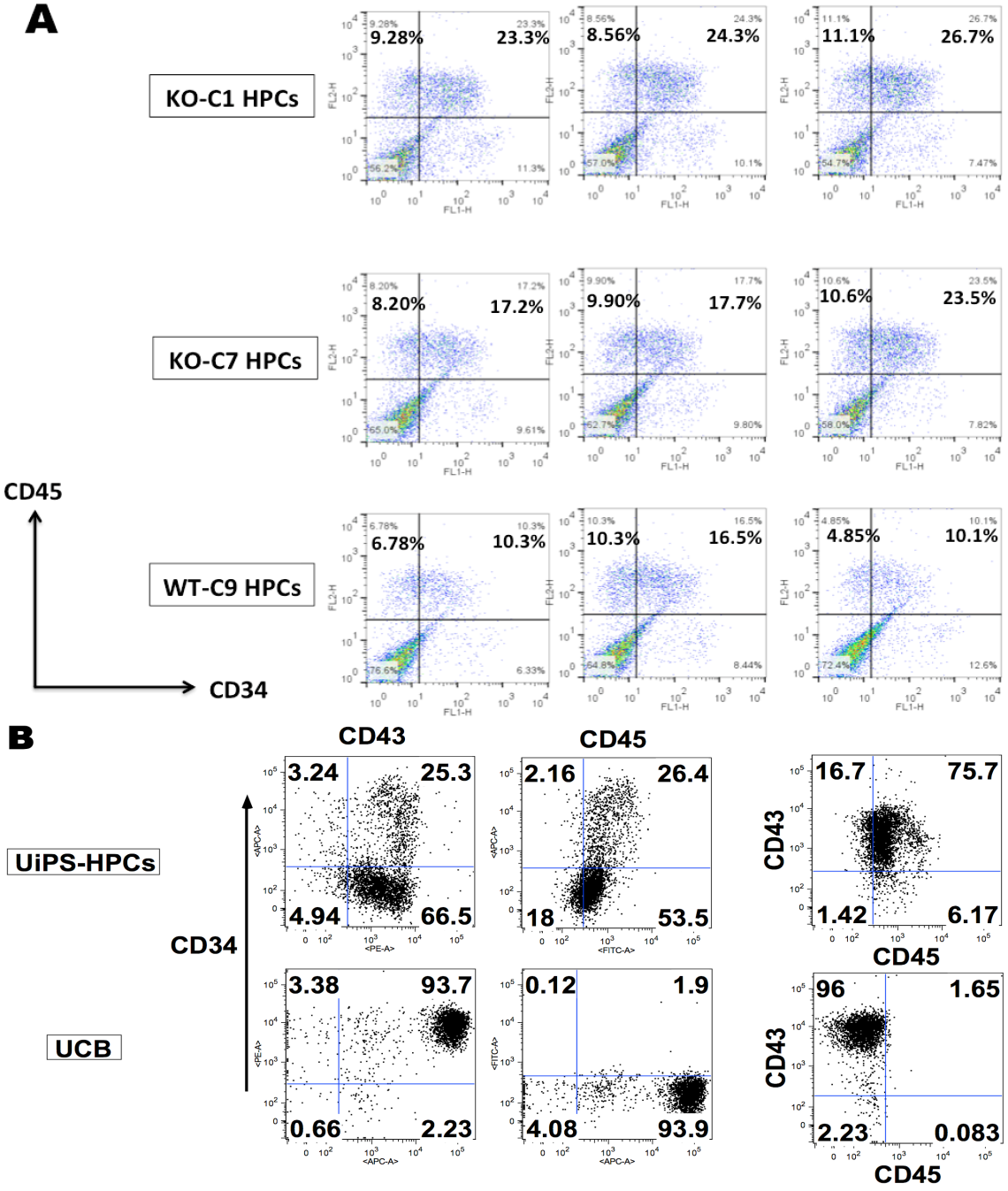
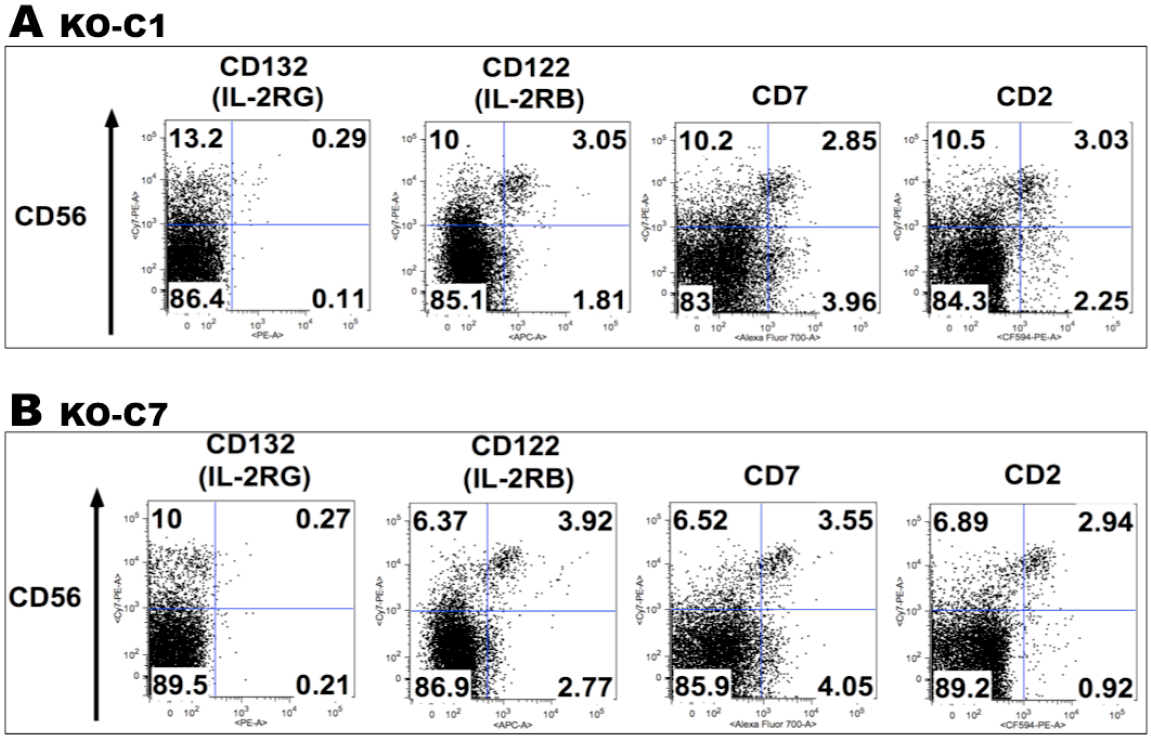
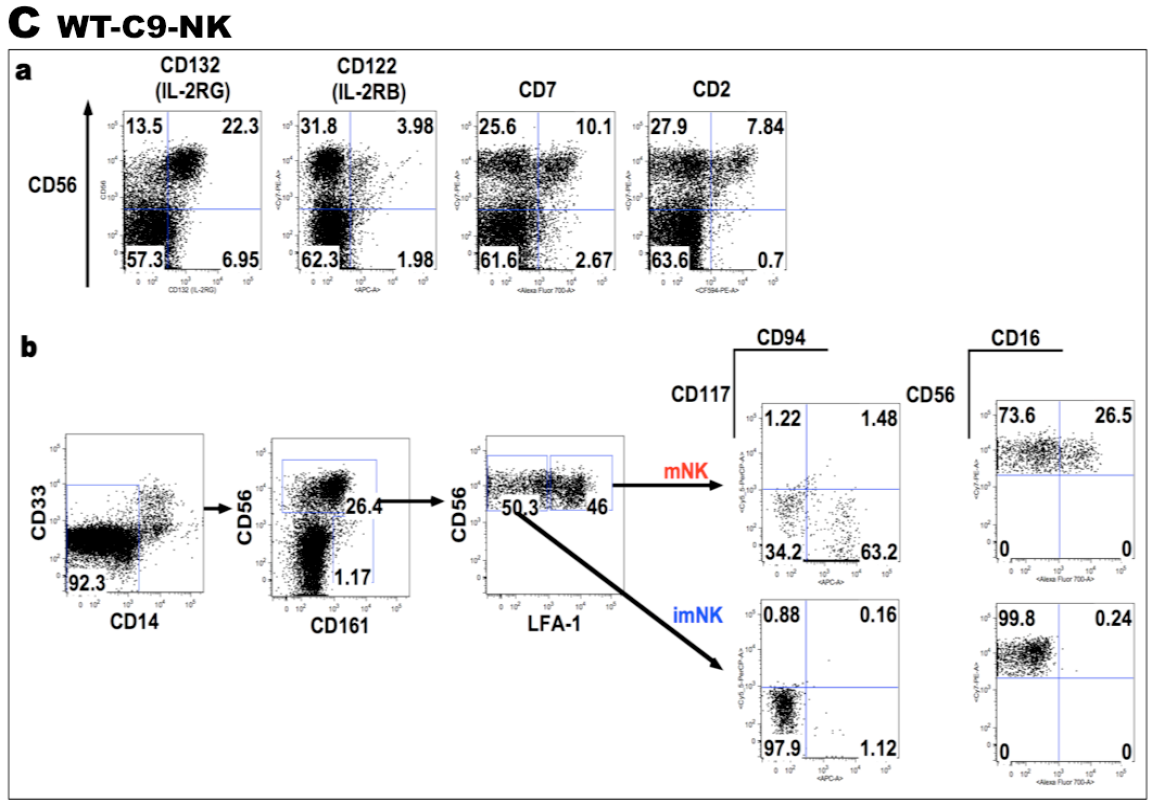
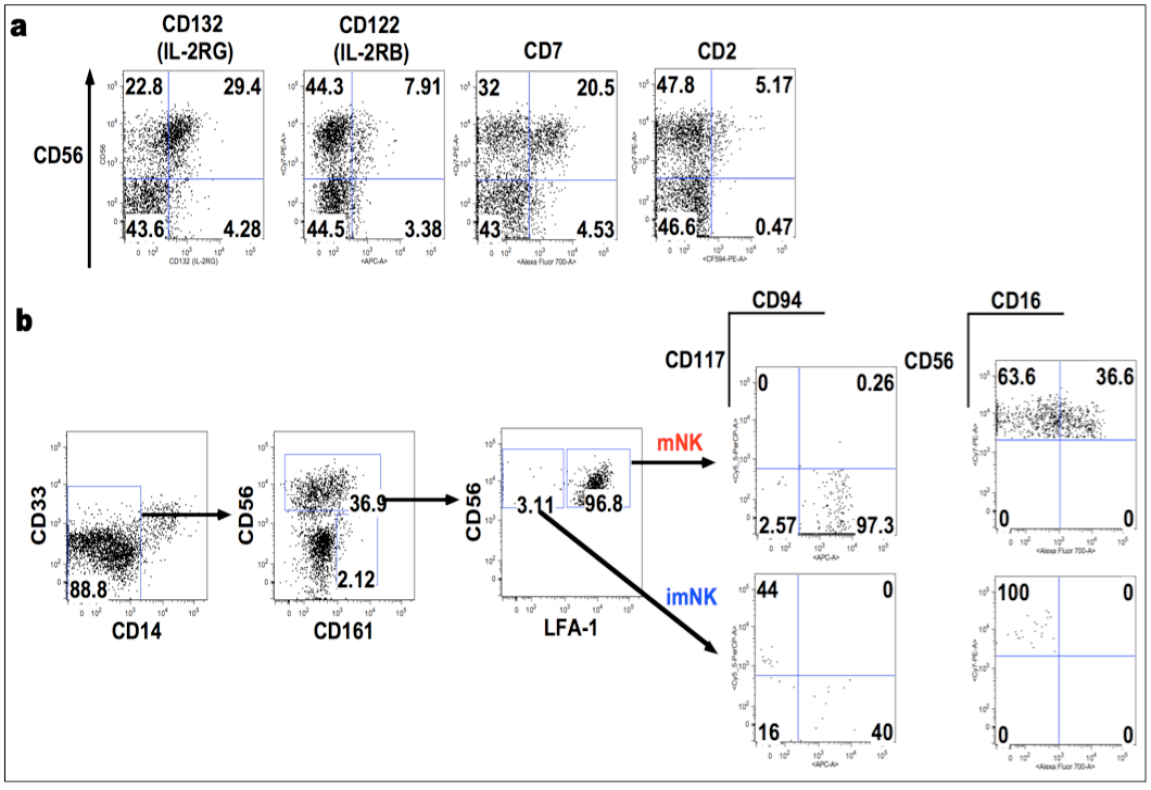


Figure 11: Derivation of NK cells from hESCs/hiPSCs in two weeks. NK cell derivation is induced with co-culture of EL08-1D2 stromal cells in NK differentiation media. The expression of CD56 and CD132 was used to define the NK cells expressing IL-2RG. Mature NK (mNK) cells are defined as CD14-/CD56+/CD161+/LFA-1+/CD117-/CD94+/CD16+. Immature NK (iNK) cells are defined as CD14-/CD56+/CD161+/LFA-1-/CD117+/-/CD94-/CD16-. The cells were analyzed by flow cytometry. **A.** KO-C1: KO-C1 HPCs derived cells; **B.** KO-C7: KO-C7 HPCs derived cells; **C.** WT-C9-NK: WT-C9 HPCs derived NK cells; **D.** UiPS-NK: UiPS-HPCs derived NK cells; **E.** UCB-NK: UCB derived NK cells.




C WT-C9-NK
a
CD132 (IL-2RG)
CD122 (IL-2RB)
CD7
CD2
CD56
13.5
22.3
57.3
6.95
31.8
3.98
62.3
1.98
25.6
10.1
61.6
2.67
27.9
7.84
63.6
0.7
b
CD33
92.3
CD14
CD56
26.4
1.17
CD161
CD56
50.3
46
LFA-1
mNK
imNK
CD117
CD94
1.22
1.48
34.2
63.2
0.88
0.16
97.9
1.12
CD56
CD16
73.6
26.5
0
0
99.8
0.24
0
0


D UiPS-NK
a
CD132 (IL-2RG)
CD122 (IL-2RB)
CD7
CD2
CD56
22.8
29.4
43.6
4.28
44.3
7.91
44.5
3.38
32
20.5
43
4.53
47.8
5.17
46.6
0.47
b
CD33
88.8
CD14
CD56
36.9
2.12
CD161
CD56
3.11
96.8
LFA-1
mNK
imNK
CD117
CD94
0
0.26
2.57
97.3
44
0
16
40
CD56
CD16
63.6
36.6
0
0
100
0
0
0

E UCB-NK

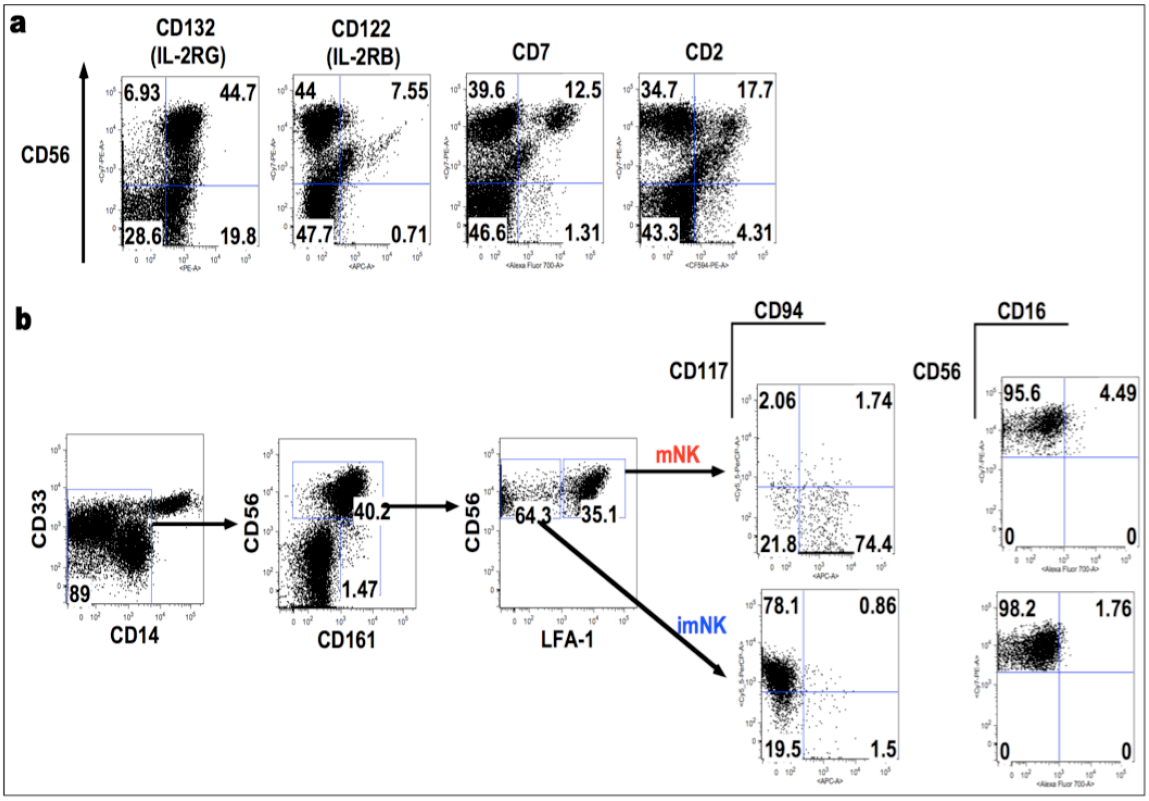
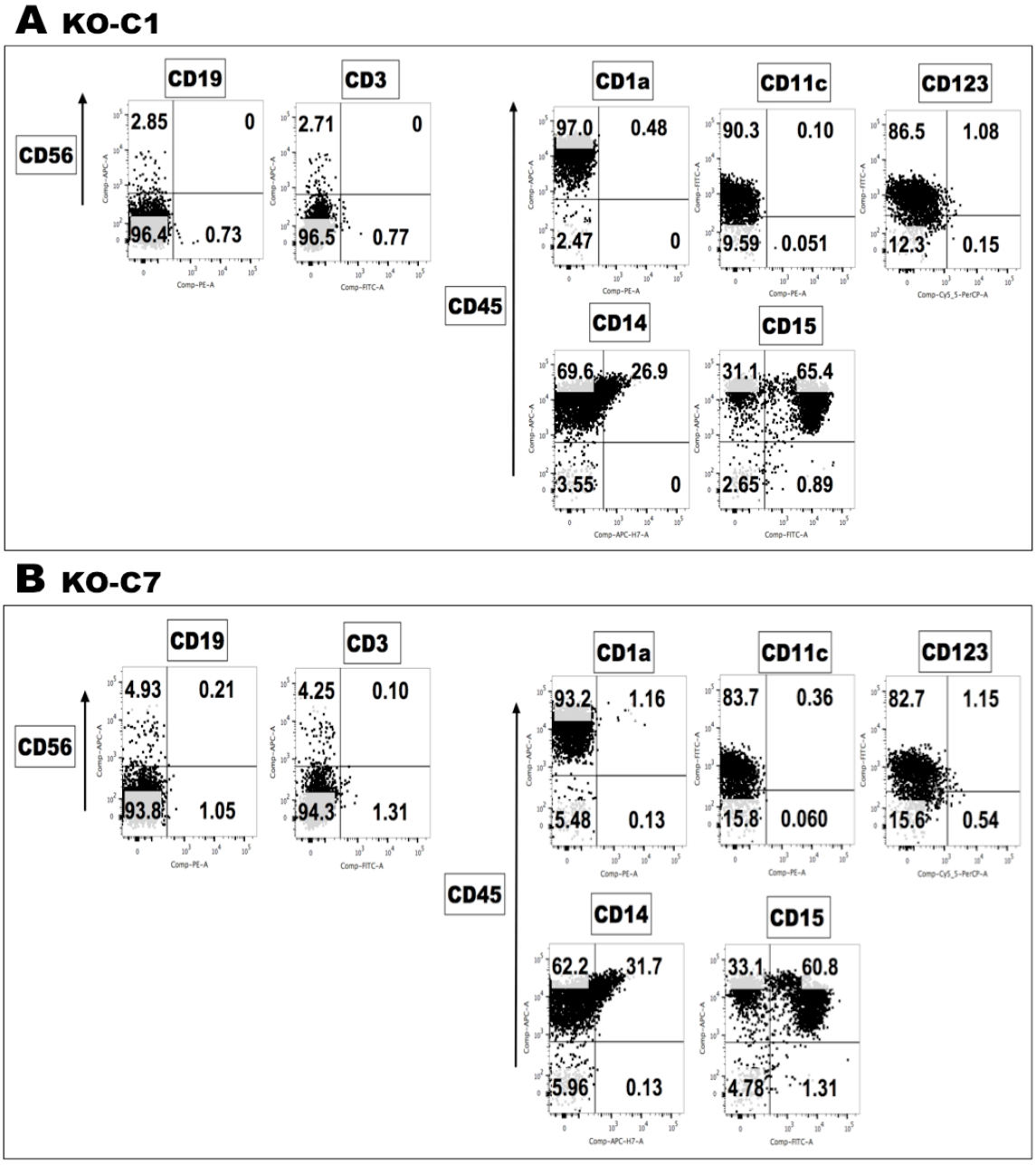
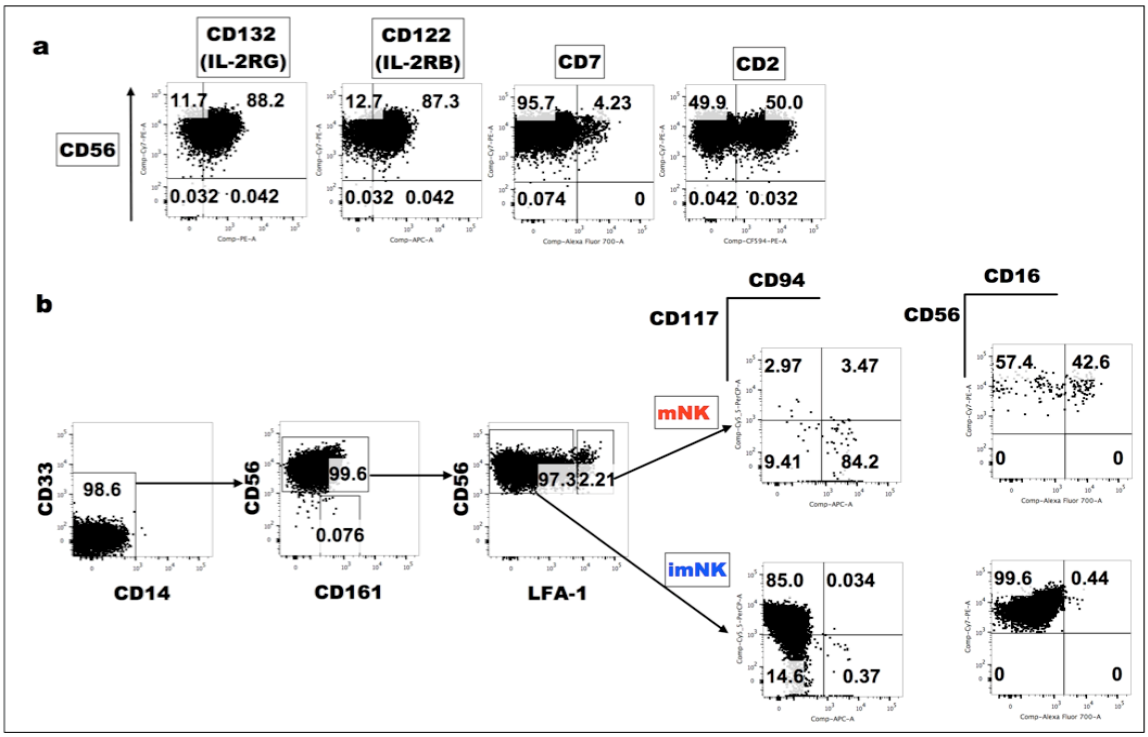
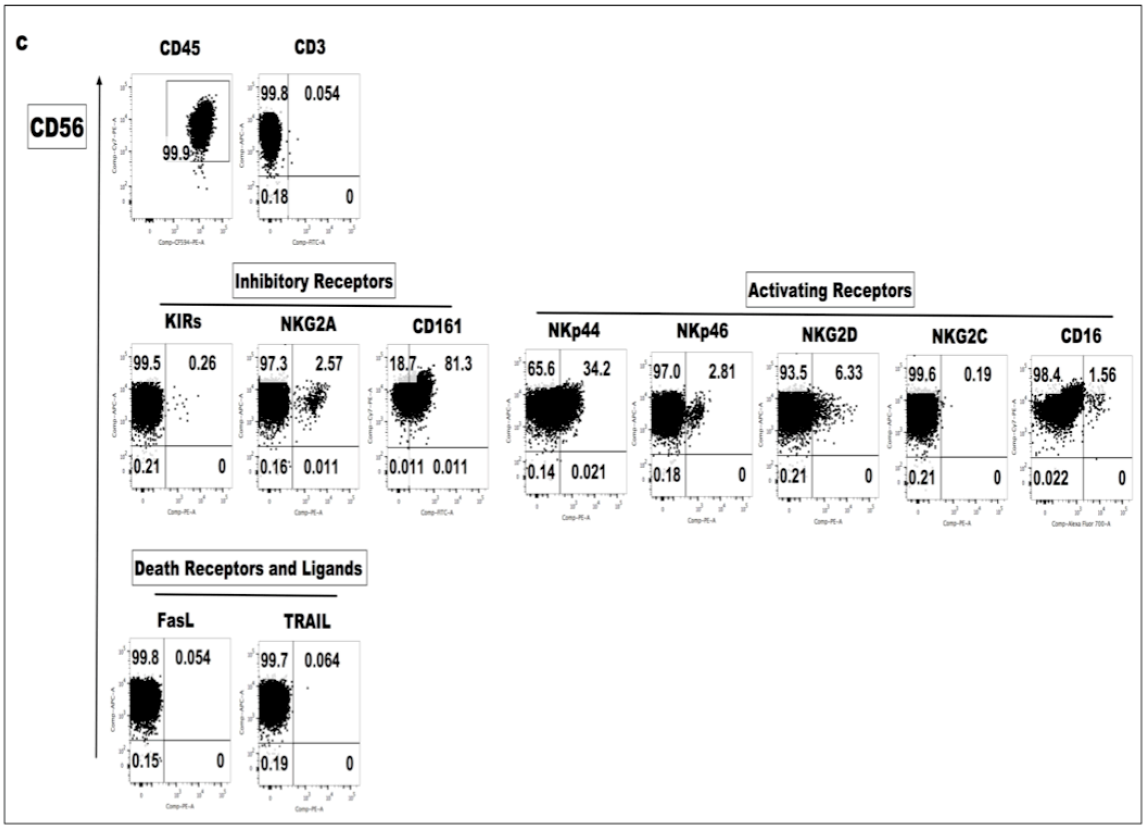

a
CD132 (IL-2RG)
CD122 (IL-2RB)
CD7
CD2
CD56
6.93
44.7
28.6
19.8
44
7.55
47.7
0.71
39.6
12.5
46.6
1.31
34.7
17.7
43.3
4.31
b
CD33
89
CD14
CD56
40.2
1.47
CD161
CD56
64.3
35.1
LFA-1
mNK
imNK
CD94
CD117
2.06
1.74
21.8
74.4
78.1
0.86
19.5
1.5
CD16
CD56
95.6
4.49
0
0
98.2
1.76
0
0

Figure 12: Derivation of NK cells from hESCs/hiPSCs in four weeks. Derivation of NK cells is continued with co-culture of EL08-1D2 stromal cells in NK differentiation media. The expression of CD56 and CD132 is used to define the NK cells expressing IL-2RG. NK inhibitory receptors are KIRs, NKG2A and CD161, activating receptors are NKp44, NKp46, NKG2D, NKG2C and CD16, death ligands are FasL (CD95L) and TRAIL. The cells were analyzed by flow cytometry. **A.** KO-C1: KO-C1 HPCs derived cells; **B.** KO-C7: KO-C7 HPCs derived cells; **C.** WT-C9-NK: WT-C9 HPCs derived NK cells; **D.** UiPS-NK: UiPS-HPCs derived NK cells; **E.** UCB-NK: UCB derived NK cells.

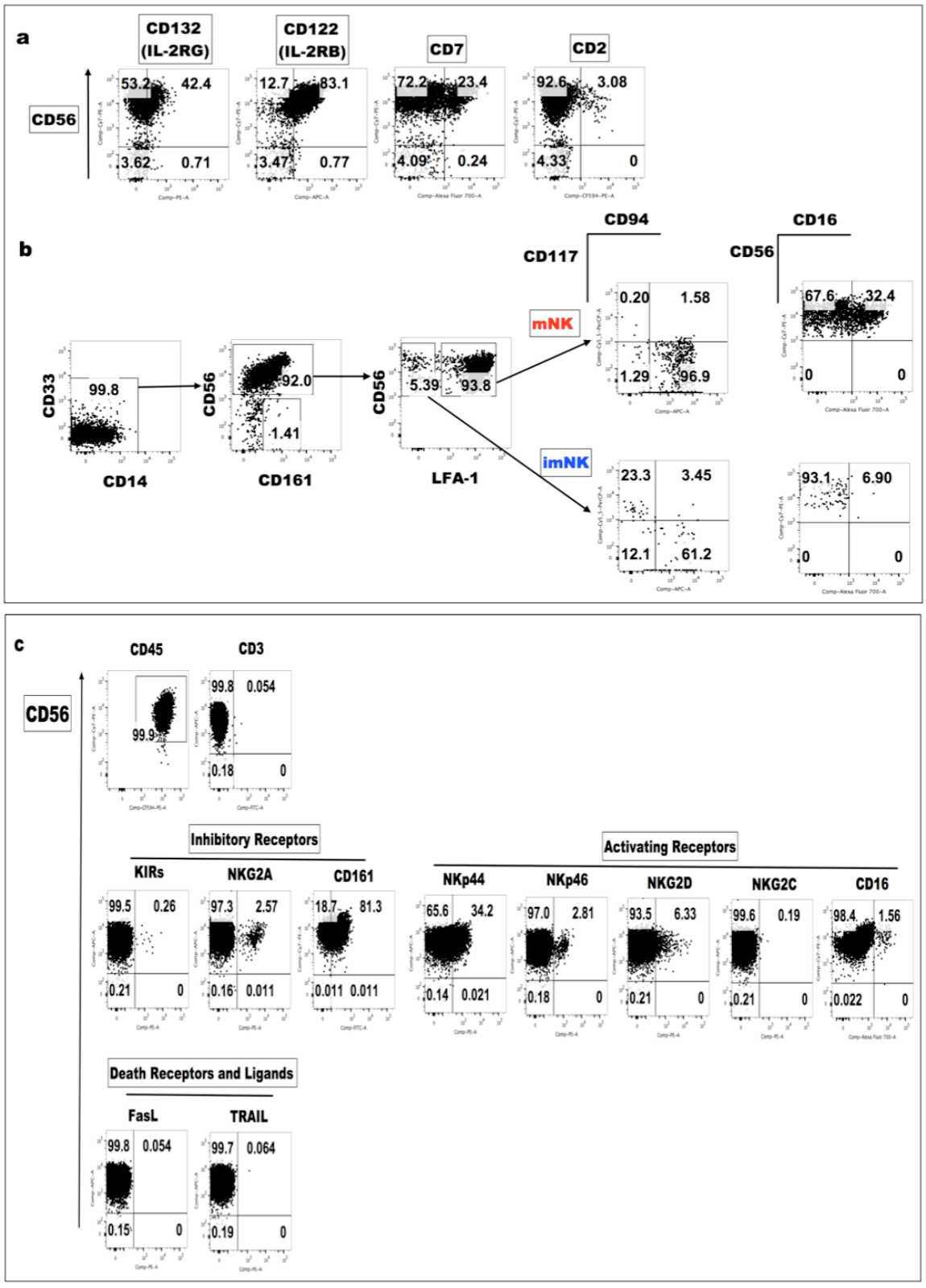


C WT-C9-NK


a
CD132 (IL-2RG)
CD122 (IL-2RB)
CD7
CD2
CD56
11.7 88.2
0.032 0.042
12.7 87.3
0.032 0.042
95.7 4.23
0.074 0
49.9 50.0
0.042 0.032
b
CD94
CD117
CD16
CD56
mNK
imNK
CD33
98.6
CD14
CD56
99.6
0.076
CD161
CD56
97.3 2.21
LFA-1
2.97 3.47
9.41 84.2
57.4 42.6
0 0
85.0 0.034
14.6 0.37
99.6 0.44
0 0


c
CD45
CD3
CD56
99.9
99.8 0.054
0.18 0
Inhibitory Receptors
KIRs
NKG2A
CD161
99.5 0.26
0.21 0
97.3 2.57
0.16 0.011
18.7 81.3
0.011 0.011
Activating Receptors
NKp44
NKp46
NKG2D
NKG2C
CD16
65.6 34.2
0.14 0.021
97.0 2.81
0.18 0
93.5 6.33
0.21 0
99.6 0.19
0.21 0
98.4 1.56
0.022 0
Death Receptors and Ligands
FasL
TRAIL
99.8 0.054
0.15 0
99.7 0.064
0.19 0

D UiPS-NK


a
CD132 (IL-2RG)
CD122 (IL-2RB)
CD7
CD2
CD56
53.2
42.4
3.62
0.71
12.7
83.1
3.47
0.77
72.2
23.4
4.09
0.24
92.6
3.08
4.33
0
b
CD94
CD117
CD16
CD56
mNK
imNK
CD33
99.8
CD14
CD56
92.0
1.41
CD161
CD56
5.39
93.8
LFA-1
0.20
1.58
1.29
96.9
67.6
32.4
0
0
23.3
3.45
12.1
61.2
93.1
6.90
0
0
c
CD45
CD3
CD56
99.9
99.8
0.054
0.18
0
Inhibitory Receptors
KIRs
NKG2A
CD161
99.5
0.26
0.21
0
97.3
2.57
0.16
0.011
18.7
81.3
0.011
0.011
Activating Receptors
NKp44
NKp46
NKG2D
NKG2C
CD16
65.6
34.2
0.14
0.021
97.0
2.81
0.18
0
93.5
6.33
0.21
0
99.6
0.19
0.21
0
98.4
1.56
0.022
0
Death Receptors and Ligands
FasL
TRAIL
99.8
0.054
0.15
0
99.7
0.064
0.19
0

E UCB-NK

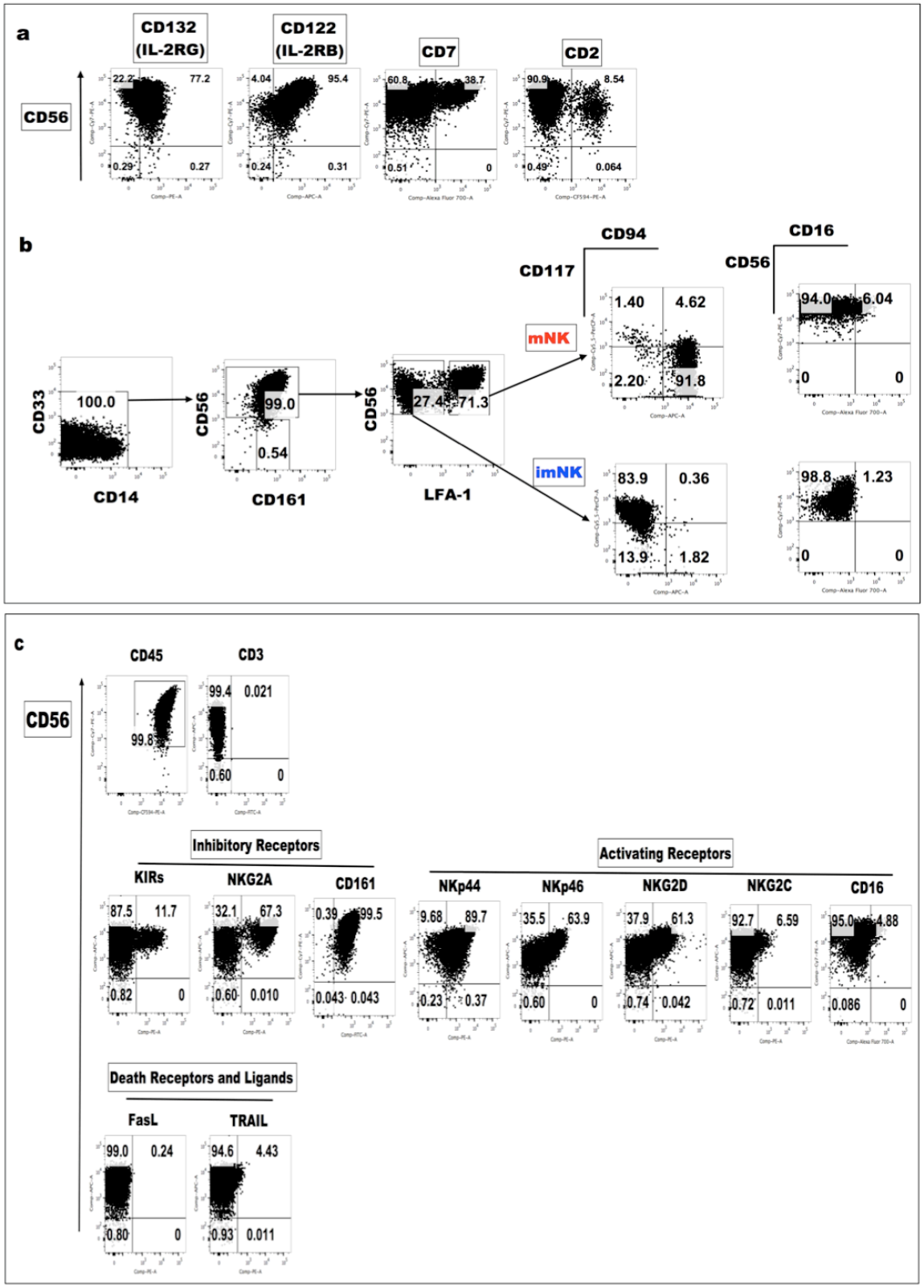

a
CD132 (IL-2RG)
CD122 (IL-2RB)
CD7
CD2
CD56
22.2
77.2
0.29
0.27
4.04
95.4
0.24
0.31
60.8
38.7
0.51
0
90.9
8.54
0.49
0.064
b
CD94
CD117
CD16
CD56
mNK
imNK
CD33
CD14
CD161
LFA-1
100.0
99.0
0.54
27.4
71.3
1.40
4.62
2.20
91.8
94.0
6.04
0
0
83.9
0.36
13.9
1.82
98.8
1.23
0
0
c
CD45
CD3
CD56
99.8
99.4
0.021
0.60
0
Inhibitory Receptors
KIRs
NKG2A
CD161
87.5
11.7
0.82
0
32.1
67.3
0.60
0.010
0.39
99.5
0.043
0.043
Activating Receptors
NKp44
NKp46
NKG2D
NKG2C
CD16
9.68
89.7
0.23
0.37
35.5
63.9
0.60
0
37.9
61.3
0.74
0.042
92.7
6.59
0.72
0.011
95.0
4.88
0.086
0
Death Receptors and Ligands
FasL
TRAIL
99.0
0.24
0.80
0
94.6
4.43
0.93
0.011

Figure 13: Expansion potential of hESCs derived NK cells. Total cells per well are counted every week to determine the cell number proliferation potential for each group: Clone 9-WT; Clone 1-KO; Clone 7-KO and UCB. The proliferation potential of CD14-CD56+ NK cells and CD56+/CD132+ NK cells is analyzed based on flow cytometry data. **A.** Total cell number proliferation curve. **B.** Percentage of CD14-CD56+ and CD56+CD132+ NK cell proliferation curve.

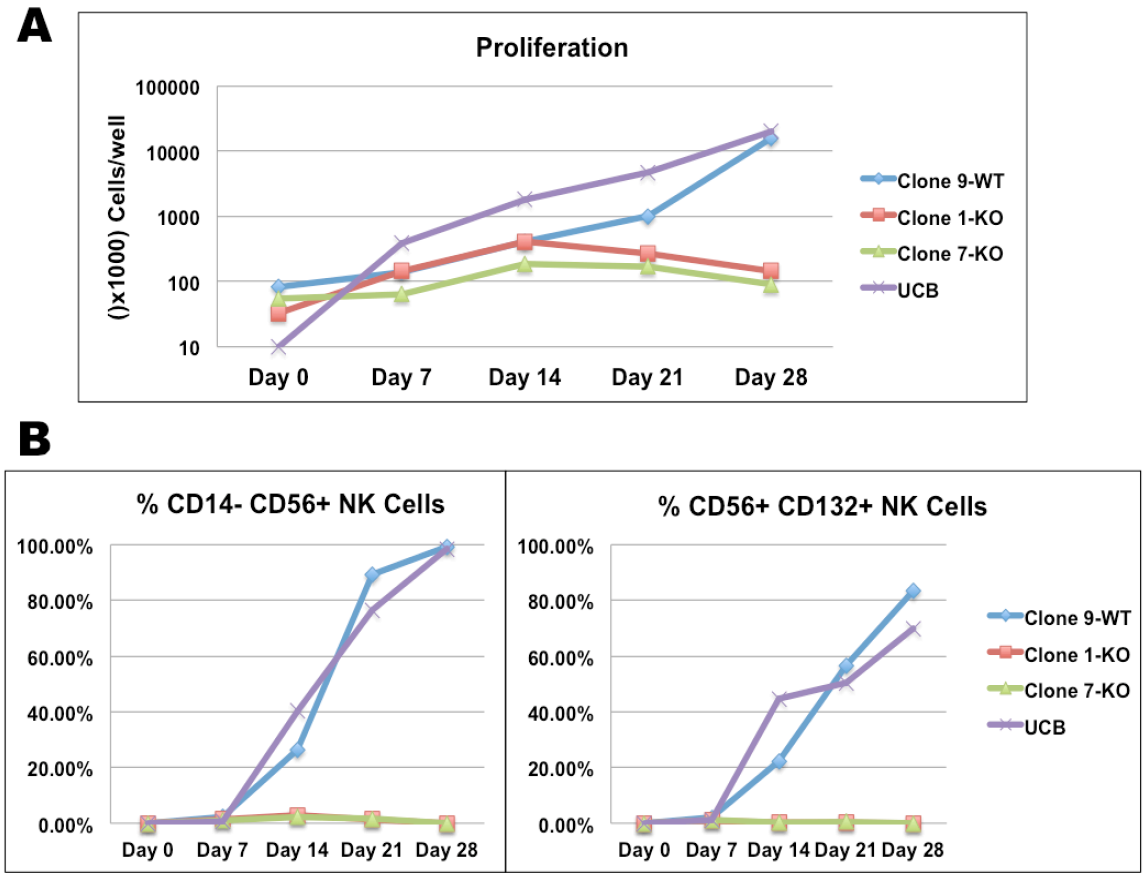
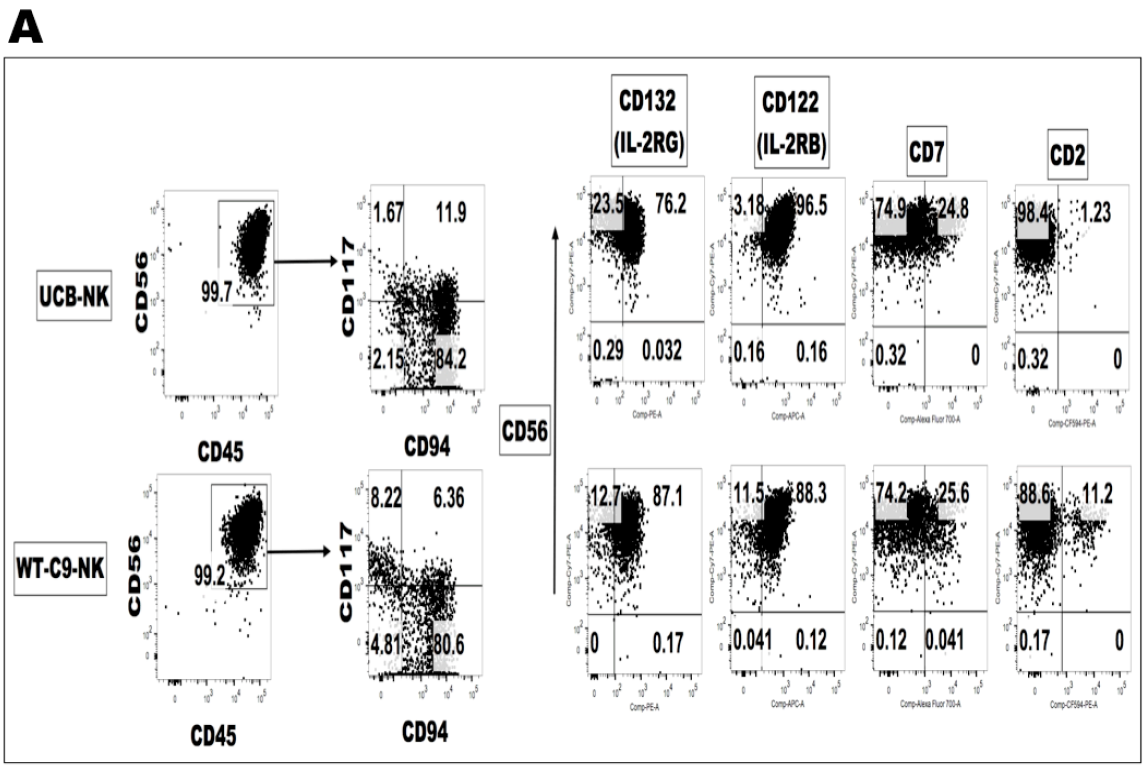
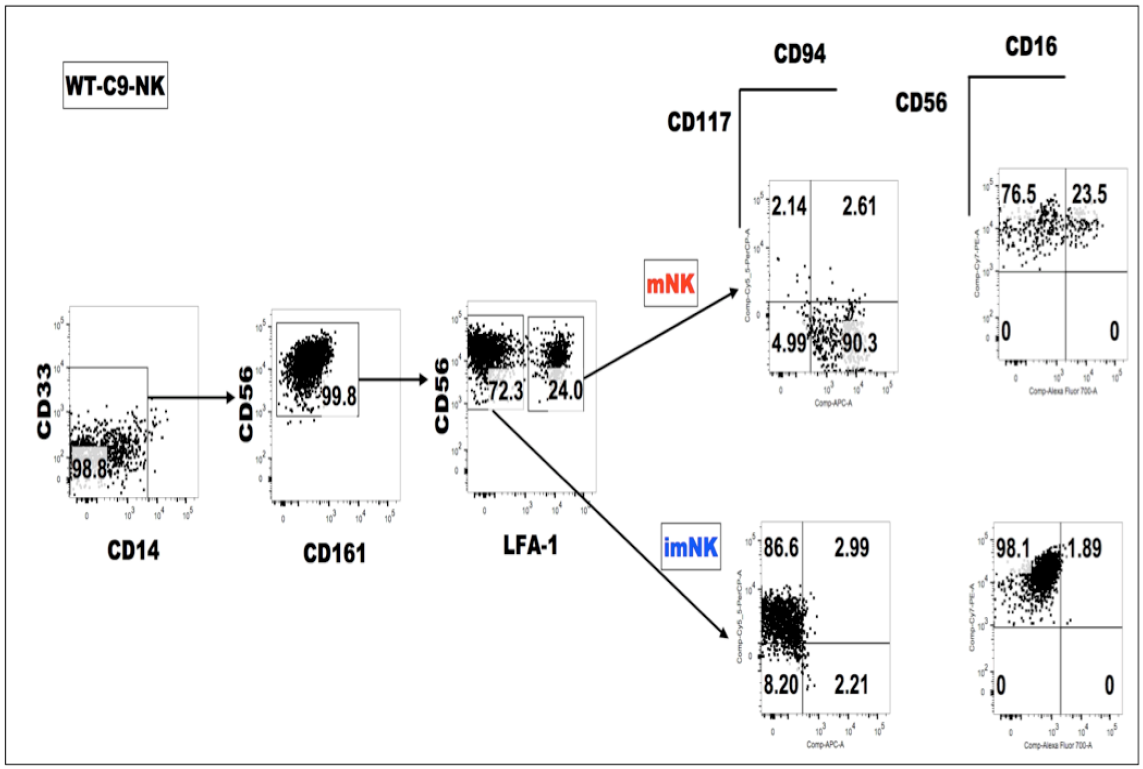
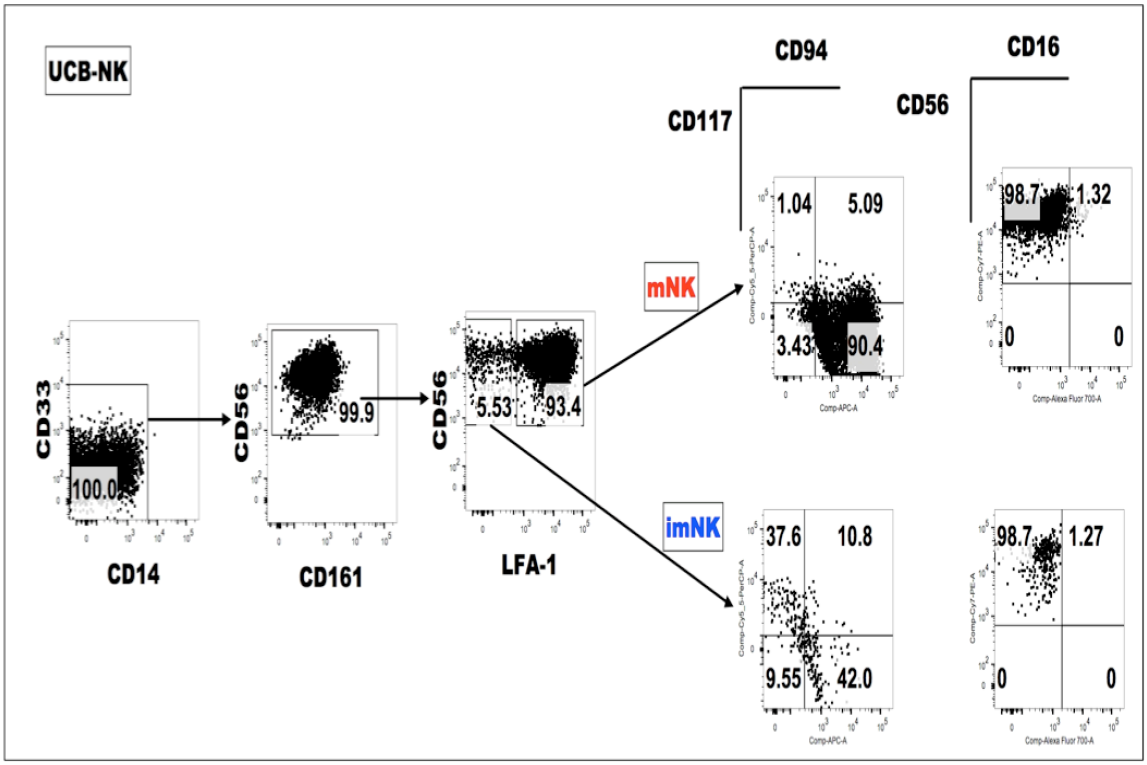


Figure 14: Stimulation of hESCs derived NK cells with aAPCs for two weeks. At the first week, irradiated aAPCs are added to NK cells (WT-C9-NK and UCB-NK) at a ratio of 2:1. At the second week, total cells are replaced with fresh aAPCs at a ratio of 1:1. After two weeks, NK cells are analyzed by flow cytometry. **A.** CD132, CD122, CD7 and CD2 expression of NK cells (CD56+); **B.** NK development stages are characterized: mature NK (mNK): CD14-/CD56+/LFA-1+/CD117-/CD94+/CD16+; immature NK (imNK): CD14-/CD56+/LFA-1-/CD117+/CD94-/CD16-; **C.** NK inhibitory receptors (KIRs, NKG2A, CD161), activating receptors (NKp44, NKp46, NKG2D, NKG2C, CD16) and death ligands (FasL, TRAIL).



B


WT-C9-NK
CD33
98.8
CD14
CD56
99.8
CD161
CD56
72.3
24.0
LFA-1
mNK
imNK
CD94
CD117
2.14
2.61
4.99
90.3
86.6
2.99
8.20
2.21
CD16
CD56
76.5
23.5
0
0
98.1
1.89
0
0


UCB-NK
CD33
100.0
CD14
CD56
99.9
CD161
CD56
5.53
93.4
LFA-1
mNK
imNK
CD94
CD117
1.04
5.09
3.43
90.4
37.6
10.8
9.55
42.0
CD16
CD56
98.7
1.32
0
0
98.7
1.27
0
0

C

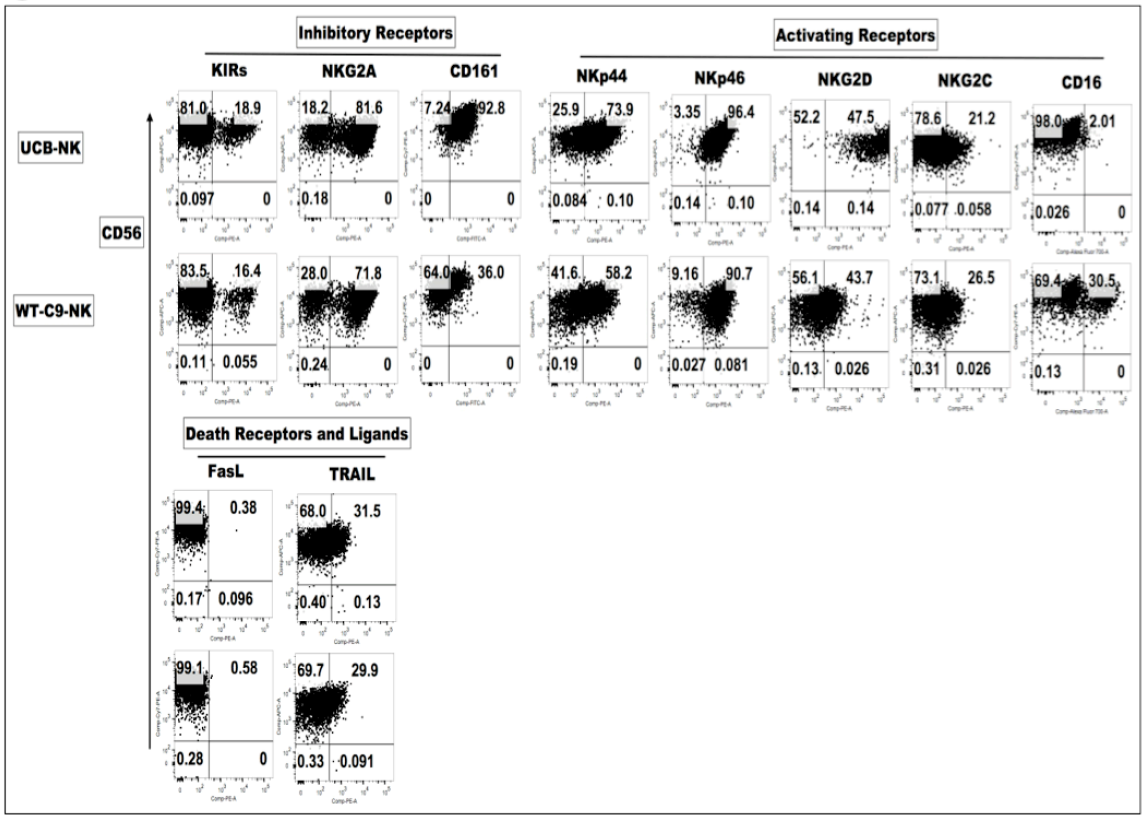

Inhibitory Receptors
Activating Receptors
KIRs
NKG2A
CD161
NKp44
NKp46
NKG2D
NKG2C
CD16
UCB-NK
WT-C9-NK
CD56
81.0 18.9 0.097 0
18.2 81.6 0.18 0
7.24 92.8 0 0
25.9 73.9 0.084 0.10
3.35 96.4 0.14 0.10
52.2 47.5 0.14 0.14
78.6 21.2 0.077 0.058
98.0 2.01 0.026 0
83.5 16.4 0.11 0.055
28.0 71.8 0.24 0
64.0 36.0 0 0
41.6 58.2 0.19 0
9.16 90.7 0.027 0.081
56.1 43.7 0.13 0.026
73.1 26.5 0.31 0.026
69.4 30.5 0.13 0
Death Receptors and Ligands
FasL
TRAIL
99.4 0.38 0.17 0.096
68.0 31.5 0.40 0.13
99.1 0.58 0.28 0
69.7 29.9 0.33 0.091

Figure 15: Cytotoxicity assay of hESCs derived NK cells before and after aAPC stimulation. The ^{51}Chromium release assay is used to test the cytotoxicity of the WT-C9-NK cells before and after stimulated with aAPCs. The targets are K562 cells. Before stimulation, the effectors are WT-C9-NK and UCB-NK cells at four weeks from the co-culture on EL08-1D2 stromal cells. After stimulation with aAPCs for two weeks, the activated WT-C9-NK and UCB-NK cells serve as the effectors after stimulation. Peripheral blood NK cells (PB-NK) are initially sorted from human peripheral blood NK cells but are routinely maintained with aAPCs in vitro. The E:T ratio is 10:01, 5:01 and 2.5:01.

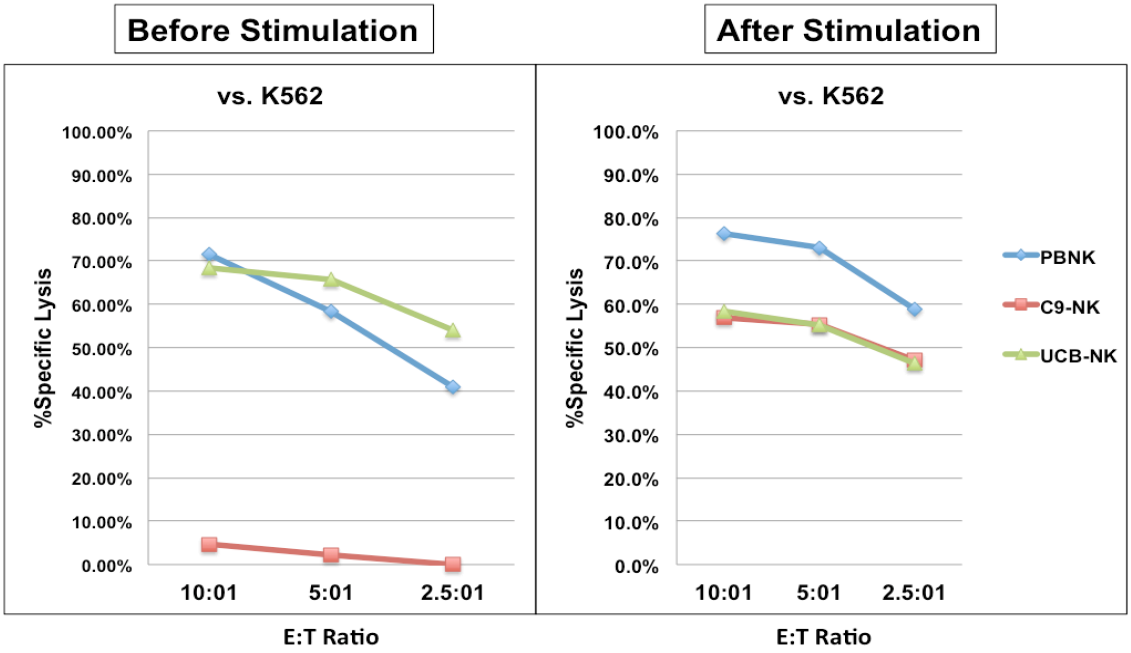


Figure 16: Human iPSCs/ESCs can be differentiated into HPCs. The expression of CD34, CD43 and CD45 is analyzed by flow cytometry. cWAS-HPCs: WAS gene corrected iPSCs derived HPCs; WAS-HPCs: WAS patient iPSCs derived HPCs; H9-HPCs: hESCs (H9 cell line) derived HPCs; UCB: human umbilical cord blood sorted CD34+ cells. cWAS-HPCs, WAS-HPCs and H9-HPCs were sorted on day 14 of hematopoietic differentiation and shipped to us overnight.

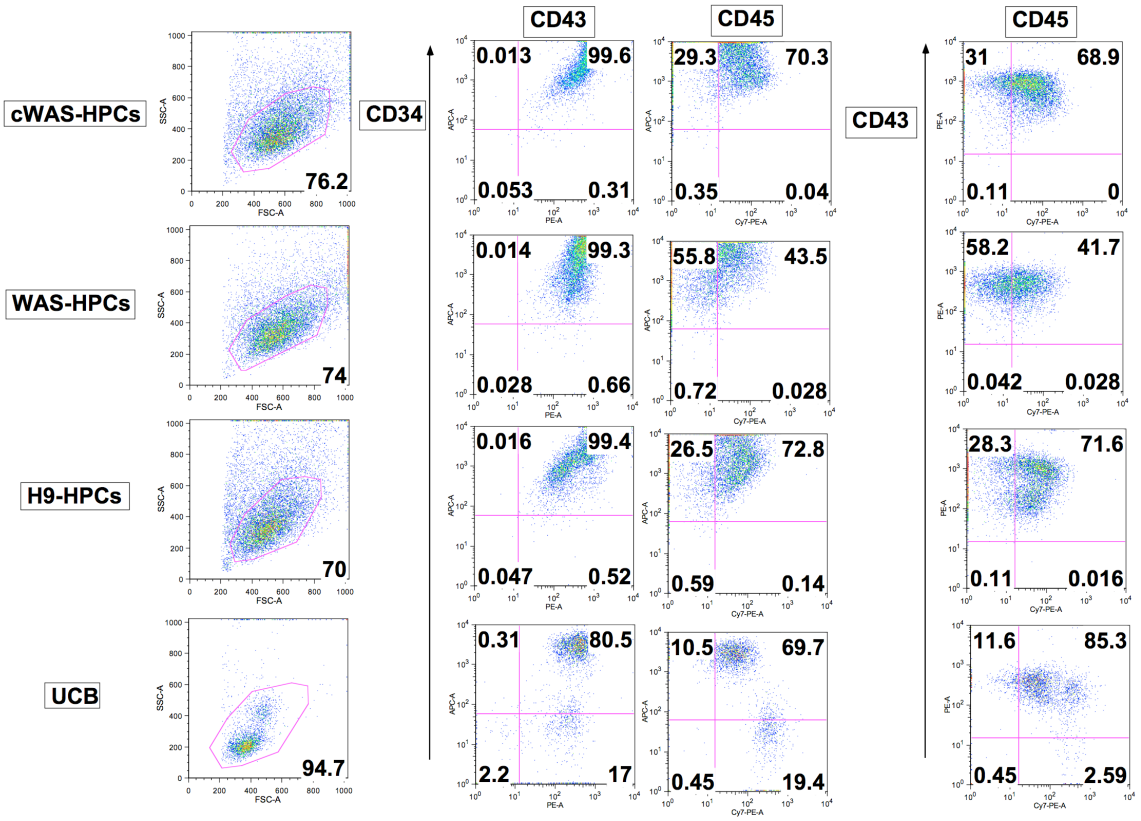
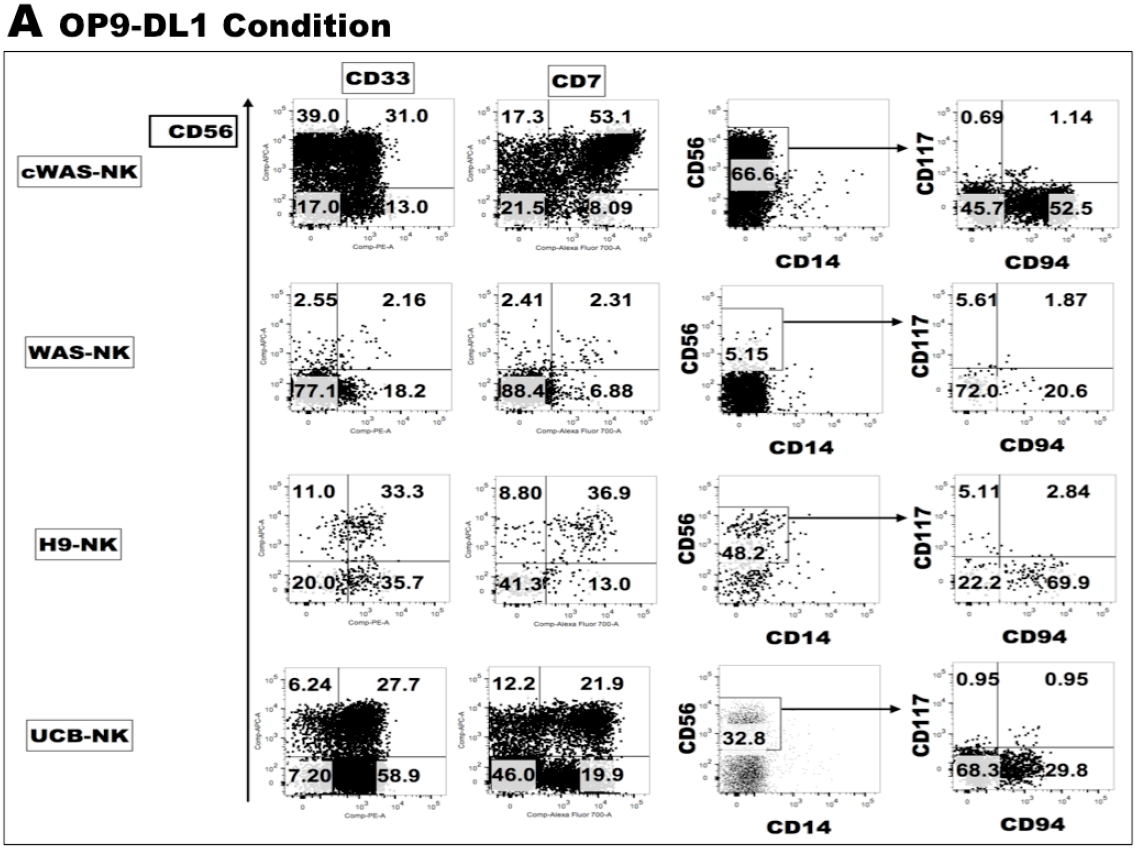


Figure 17: Derivation of NK cells from hiPSCs/hESCs on Day 13. Flow cytometric analysis for cell surface antigen expression (CD56, CD33, CD7, CD14, CD117 and CD94) in two stromal cell co-culture conditions: **A.** OP9-DL1 Condition **B.** OP9-DL4 Condition. cWAS-NK: cWAS-HPCs derived NK cells; WAS-NK: WAS-HPCs derived NK cells; H9-NK: H9-HPCs derived NK cells; UCB-NK: UCB derived NK cells.



B **OP9-DL4 Condition**

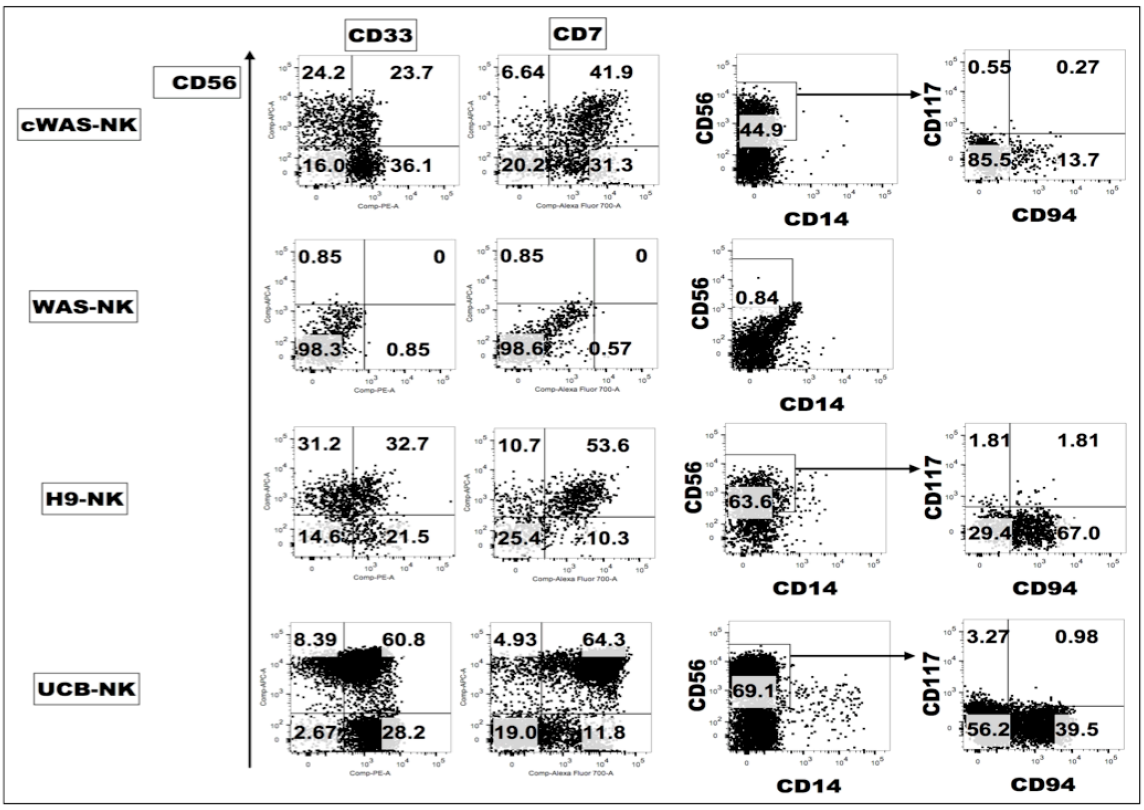
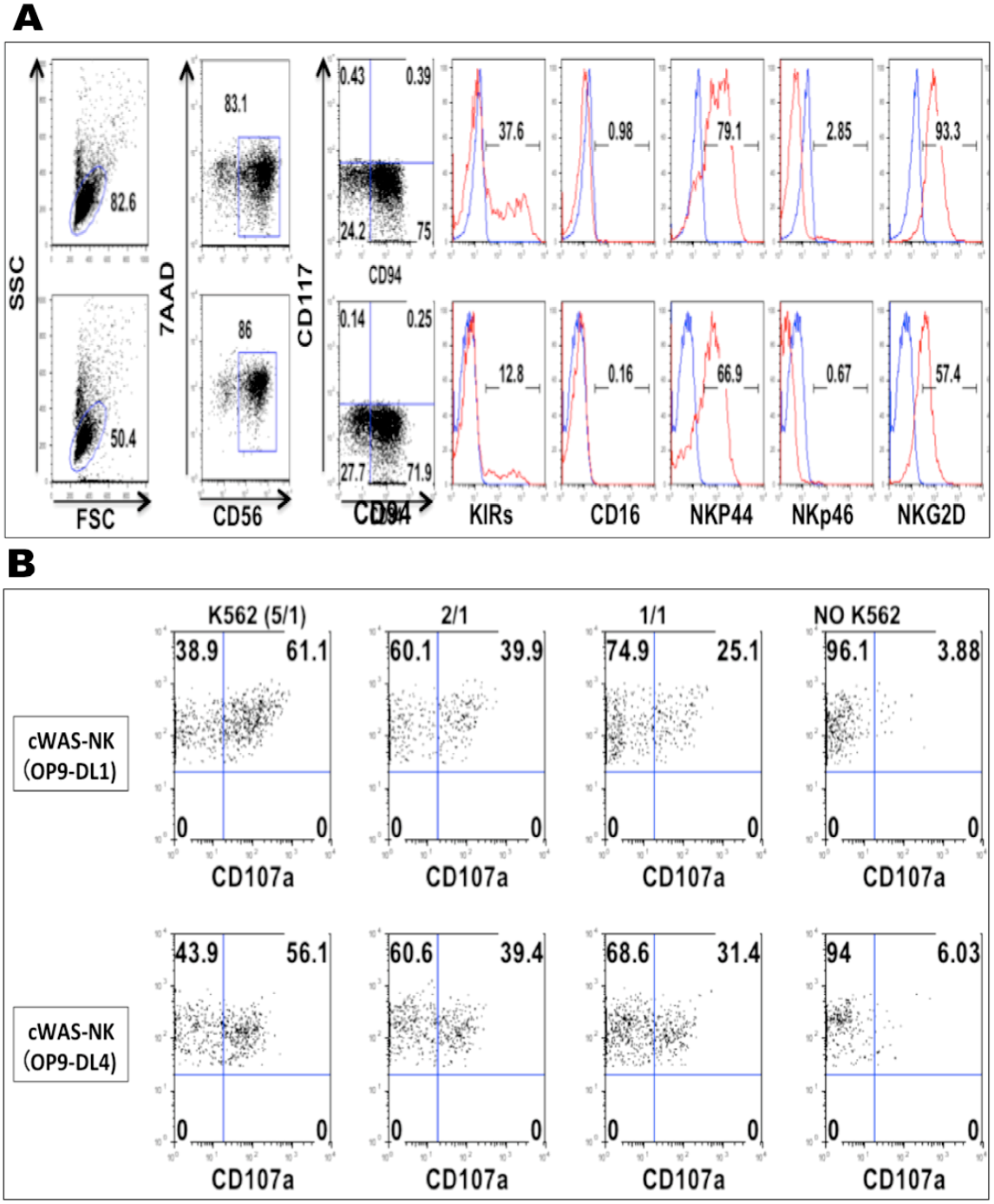


Figure 18: The characterization and function test of mature NK cells derived from WAS gene corrected hiPSCs. A. Flow cytometric analysis for mature cWAS-NK cell surface antigens on Day 34 in both OP9-DL1 condition (upper lane) and OP9-DL4 condition (lower lane). **B.** cWAS-NK cells were tested against K562 targets for CD107a expression. Effector cells were incubated with targets for 5 hours at a ratio of 1:5, 1:2 and 1:1. The results were analyzed by flow cytometry.



Discussion

Human pluripotent stem cells, consisting of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), provide unlimited cellular sources to establish human in vitro developmental models and offer the potential for transplantation therapy, disease modeling and drug screening [96]. Although hESCs provide the gold standard for human pluripotency, hiPSCs are almost identical to hESCs in morphology and function [55, 56]. Compared to hESCs, hiPSCs have more advantages in terms of cell source availability and patient specificity, thus making hiPSCs a more exciting tool for clinical applications in regenerative medicine [96]. Especially it has been proposed that when iPSC technology is combined with gene editing techniques, diseases with somatic mutations will be cured when patients' hiPSCs are gene corrected in vitro first and then transplanted back to the patients with required cell types derived from the gene corrected hiPSCs [58]. Therefore, hiPSCs have advantages over hESCs in terms of eliminating the need for immunosuppression and providing autologous transplantable cells to the specific patients. Defects in lymphocyte development can cause different immunodeficiency diseases, with a combination of iPSC and gene editing technology, if lymphocytes can be readily derived from hiPSCs, then the patients with genetic immunodeficiency diseases can be potentially transplanted with these immune cells for immunotherapy.

Derivation of lymphocytes from human pluripotent stem cells has been a goal since the derivation of hESCs. Human lymphocytes, including B cells, T cells and NK cells, are important immune cell populations in mediating both adaptive and innate immunity and can be applied in immunotherapy, such as killing tumor cells and virally infected cells. It has been a long goal for researchers to generate lymphocytes from hESCs/hiPSCs. Previously, our lab tried to use hESC derived CD34+CD45+ hematopoietic progenitor cells for B cell, T cell and NK cell derivation, and found that they could generate NK cells but not B cells and T cells [17]. This is likely due to ID proteins (ID2, ID3), which were highly expressed in these hematopoietic progenitor cells and could promote NK cell development and block B and T cell development [17, 18]. B cell derivation from

hESCs/hiPSCs has been severely restricted, regardless of some limited progress has been done [74]. T cell derivation from hESCs/hiPSCs has been made possible among some groups, but in terms of consistency and efficiency, these T cell co-cultures have become very difficult to repeat in other labs [79-83]. Thus, the difficulty in the derivation of B cells and T cells from human pluripotent stem cells remains the main hindrance to promote the use of hESCs/hiPSCs derived B and T cell for immunotherapies.

Unlike the limited derivation of B cells and T cells from hESCs/hiPSCs, the generation of NK cells with a mature phenotype that is secreting cytokines and cytotoxic against tumor cells in vitro and in vivo has been achieved in our lab [69-71]. NK cells have become an alluring option for immunotherapy due to their killing ability for tumor and virally infected cells without prior sensitization. The use of NK cells for immunotherapy relies on the availability of a great number of NK cells with optimal cytotoxic function. By combining a novel defined embryoid body formation condition to efficiently derive hematopoietic progenitor cells from hESCs/hiPSCs, as well as using membrane-bound interleukin 21-expressing artificial antigen-presenting cells, the production of enough mature and cytotoxic NK cells to treat a single patient can be derived from fewer than 250,000 hESCs/hiPSCs [71]. In allogenic hematopoietic stem cell transplantation, T cell immunotherapy can cause graft versus host disease (GVHD), while NK cells do not [97, 98]. The hESCs/hiPSCs derived NK cells cannot only be potentially used for treating leukemia upon HSC transplantation, but also possibly be used for killing solid tumors like ovarian, pancreatic, breast and prostate cancers [71]. The hESCs/hiPSCs derived NK cell treatment for HIV infection is also possible [89, 93].

The overall goal of this research is to use human induced pluripotent stem cells as a novel tool to study the human lymphocyte development in vitro as well as modeling immunodeficiency diseases. The research contains three projects.

Project I aims to derive human lymphocytes (NK, B and T cells) by comparing different iPSC lines. Two sources of iPSCs gave rise to hematopoietic progenitor cells (HPCs),

iCell-HPCs and UiPS-HPCs both with a high expression of CD34 (99% and 84%, respectively) (Figure 3). They were then plated in different co-cultures for lymphocyte (B, T and NK cells) differentiation. In NK conditions, murine fetal liver stromal cells (EL08-1D2) and murine bone marrow stromal cells (OP9, OP9-DL1 and OP9-DL4) were used. As early as in two weeks, UiPS-HPCs became NK cells in OP9-DL1 and OP9-DL4 conditions at 16.4% and 19.2% respectively, while only 1.21% and 1.2% of iCell-HPCs became NK cells in these two conditions. (Figure 4). Four week co-culture showed that both iCell-HPCs and UiPS-HPCs gave rise to mature NK cells (more than 95%) with typical inhibitory and activating receptors but with varied expression level on different stromal cells. Among these different conditions, OP9-DL1 condition maintained the best (Figure 5.1, 5.2). And in the OP9-DL1 condition, both iCell-NK and UiPS-NK experienced a 20 fold of increase in proliferation from 50,000 cells expanded to 1,000,000 cells (Figure 6.1, 6.2). The ^{51}Chromium release assay showed that OP9-DL1 condition derived iCell-NK and UiPS-NK both obtained normal cytotoxicity, while UiPS-NK was 20% higher than the average killing ability of iCell-NK (Figure 7). In terms of the B cell co-culture with MS5 stromal cells, both iCell-HPCs and UiPS-HPCs could not generate any live cells with B cell marker expression (Figure 8B). In addition, in the T cell co-culture with OP9-DL1 stromal cells, both iCell-HPCs and UiPS-HPCs could not generate any live cells with T cell marker expression (Figure 9B). UCB CD34+ cells, when plated in NK, B and T cell conditions, made NK cells, B cells and T cells, indicating that they could always serve as a positive control in deriving these lymphocytes in vitro. Therefore, derivation of NK cells from these two sources of hiPSCs was achieved and a comprehensive panel of mature phenotype of NK cells with normal expansion potential and cytotoxicity was characterized. However, the derivation of B and T cells from the two sources of hiPSCs was severely limited.

Project II aims to use IL-2RG gene targeted human ESCs to establish an in vitro lymphocyte development model to study X-SCID. NK cells were generated from different clones of IL-2RG targeted hESCs on EL08-1D2 stromal cells. Both IL-2RG knock out hESCs (KO-Clone 1 and KO-Clone7) and wild type hESCs (WT-Clone 9)

underwent successful in vitro hematopoiesis (Figure 10). Once they were plated in NK condition for two weeks, WT-C9 HPCs gave rise to 22.3% of CD56+/CD132+ NK cells, while KO-C1 and KO-C7 HPCs did not make any NK cells (Figure 11). By four weeks co-culture, 97% of the WT-C9 HPCs became a homogenous immature NK cell population, while in the co-cultures of KO-C1 and KO-C7 HPCs, only a few monocytes and neutrophils survived (Figure 12). During four weeks' NK co-culture, WT-C9-NK cells with a phenotype of CD56+/CD132+ proliferated very well, from 0% to 80% (Figure 13). Once stimulated with aAPCs, these immature WT-C9-NK cells became mature and functional, with a significant increase of cytotoxicity (Figure 14, 15). Therefore, IL-2RG wild type hESCs could initially generate immature NK cells and then became mature and functional upon aAPCs stimulation. But IL-2RG knock out hESCs could not generate any NK cells. Thus, the common γ chain mutation caused X-SCID disease model is established by manipulating human pluripotent stem cells in the derivation of NK cells.

Project III establishes an in vitro NK cell development model by using gene corrected WAS-patient iPSCs (cWAS-iPSCs). Both cWAS-iPSCs and WAS-iPSCs (without WAS gene corrected) underwent hematopoietic differentiation and were successfully derived into a 99% pure population of CD34+/CD43+ cells. UCB CD34+ cells and hESCs derived HPCs both served as controls (Figure 16). As early as in 13 days of co-culture, cWAS-iPSCs generated NK cells with CD14-/CD56+ expressing 66.6% in OP9-DL1 condition and 45% in OP9-DL4 condition, while WAS-iPSCs did not generate any NK cells (Figure 17). Both UCB CD34+ cells and hESCs derived HPCs served good control for generating NK cells. After 34 days, more than 80% of cWAS-NK cells were found in OP9-DL1 and OP9-DL4 conditions, and more than 70% of these NK cells had a mature phenotype (CD117-/CD56+) with NK inhibitory and activating receptors expression (Figure 18). To test NK degranulation, cWAS-NK cells were stimulated with K562 cells in the CD107a assay. The increased expression of CD107a on the cWAS-NK cell surface was in a K562 dose dependent pattern (Figure 18). Thus, the NK cell development was observed in the cWAS-iPSCs, but not in the WAS-iPSCs. Taken together, these results

demonstrate that the targeted correction of WAS gene in WAS-iPSCs results in the restoration of the lymphoid development defect of the WAS disease.

Based on the above findings, we find that both hESCs and hiPSCs can be differentiated into NK cells. Different sources of hiPSCs can become NK cells. The iPSC-derived NK cells display mature phenotype with typical expression levels of inhibitory and activating receptors on the cell surface. Also these NK cells acquire killing ability against tumor cells. The challenge for the hiPSCs is that they cannot produce any B and T cells, even in a low efficiency. This has been previously attributed to ID2 and ID3 enrichment in the HPCs, which promotes NK cell development while inhibiting E proteins that mediate B and T cell development [17, 18]. This may also suggest that hiPSCs derived HPCs are already a sub-population of downstream progenitors following the common lymphoid progenitors (CLPs), losing their ability to become B and T cells. More studies should be performed to elucidate this. Although there is a lack of B and T cell derivation, hiPSCs can be differentiated into functional mature NK cells. Thus, hiPSCs can provide a novel model to study the normal development of NK cells in vitro and promote NK cell based immunotherapy.

Thus, two “proof of concept” projects were performed. By collaborating with other groups, we established two immunodeficiency disease models to study the in vitro NK cell development from hESCs/hiPSCs. With David Russell’s group, by targeting IL-2RG gene in hESCs, hESCs with IL-2RG gene knock out could resemble iPSCs derived from X-SCID patients caused by IL-2RG mutation. The results showed that wild type IL-2RG hESCs clone could generate mature and functional NK cells, but the IL-2RG knock out hESCs clones could not make NK cells due to lack of IL-2RG mediated signaling required for NK cell development. It suggests that once the X-SCID patient iPSCs have IL-2RG gene corrected in vitro, they can be derived into mature NK cells, promoting the possibility of autologous NK cell transplantation to the patients. Then this idea was actually tested in project III. By collaborating with Brian Davis’ group, WAS patient iPSCs first had WAS gene corrected by ZFNs in vitro, then both corrected and non-

corrected WAS-iPSCs were differentiated into HPCs. Finally NK cells were derived from corrected WAS-iPSCs, but were not found from the non-corrected WAS-iPSCs. These NK cells were also phenotypically mature and obtained normal cytotoxicity ability upon stimulation. Therefore, restoration of NK cell development can be observed in vitro following gene correction of WAS-iPSCs, making it possible that autologous transplantation of these hematopoietic progenitor cells or NK cells into the WAS patients.

To establish NK cell therapy, the function of in vitro derived NK cells from patient hiPSCs should be further evaluated by transplanting them to humanized mouse models with the patient disease. Also, in order to overcome the hindrance of using hiPSC derived B cells and T cells for immunotherapy, sequential over-expression of specific B and T cell transcription factors as well as suppressing ID2 and ID3 expressions during the hiPSC derived hematopoiesis could potentially provide a method to promote B and T cell development.

Based on the derivation of NK cells from iPSCs, we can provide a novel approach to establish certain gene mutation caused immunodeficiency disease models in vitro. We believe this will promote the NK cell adoptive therapy to the patients. First, iPSCs can be obtained from the specific patient, then the iPSCs contain the mutation genes can be corrected by in vitro gene targeting techniques. Then following the in vitro derivation, activation and expansion of mature and functional NK cells, NK cells can be injected back to the patient to treat the disease. Therefore, by combing iPSCs and gene editing techniques, autologous immunotherapy can be conducted by the derivation of NK cells from iPSCs.

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