Chapter

Immune Cell Generation from Human-Induced Pluripotent Stem Cells: Current Status and Challenges

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Abstract

The immune system plays a crucial role in recognizing and eliminating foreign antigens, working in conjunction with other bodily systems to maintain the stability and physiological balance of the internal environment. Cell-based immunotherapy has revolutionized the treatment of various diseases, including cancers and infections. However, utilizing autologous immune cells for such therapies is costly, time-consuming, and heavily reliant on the availability and quality of immune cells, which are limited in patients. Induced pluripotent stem cell (iPSC)-derived immune cells, such as T cells, natural killer (NK) cells, macrophages, and dendritic cells (DCs), offer promising opportunities in disease modeling, cancer therapy, and regenerative medicine. This chapter provides an overview of different culture methods for generating iPSC-derived T cells, NK cells, macrophages, and DCs, highlighting their applications in cell therapies. Furthermore, we discuss the existing challenges and future prospects in this field, envisioning the potential applications of iPSC-based immune therapy.

Keywords: NK cells, macrophage, iPSC-derived cells, cellular therapy, T cells

1. Introduction

Immunotherapy has emerged as a highly promising therapeutic approach, particularly in the field of anticancer treatment, showcasing remarkable clinical efficacy. It encompasses various strategies, such as adoptive cell transfer (ACT) and immune checkpoint inhibitors (ICIs). Immune cells, being central players in the pathogenesis and progression of numerous diseases, serve as the fundamental components underlying the effectiveness of immunotherapy [1, 2]. Currently, a Phase I/IIa clinical trial (NCT03666000) is underway, investigating the efficacy of allogeneic CD19 chimeric antigen receptor (CAR)-T cell therapy in the treatment of relapsed/refractory B-non-Hodgkin lymphoma (NHL) and B-cell acute lymphoblastic leukemia (ALL). Furthermore, a Phase I clinical trial (NCT04220684) is evaluating the potential of allogeneic natural killer (NK) cell therapy for acute myeloid leukemia (AML). However, the use of autologous immune cells in such immunotherapy approaches is associated with significant drawbacks, including

high costs, time-intensive procedures, and a heavy reliance on the availability and quality of immune cells, which are limited in patients. Consequently, there is an urgent need to develop an "off the shelf" strategy in an allogeneic setting, allowing for the unlimited proliferation of immune cells to address these challenges and facilitate clinical advancements.

In 2006, the Takahashi and Yamanaka research group achieved a groundbreaking milestone by generating induced pluripotent stem cells (iPSCs) from fibroblasts through the transfection of key factors known as the "Yamanaka factors." This pioneering approach involved the introduction of OCT3/4, SOX2, KLF4, MYC, NANOG, and LIN28 into the cells, resulting in their reprogramming into a pluripotent state [3, 4]. These iPSCs possess similar properties to those of embryonic stem cells (ESCs) in terms of morphology, growth characteristics, and developmental potential [5, 6]. iPSCs offer distinct advantages in the production of immunotherapeutic cells, primarily due to their ability to undergo unlimited reproduction *in vitro* and their ease of genetic modification. These characteristics make iPSCs highly valuable in the generation of immunotherapeutic cells for various applications [7, 8]. Furthermore, unlike ESCs, the clinical use of iPSCs does not raise ethical concerns. As a result, iPSC technology holds great potential for the development of allogeneic "off-the-shelf" cellular therapeutics that can benefit a larger number of patients. This approach is anticipated to be the most effective method for treating various types of malignancies. The advent of iPSC-derived immune cells signifies the beginning of a new era in immunotherapy, paving the way for innovative advancements in the field [9]. In this chapter, we will provide an overview of the key methods utilized for generating immune cells from iPSCs, along with their potential clinical applications and inherent limitations.

2. iPSCs-T cells

T lymphocytes are derived from hematopoietic stem cells (HSCs) located within the bone marrow. Following their production in the bone marrow, hematopoietic progenitors migrate to the thymus, where they undergo maturation into functional T cells under the influence of thymic hormones. Matured T cells are then distributed throughout the body via the bloodstream, reaching thymus-dependent regions of peripheral immune organs. They can also circulate through lymphatic vessels, peripheral blood, and tissue fluid, performing essential functions in cellular immunity and immune regulation [10]. Adoptive T-cell immunotherapy has emerged as a promising therapeutic strategy for treating various cancers and viral infections [11–14]. However, the current processes involved in generating T-cell lines from donors or genetically modifying autologous T cells for each patient are time-consuming and expensive. These limitations hinder the widespread and convenient utilization of T cells with antigen specificity. Moreover, the exhaustion of antigen-specific T cells remains a significant challenge in this approach. There is an urgent need for an unlimited supply of T lymphocytes with antigen-specific characteristics to enhance the effectiveness of T-cell therapies. In this regard, potential sources for such T cells include peripheral blood T cells from healthy donors and T cells generated from iPSCs. iPSC technology, as an "off-the-shelf" source of T cells, holds the potential to generate antigen-specific T cells that not only fulfill the requirements for large-scale clinical applications but also ensure the expression of identical T-cell receptor (TCR) genes [15, 16].

2.1 Generation

In 2002, Hochedlinger and Jaenisch conducted a groundbreaking experiment, demonstrating the successful transfer of mature lymphocyte nuclei into oocytes. This pioneering approach enabled them to establish ESCs from cloned blastocysts. To advance their research, they further injected these ESCs into tetraploid blastocysts, leading to the generation of monoclonal mice. These significant findings provided compelling evidence that a fully differentiated cell possesses the capacity for reprogramming and can give rise to an adult cloned animal [17]. In nearly all protocols for iPSC-T cell differentiation, the initial step involves reprogramming T-iPSCs from sorted $CD4^+$ helper T cells and $CD8^+$ cytolytic T cells obtained from a healthy donor. This reprogramming process is typically achieved through the transfection of Sendai virus vectors or episomal plasmid vectors carrying the four Yamanaka factors [15, 18–21]. During the process of reprogramming, it is possible to retain the antigenic specificity of T cells. This is because T-iPSCs inherit the same rearranged TCR genes at the T-cell receptor loci as the original T cells, which allows for the generation of functional T cells with the desired antigenic specificity [15, 22].

To differentiate T-iPSCs into iPSC-T cells, three different methods have been employed. These methods include the two-dimensional (2D) Delta-like ligand (DLL)1/DLL4-expressing stroma system [22–26], 2D stroma-free system [27, 28], and three-dimensional (3D) artificial thymus organoid (pluripotent stem cell-artificial thymus organoid (PSC-ATO)) system [29, 30]. In 2013, three separate research groups pursued a similar approach by employing feeder cells to differentiate iPSC-T cells. Typically, T-iPSCs were initially cultured on mouse embryonic feeder (MEF) cells to expand pluripotent stem cells *ex vivo*. Subsequently, they were redifferentiated into hematopoietic progenitors using OP9 or C3H10T1/2 feeder cells. It is worth noting that the Notch pathway plays a critical role in the generation of HSCs during this process [31–33]. In mammals, the Notch signaling pathway comprises four Notch receptors (Notch 1–4) and five Notch ligands, including Delta-like ligands (Dll) 1, 3, and 4, and Jagged 1 and 2 (JAG 1 and 2). This pathway is highly conserved and plays a crucial role in various developmental processes, particularly in hematopoiesis [34, 35]. Dar Heinze et al. conducted a study where they discovered that early stimulation of the Notch pathway, achieved through the use of OP9-hDLL4 feeder cells or hDLL4-coated plates, directed hematopoietic progenitors toward differentiation into NK cells and T cells. This finding highlights the critical role of Notch signaling in guiding the fate determination of hematopoietic progenitors toward these specific immune cell lineages [36]. The final step in the differentiation of iPSC-T cells involved seeding the cells onto OP9-DL1 feeder cells and utilizing a combination of cytokines to promote the production of functional T cells [23, 24, 26]. This standardized approach proved successful in generating a significant number of $CD8^+$ T cells, with over 90% of these cells originating from the same T-iPSC source. The results demonstrated that iPSCs are a potent tool for generating and developing T-cell lineages *in vitro*. This advancement holds great potential in the field of regenerative medicine, particularly for the progress of allogeneic therapies [37].

Feeder cells were employed in the generation of iPSC-derived T cells at various stages of the process. Nevertheless, the use of murine-derived stroma feeder layers raises concerns about potential cross-species contamination [27, 38]. Moreover, the utilization of different feeder cells necessitates distinct combinations of serum and basal media for maintenance culture. This complexity in culturing conditions can

increase the risks of uncontrolled differentiation and pose challenges for ensuring quality control of feeder cells and serum. To address this issue, Iriguchi et al. devised a feeder-free and serum-free culture system for differentiating iPSC-T cells [27]. First, they induced hematopoietic progenitors from T-iPSC cell lines. Embryoid bodies (EBs) were generated from single cells without the need for feeder cells and serum. Next, hematopoietic cells were induced in the presence of CHIR99021, bone morphogenetic protein 4 (BMP-4), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF), and they underwent proliferation in the presence of hematopoietic cytokines, specifically SB431542. Second, to enhance the production of T cells, a combination of CXCL12-SDF1α and a p38 inhibitor, SB203580, was utilized to generate $CD4^+CD8\alpha\beta^+$ double-positive (DP) cells. This innovative approach demonstrates remarkable efficiency and scalability in generating fully functional CD8αβ T cells from iPSCs. More recently, a 3D organoid culture system was reported to successfully generate CAR T cells for "off-the-shelf" manufacturing strategies [29]. Montel-Hagen et al. introduced a 3D artificial thymus organoid (ATO) culture system for the *in vitro* differentiation of human hematopoietic stem and progenitor cells (HSPCs) into functional, mature T cells. They achieved this by utilizing a standardized stromal cell line expressing Notch ligands in a serum-free environment [39]. This innovative continuous culture system facilitated both the specification of hematopoietic cells and their subsequent terminal differentiation into naïve $CD3^+CD8\alpha\beta$ + and $CD3^+CD4^+$ conventional T cells.

While the redifferentiation strategy allows for the realization of "off-the-shelf" T cells [8, 40, 41], there is a risk of rejection by the patient's immune system when using allogeneic T cells. Additionally, a significant concern arises from the production of heterogeneous T cells, which can lead to the generation of potentially harmful alloreactive T cells at varying frequencies. In order to circumvent immune rejection and the production of polyclonal T cells, T-iPSCs can also be derived from patients themselves. Daopeng Yang conducted a study where T-iPSCs were generated from cytotoxic T lymphocytes infiltrating hepatocellular carcinoma (HCC). This was achieved using an integrative Sendai virus vector. The resulting pluripotent cell line exhibited a normal karyotype and could be redifferentiated into rejuvenated CTLs specifically targeting HCC [42]. Munenari Itoh conducted a study where T-iPSCs were generated from monocytes of a melanoma patient. $CD8^+$ T cells were sorted after stimulation with tumor antigens, and then reprogrammed into iPSCs through the exogenous expression of reprogramming factors, utilizing the Sendai virus vector [43].

The generation of T lymphocytes from induced pluripotent stem cells (iPSCs) *in vitro* holds promise for adoptive T-cell therapy. However, the yield and efficiency of lymphoid cells have been limited, and their properties are still only partially understood [7, 44]. Studying T cells derived from ESCs and iPSCs faces challenges due to a limited understanding of their antigen specificity and human leukocyte antigen (HLA) restriction. T cells generated in the laboratory from ESCs or iPSCs exhibit unpredictable T-cell receptor (TCR) repertoires due to random rearrangements of TCR genes, and the mechanisms involved in their selection during *in vitro* differentiation are not yet well understood. Nevertheless, this limitation can be overcome by utilizing iPSCs that possess an endogenous TCR with known antigen specificity [23, 24]. However, this approach requires a time-consuming procedure of cloning T cells specific to the antigen, and it is limited to antigens that can be identified using unique T cells from each patient. Additionally, the clinical use of T cells that recognize antigens through their inherent TCR is constrained by the need to match their specificity with the HLA molecules of the recipient patient.

2.2 Translation

One of the notable advantages of iPSCs is their versatility in genetic modification. iPSC-derived T cells have emerged as a prominent tool in cancer immunotherapy. By introducing a chimeric antigen receptor (CAR) or transgenic T-cell receptor (TCR) gene, iPSCs can be transformed into antigen-specific T cells capable of effectively targeting and eliminating cancer cells *in vitro* and *in vivo* [23, 24, 26]. The incorporation of CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9) technology further enhances the efficacy and safety of this approach. Through CRISPR-Cas9-mediated insertion of CAR genes into the TRAC locus of the endogenous TCRα constant (TRAC) gene, the risk of host-related alloreactions can be minimized, thus improving immune compatibility [45]. Genetic editing of iPSCs has proven to be a valuable strategy in broadening immune compatibility, supporting the potential for developing "off-the-shelf" cell products. In the context of T-iPSCs (T cell-derived iPSCs), reprogrammed iPSC clones can inherit the original TCR and subsequently be redifferentiated into iPSC-T cells (T cells redifferentiated from iPSCs). However, this approach necessitates time-consuming cloning of antigen-specific T cells and is limited to antigens that can be identified from patient-specific T cells. Furthermore, the therapeutic application of iPSC-T cells is restricted by the need for HLA compatibility between the donors and recipients, significantly limiting the universality of potential "off-the-shelf" applications [16, 25].

Engineering strategies for iPSC-derived T cells involve the introduction of TCR and CAR constructs. TCR-mediated therapies using iPSC-T cells have been carried out by either inheriting the endogenous TCR genes from antigen-specific T cells [22–24] or genetically incorporating exogenous TCRs [25, 29]. Clinical trials have shown the effectiveness of TCR-engineered primary T cells targeting melanoma antigen MART1 [46] and germline antigen New York esophageal squamous cell carcinoma 1 (NY-ESO1) [47]. In these studies, TCR-engineered iPSC-T cells have demonstrated promising results in clinical settings, validating their potential as therapeutic agents for cancer treatment.

Successful outcomes have been observed when introducing a CAR into iPSCs or directly into iPSC-derived T cells to generate antigen-specific T cells [26–28, 30, 48]. CAR engineering effectively redirects T-cell specificity in an HLA-independent manner, eliminating the need for HLA restriction and enhancing the antitumor properties. In a study conducted by Themeli et al. in 2013, T-iPSC clones were generated by reprogramming peripheral T cells from a healthy donor. Subsequently, a second-generation CAR specific for CD19 was transduced into the selected T-iPSC clone. The resulting CAR-expressing iPSC-T cells exhibited remarkable antitumor efficacy in a xenograft model, although they exhibited phenotypic similarities to innate γδT cells [26]. This method effectively generated an innate type of T cell expressing a CD8αα homodimer, which influenced the antigen-specific cytotoxic capacity of the redifferentiated T cells in a manner akin to MART-1-specific T cells. To improve upon the conventional approach, Maeda et al. modified the method by purifying differential pressure (DP) cells, which were subsequently stimulated with monoclonal anti-CD3 antibodies to generate CD8αβ T cells. These modified T cells displayed comparable antigen-specific cytotoxicity to the original cytotoxic T lymphocytes (CTLs) [15].

In recent years, iPSC-T cells have emerged as a promising approach in the treatment of various diseases. One notable example is their application in highly aggressive lymphoma, specifically extranodal NK/T-cell lymphoma of the nasal type (ENKL). iPSC-derived cytotoxic T lymphocytes, specifically designed to target the EBV

antigen, have exhibited remarkable results. These cytotoxic T lymphocytes have significantly prolonged patient survival, demonstrated potent tumor-suppressive effects, and persisted as central memory T cells *in vivo* for a minimum of 6 months [49–55]. Rejuvenated cytotoxic T lymphocytes have also exhibited promising outcomes in the treatment of various solid tumors, including cervical cancer associated with human papillomavirus (HPV) infection [56] and renal cell carcinoma [57]. For instance, in cervical cancer cases, T-iPSCs derived from human papillomavirus type 16 (HPV16) E6- or E7-specific cytotoxic T lymphocytes efficiently differentiated into rejuvenated CTLs that specifically targeted HPV16. These rejuvenated CTLs not only displayed prolonged survival compared to the original CTLs but also induced increased tumor shrinkage and significantly prolonged survival in an *in vivo* mouse model [56]. Similarly, in renal cell carcinomas, T-iPSCs established from Wilms tumor 1-specific CTLs effectively suppressed tumor growth in an *in vivo* mouse model [57]. These findings underscore the potential of iPSC-T cells as a valuable therapeutic strategy for targeting and treating various types of cancers, opening new avenues for therapeutic interventions.

3. iPSCs-NK cells

Natural killer cells are a type of lymphocyte that originates from common lymphoid progenitors (CLPs) during hematopoiesis [58]. They are predominantly found in the bone marrow, peripheral blood, liver, spleen, lung, and lymph nodes. Unlike T and B cells, NK cells possess the unique ability to eliminate tumor cells and virus-infected cells without prior sensitization. NK cells play a crucial role in immune responses, including antitumor activity, defense against viral infections, immune regulation, and even involvement in hypersensitivity and autoimmune diseases in certain cases. They possess the ability to recognize target cells and mediate their killing. When compared to T cells, allogeneic NK cell transfers have a lower risk of graft-versus-host disease (GVHD) and may even decrease the overall risk [59, 60]. However, current strategies dependent on donor cells can only provide a limited supply of custom-made therapeutic NK cells for a restricted number of patients. To overcome this limitation and offer a more accessible treatment option, induced pluripotent stem cells (iPSCs) have been employed in immunotherapy to enable the mass production of NK cells from iPSCs. This approach holds the potential to provide an unlimited supply of "off-the-shelf" NK cells, benefiting a larger number of recipients.

3.1 Generation

In contrast to the redifferentiation process of iPSCs into iPSC-T cells, NK cells can be reprogrammed directly from iPSC cell lines [61–63] or generate from peripheral blood cells [64] and human fibroblasts [65]. The iPSC cell lines used in these approaches were established from various sources, including umbilical cord blood CD34+ cells and newborn human foreskin fibroblasts [66, 67].

Two methods for the production of iPSC-derived NK cells can be categorized based on the use of feeder cells. In the standard protocol, iPSC cell lines were cultured on MEFs and differentiated into hematopoietic progenitors using M210-B4 cells. To generate spin EBs suitable for aggregation, iPSCs were passaged in TrypLE Select on a low-density MEF layer. Subsequently, the spin EBs were seeded onto plates either with or without EL08-1D2 (a murine embryonic liver cell line) for NK cell differentiation.

This differentiation process was carried out in the presence of specific NK cell initiating cytokines, including interleukin (IL)-3, IL-7, IL-15, stem cell factor (SCF), and fms-like tyrosine kinase receptor-3 ligand (FLT3L) [62, 63, 68].

A method developed by Frank Cichocki et al. eliminates the need for spin EB generation. In this approach, iPSCs were cultured in a combination of small molecules and cytokines to generate $CD34^+$ hematopoietic progenitor cells. These $CD34^+$ cells were then cocultured with stromal cells that were transduced with Notch ligand and supplemented with cytokines that support the proliferation and differentiation of hematopoietic progenitor cells toward the NK cell lineage. Subsequently, the cells were cocultured with modified K562 cells to further expand the differentiation of iPSC-derived NK cells. This method offers a streamlined process for the efficient production and expansion of iPSC-NK cells [65].

In 2021, Kyle B Lupo et al. developed a serum- and feeder-free system for differentiating iPSCs into NK cells [69]. The differentiation process involved several key steps. First, iPSCs were differentiated into hematopoietic cells using a hematopoietic differentiation medium comprising STEMdiff APEL 2, SCF, bone morphogenetic protein 4, vascular endothelial growth factor, and a rho-associated protein kinase (Rock) inhibitor. To facilitate the formation of EBs, the cells were subjected to a spinning step. After 11 days, hematopoietic progenitor cells were collected from the EBs and seeded in a specialized NK cell differentiation medium containing STEMdiff APEL 2, SCF, IL-7, IL-15, and FLT3L to initiate the differentiation into NK cells. This novel system offers a serum- and feeder-free approach for efficient and controlled differentiation of iPSCs into functional NK cells.

3.2 Translation

Natural killer cells derived from iPSCs offer the advantage of not being HLA restricted, making iPSC-NK cells an excellent candidate for allogeneic "off-the-shelf" immunotherapy [66]. These iPSC-NK cells serve as a readily available source of cells for immunotherapy, capable of targeting tumors and activating the adaptive immune system to transform a "cold" tumor into a "hot" one by facilitating the recruitment of activated T cells, thus enhancing the efficacy of checkpoint inhibitor therapies [65]. The ability to produce iPSC-NK cells under defined conditions and their demonstrated functional responses indicate their potential as effective therapeutic agents in adoptive transfer settings for treating solid tumors. They offer a renewable source of donor-independent NK cells for immunotherapy, holding great promise in clinical applications [69].

Moreover, iPSC-NK cells, being derived from iPSCs, possess the characteristic feature of being amenable to genetic editing. One strategy for genetic modification of iPSC-NK cells is the incorporation of CARs to enhance their antitumor cytotoxicity. Reports have shown that CARs effectively reprogram NK cell specificity [70]. Notably, Laurent Boissel et al. observed that CAR-NK cells exhibited enhanced elimination of primary chronic lymphocytic leukemia (CLL) cells through antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by anti-CD20 monoclonal antibodies [71]. iPSC-NK cells engineered with CARs offer several advantages: (1) they have fewer complications such as cytokine release syndrome (CRS), neurotoxicity, or GVHD; (2) they are not restricted by HLA; and (3) they can activate cytotoxic effects independently of the CAR itself [72–77]. In a study by Dan Kaufman's group, a first-generation CAR incorporating CD4/CD3ζ was introduced into iPSC-NK cells, demonstrating their ability to suppress human immunodeficiency virus (HIV)

replication in $CD4^+$ T cells [68]. Furthermore, Li et al. tested a series of specialized CARs incorporating costimulatory molecule intracellular domains and found that iPSC-derived NK cells expressing CAR (NK-CAR-iPSC-NK cells) exhibited a typical NK cell phenotype and demonstrated superior antitumor activity compared to iPSC-derived NK cells expressing T-cell CARs (T-CAR-iPSC-NK cells) or non-CAR-expressing cells, both *in vitro* and *in vivo* [78].

The persistence and enhanced functional activity of NK cells rely on their interaction with various immune cells that release different cytokines. Among these cytokines, IL-15 plays a crucial role in the differentiation of NK cells [79]. However, during *in vitro* culturing, the frequent addition of IL-15 is necessary due to its short half-life [80]. To overcome this limitation, innovative approaches have been explored, including the use of IL-15 constructs such as secreted IL-15 or an IL-15/IL-15-receptor fusion construct (IL-15RF). Woan et al. developed triple-gene-edited iPSC-NK cells with a high-affinity, noncleavable version of the Fc receptor CD16a, a membrane-bound interleukin (IL)-15/IL-15R fusion protein, and a knockout of the ecto-enzyme CD38, which hydrolyzes NAD^+. They discovered that these engineered iPSC-NK cells exhibited enhanced anticancer effects in leukemia and multiple myeloma [81]. Another important negative regulator of IL-15 signaling in NK cells is cytokine-inducible SH2-containing protein (CIS), encoded by the CISH gene. Huang Zhu et al. found that knockout of CISH in iPSC-NK cells improved the expansion capacity of NK cells and increased their cytotoxic activity against multiple tumor cell lines when maintained at low cytokine concentrations [82]. These modified IL-15 forms provide sustained proliferation signals, thereby augmenting the antitumor efficacy of NK cells both in laboratory settings and in living organisms [83, 84].

Antibody-dependent cell-mediated cytotoxicity is a mechanism by which NK cells exert cytotoxicity through the Fc receptor CD16a. However, CD16a has a low affinity for tumor-bound IgG antibodies and is susceptible to cleavage by a disintegrin and metalloprotease 17 (ADAM17) upon NK cell activation. To address these limitations, Kristin M Snyder et al. enhanced the binding ability of NK cells to antitumor monoclonal antibodies (mAbs) by constructing a fusion protein comprising CD64, the highest-affinity Fc-gamma receptor (FcγR) expressed by leukocytes, and CD16A. This CD64/16A fusion protein lacked the ADAM17 cleavage region in CD16A, preventing downregulation of expression following NK cell activation during ADCC. The CD64/16A iPSC-NK cells exhibited enhanced conjugation to antibody-treated tumor cells, improved ADCC, cytokine production, and ultimately mediated effective tumor cell killing [85]. Another strategy involves mutating CD16a to produce a high-affinity noncleavable variant known as hnCD16. When hnCD16 was incorporated into iPSC-NK cells, the resulting hnCD16-iPSC-NK cells exhibited functional maturity and demonstrated enhanced ADCC against multiple tumor targets. In *in vivo* xenograft studies using a human B-cell lymphoma model, the combination of hnCD16-iPSC-NK cells and anti-CD20 monoclonal antibodies significantly improved regression of B-cell lymphoma and increased overall survival [86]. Additionally, Fanyi Meng fused the ectodomain of hnCD16 with NK cell-specific activating domains in the cytoplasm. This fusion protein showed improved ADCC and cytotoxicity *in vitro* and *in vivo*, as observed in coculture experiments with tumor cell lines and in a xenograft mouse model bearing human B-cell lymphoma [87].

As mentioned above, iPSC-NK cell technology has been utilized for the treatment of hematologic malignancies. However, its applications extend beyond that and encompass the field of solid tumors and viral infections as well. Studies have demonstrated the efficacy of iPSC-derived NK cells in various contexts. For instance,

Hermanson DL found that iPSC-derived NK cells enhanced the antitumor effect and prolonged survival in ovarian cancer [66]. Furthermore, iPSC-derived NK cells have shown promise as an improved approach for treating HIV infection [61, 68] and COVID-19 [88]. These findings highlight the broad potential of iPSC-NK cells in combating a range of diseases, including both cancers and viral infections.

4. iPSCs-macrophages

Macrophages are a type of white blood cells that reside within tissues and are derived from monocytes, which themselves originate from precursor cells in the bone marrow. Macrophages, along with monocytes, function as phagocytes involved in both nonspecific defense (innate immunity) and specific defense (cellular immunity) in vertebrates. Their primary role is to engulf and digest cell fragments and pathogens, whether in the form of stationary or free cells, and to activate lymphocytes or other immune cells to mount a response against pathogens. Macrophages are immune cells with diverse functions, making them crucial subjects for the study of cellular immunity and molecular immunology. These nonreproductive cells can survive for 2–3 weeks under favorable conditions. While primary cultures of macrophages are often used, they are challenging to maintain for extended periods. Immortalized macrophage cell lines are not suitable for clinical applications, and engineering bone marrow or peripheral blood mononuclear cell (PBMC)-derived primary macrophages is not efficient. Therefore, iPSC-derived macrophages represent a valuable source for myeloid cell-based immunotherapy, offering great potential in the field of immunotherapy [89].

4.1 Generation

Similar to the production of iPSC-NK cells, iPSCs utilized for differentiating into macrophages originate from iPSC cell lines derived through the reprogramming of fibroblasts using iPSC reprogramming vectors such as OCT4, SOX2, KLF4, and c-MYC [90–92], $CD34^+$ bone marrow cells [93] or peripheral blood monocytes [94, 95]. The methods employed to generate iPSC-macrophages can also be categorized based on the use of feeder cells.

In standard protocols, iPSC cells are initially cocultured with feeder cells such as OP9 mouse stromal cells, in the presence of bone morphogenetic protein 4. This culture condition leads to the differentiation of iPSCs into either 37.8% CD133 HSCs or 9–17% $CD43^+$ hematopoietic progenitors. To generate macrophages, myelomonocytic colonies are cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF). The resulting iPSC-derived macrophages exhibit functionality and, upon stimulation, secrete substantial amounts of IL-6, IL-10, and tumor necrosis factor alpha (TNF-α) compared to nonstimulated macrophages [90]. IL-3 plays a crucial role in promoting the proliferation of various types of hematopoietic cells during early primitive hematopoiesis and definitive hematopoietic specification. Lachmann, N. et al. combined the use of IL-3 with M-CSF or G-CSF to achieve prolonged and large-scale production of functional granulocytes as well as monocytes/macrophages through EB-based hematopoietic *in vitro* differentiation [96]. They initiated EB formation in ESC medium supplemented with basic fibroblast growth factor (bFGF) and a Rock inhibitor. Subsequently, an intermediate myeloid-cell-forming complex (MCFC) was generated by culturing the EBs in albumin polyvinylalcohol essential lipid (APEL) medium supplemented

with human IL-3, human M-CSF, human G-CSF, or human GM-CSF for a period of 7 days. From day 10 to day 15 onward, monocytes/macrophages or granulocytes were generated. To further promote maturation, the generated monocytes/macrophages or granulocytes were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 10% fetal serum, L-glutamine, human M-CSF, human G-CSF, or human GM-CSF for 7–10 days.

The use of feeder cells or serum in the culture system adds additional biological and regulatory complexities, which may limit the clinical utility of iPSC-derived monocytes and macrophages. To overcome these challenges, a fully chemically defined, serum- and feeder-free protocol has been developed, significantly improving reproducibility [97, 98]. In this protocol, iPSC cells are used to generate spin embryoid bodies (EBs) in a culture medium supplemented with BMP4, VEGF, and SCF. Subsequently, the EBs are collected and passed through a 40-μm strainer before being transferred to a "factory" medium. This medium, known as X-VIVO 15, is supplemented with Glutamax, 1% penicillin/streptomycin, mercaptoethanol, M-CSF, and IL-3. Utilizing this serum-free protocol, differentiation cultures are established, which continue to produce harvestable and uniform monocytes for extended periods, often lasting up to 1 year [98]. López-Yrigoyen, M. also successfully generated macrophages from an iPSC line using this method [99].

During the differentiation process, the generation of EBs typically involves reseeding and size control steps. However, alternative protocols modified by Cao et al. have been developed to eliminate the need for EB generation by employing serum-free culture conditions. Despite this improvement, the yield obtained from these protocols is relatively low, thereby limiting the scalability of the studies [100]. To address this limitation, Cui, D. et al. present a fully optimized differentiation protocol that incorporates precise timing of steps and the addition of specific cytokines, chemokines, or chemicals. This optimized protocol enables large-scale production of macrophages under serum- and feeder-free conditions without the need for the EB generation step [101]. The development of this fully optimized differentiation protocol represents a significant advancement in the field, providing a reliable and scalable method for generating macrophages from iPSCs. By incorporating precise timing and additional factors, this protocol enhances the efficiency and yield of macrophage production, enabling large-scale studies and expanding the potential applications of iPSC-derived macrophages.

4.2 Translation

Induced pluripotent stem cell (iPSC)-derived macrophages can be engineered with CARs through gene modification. Klichinsky et al. engineered human primary macrophages with an anti-CD19 CAR containing a CD3ζ intracellular domain. These CAR-macrophages exhibited M1-like pro-inflammatory phenotypes and were resistant to the immunosuppressive effects of the tumor microenvironment (TME) through stimulation by the adenovirus vector [102]. Zhang et al. established a platform to engineer iPSCs with a CAR and differentiate them into macrophages, referred to as CAR-iPSCs-macrophages [89]. CAR expression conferred antigen-dependent macrophage functions, including cytokine expression and secretion, polarization toward a pro-inflammatory/antitumor state, enhanced phagocytosis of tumor cells, and demonstrated *in vivo* anticancer activity [89]. Fusing a CD20 single-chain variable fragment (scFv) to FcγR1 in iPSC-macrophages enhanced their ability to engulf and eliminate B-cell leukemic cells both *in vitro* and *in vivo* [103]. Zhang et al. successfully developed iPSC-derived CAR macrophages expressing either a CD19-specific

or a mesothelin-specific fusion receptor, utilizing two distinct endodomain configurations [89]. These iPSC-derived CAR macrophages exhibited antigen-dependent anticancer functions, including cytokine expression and secretion, polarization toward a pro-inflammatory/antitumor state, enhanced phagocytosis of tumor cells, and demonstrated anticancer activity *in vivo* [89].

Macrophages are terminally differentiated cells with limited capacity for expansion. To overcome this limitation and enable the large-scale clinical use of iPSC-derived macrophages, Azusa Miyashita et al. employed gene transduction techniques to introduce genes involved in cell growth or senescence suppression, such as c-MYC, in combination with BMI1, murine double minute (MDM2), or enhancer of zeste homolog 2 (EZH2). This approach resulted in the production of human iPSC-derived macrophages that could be propagated for extended periods, functioning as primary macrophages [104]. The engineered cell lines demonstrated a low risk of tumorigenicity, as they exhibited cytokine-dependent proliferation *in vitro*. Importantly, the cytokine-rich conditions required for their growth could not be replicated in the physiological environment *in vivo*.

Patient-derived iPSCs provide valuable cellular models for studying disease pathogenesis and evaluating potential treatments. iPSC-derived macrophages, in particular, hold great promise for investigating various diseases, including cancer. Both macrophages derived from patients' monocytes and iPSCs derived from patients' fibroblasts have been utilized as models in diseases such as Gaucher disease [93]. In Gaucher disease, iPSC-derived macrophages generated from fibroblast lines obtained from patients with type 1 or type 2 Gaucher disease displayed similar characteristics. These macrophages exhibited reduced glucocerebrosidase activity and increased accumulation of glucocerebrosidase and glucosylsphingosine in lysosomes, mirroring the observations in patient monocytes. Furthermore, all the macrophages demonstrated effective phagocytosis of bacteria but exhibited reduced production of intracellular reactive oxygen species (ROS) and impaired chemotaxis [91]. Another example involves iPSCs derived from a patient with hereditary pulmonary alveolar proteinosis. When differentiated into macrophages, these cells exhibited defects in GM-CSF-dependent functions, characteristic of the disease phenotype [93].

5. iPSCs-DCs

Dendritic cells (DCs) are derived from myeloid pluripotent hematopoietic stem cells and undergo differentiation through two main pathways. Myeloid dendritic cells (MDCs) are generated by stimulation with GM-CSF and differentiate from common precursor cells shared with monocytes and granulocytes. On the other hand, lymphoid dendritic cells (LDCs) or plasmacytoid dendritic cells (pDCs) arise from lymphoid stem cells and share precursor cells with T cells and NK cells. These LDCs are also known as DC2 cells. DCs exhibit widespread distribution in various tissues, including the skin, airways, and lymphatic organs, with notable heterogeneity. Consequently, different tissues have distinct names for DCs. For instance, DCs present in the basal layer of the skin epidermis and spinous cells are referred to as Langerhans cells. DCs are considered the most potent professional antigen-presenting cells (APCs) in the body. They efficiently capture, process, and present antigens to other immune cells. Mature DCs play a crucial role in the initiation, regulation, and maintenance of immune responses by effectively activating naïve T cells, which are central to the immune response.

5.1 Generation

Dendritic cells derived from iPSCs exhibit characteristics similar to those of other immune cells. Kitadani et al. utilized dermal fibroblasts transfected with Sendai virus vectors to generate iPSCs. These iPSCs were then differentiated into hematopoietic progenitors using a combination of recombinant human bone morphogenetic protein 4 (rhBMP4), recombinant human vascular endothelial growth factor (rhVEGF), growth factor (GF), and recombinant human stem cell factor (rhSCF). Following the addition of a cytokine mixture and CD14 cell sorting, monocytic cell cultures were established and further differentiated into DC cells [105]. In contrast to Kitadani et al.'s method, a feeder-dependent system was employed for the production of iPSC-derived DCs (iPS-DCs). In this approach, iPSCs were seeded onto OP9 cell layers and cultured in the presence of GM-CSF. Once the cells differentiated into hematopoietic progenitors, the floating cells were collected and transferred to Petri dishes without feeder cells. After 5–7 days, the majority of the floating cells had differentiated into iPS-DCs. To promote their maturation, the cells were transferred and cultured in RPMI-1640/10% fetal calf serum (FCS) supplemented with GM-CSF, IL-4, TNF-α, and anti-CD40 monoclonal antibody [103, 106, 107]. These two different methods demonstrate distinct approaches for generating iPSC-derived DCs. Both techniques have proven effective in producing functional DCs from iPSCs, providing valuable tools for studying the biology of DCs and their potential applications in immunotherapy and disease modeling.

5.2 Translation

Dentritic cell vaccines have been considered as a promising option for immune cell therapy against cancer. As APCs with robust T-cell stimulating activity, DCs play a pivotal role in orchestrating the immune response. Despite their potential, clinical trials utilizing DC vaccines have encountered challenges, and the outcomes have been largely disappointing [108]. One possible reason for the limited success of DC vaccines in clinical trials is the presence of immune exhaustion in cancer patients, which compromises the ability to generate a sufficient T-cell response [109].

Dentritic cells play a crucial role in immune responses, particularly in stimulating cytotoxic T lymphocytes against viral and tumor-associated antigens through the process of cross-presentation in an MHC class I-restricted manner. Under steady-state conditions, CD141 DCs, residing in interstitial tissues, are primarily involved in maintaining immune homeostasis and inducing tolerance to local antigens. However, iPSC-derived DCs have emerged as a promising avenue for antitumor immunotherapy. In a study by Junya Kitadani et al., iPSCs derived from three healthy donors were differentiated into DCs using feeder-free culturing protocol. Carcinoembryonic antigen (CEA) complementary DNA (cDNA) was then introduced into the iPSC-derived DCs through transduction. The researchers demonstrated that these genetically modified iPSC-derived DCs were capable of inducing CEA-specific cytotoxic T lymphocytes in a human model and exhibited significant antitumor effects in a CEA transgenic mouse model [105]. Building upon their previous work, the same research group, in 2023, designed iPSC-derived DCs targeting mesothelin (MSLN) and focused on enhancing the antigen-presenting ability of these cells through the ubiquitin-proteasome system. By simultaneously expressing ubiquitin and MSLN, genetically modified iPSC-derived DCs exhibited potent cytotoxicity against tumors that naturally express MSLN, thereby overcoming immune tolerance and eliciting robust antitumor immune responses [110]. These findings highlight the potential of

iPSC-derived DCs in antitumor immunotherapy. The ability to genetically modify iPSC-derived DCs to express specific antigens opens up opportunities for personalized and targeted therapies. Further research and development of iPSC-derived DC-based immunotherapies hold promise for enhancing the efficacy of cancer treatments and improving patient outcomes.

6. Limitations and challenges

Although iPSC-derived cells hold great potential for immunotherapy in clinical applications, several limitations currently hinder their widespread use. One major challenge is the low efficiency of pluripotent reprogramming across various cell types. Reprogramming adult human fibroblasts, for instance, yields a conversion rate of only 0.02–0.05% [111]. Similarly, differentiation of CD34+ mobilized human peripheral blood cells results in a conversion rate of just 0.01–0.02% [112]. In order to achieve successful cell transplantation in patients, a high yield of immune cells is required. The low reprogramming efficiency not only limits the final cell yield but also poses challenges for scaling up the process for clinical applications. Another limitation is the time-consuming nature of the differentiation process. As mentioned earlier, the differentiation of iPSCs into functional immune cells often takes 1–2 months for the development of mature properties. For iPSC-derived T cells, it typically requires 3–7 weeks to expand and reach maturity [27, 29, 38, 44]. iPSC-derived NK cell production takes at least 4 weeks [113], while iPSC-derived macrophages require around 3–7 weeks [114, 115]. The lengthy duration of expansion and differentiation not only increases costs but also prolongs the overall treatment time in clinical therapy. Furthermore, the use of murine-derived feeder cells and serum in current

	Advantages	**Limitations**
iPSC	• Ease of genetic editing • Unlimited source • Reduces cost • Time-consuming • Avoids ethical issue	• Low efficiency • Low functional maturity • Immunogenicity • Safety
iPSC-T cells	• Keep the antigenic specificity • Allogeneic therapies	• Immune rejection • Polyclone T cells to alloreactive • HLA restriction • More complications
iPSC-NK cells	• No HLA restriction • Less complications	• Less persistence *in vivo*
iPSC-macrophages	• Benefit to solid tumor therapy • Play a phagocytic role in tumor cells • Less toxicity and limited circulation time	• Undergo frequent polarization • Lack of proliferation capacity • Do not meet clinical safety requirements for oncogenes used

Table 1.
Advantages and limitation of induced pluripotent stem cell (iPSC)-immune cells.

culture approaches introduces the risk of cross-species contamination and variations in the final cell products. Moreover, iPSCs derived from fibroblasts pose challenges in terms of product heterogeneity. Although researchers have developed serum-free and feeder-free culture systems that are more suitable for industrial applications [69, 116], achieving standardization and consistency of the final immune cell products remains a challenge for large-scale industrial production and their use in real-patient applications and clinical trials. The safety of iPSCs is also a concern before large-scale clinical application. Recent studies have reported the tumorigenic potential of undifferentiated iPSCs and the potential for malignant transformation in differentiated iPSCs [117, 118]. Additionally, investigations into TCR gene usage in T cells derived from T-iPSCs and TCR-iPSCs have revealed a small portion of rearranged TCRs, raising further safety considerations [15].

Despite the limitations discussed above, iPSC-derived immune cells offer several advantages (see **Table 1**). To address the challenges associated with iPSC-based immunotherapy, it is crucial to develop standardized protocols and identify novel targets for cell therapy. Additionally, the influence of the immune microenvironment should be carefully considered and investigated to optimize the efficacy of iPSC-derived immune cells. By overcoming these limitations and leveraging the strengths of iPSC-based approaches, we can unlock the full potential of iPSC-immune cells in the field of immunotherapy.

7. Conclusion and future perspectives

The utilization of iPSC technology in immunotherapy has revolutionized traditional immune therapies, as it offers a virtually limitless supply of genetically engineered immune cells that can be readily available for patients' therapeutic needs. This approach eliminates the dependence on scarce cell sources from individual patients, which may not be sufficient for therapeutic purposes. By establishing iPSC banks, standardized protocols for immune cell differentiation can be implemented to ensure scalability and quality control of the generated cell products before administration. Currently, there are approximately 10 iPSC banks that have been established, with a focus on stem cell research and disease-specific cell lines, catering to the needs of both academic and industrial research endeavors [119].

The application of iPSC technology in cell therapy carries certain risks, including tumorigenicity and immune suppression. To address these concerns, novel strategies have been developed to enhance iPSC differentiation and modification. Various approaches can be employed to mitigate the tumorigenic risks associated with iPSCs. For instance, undifferentiated cells can be selectively sorted out using antibodies that target surface biomarkers [120] or eliminated through the use of cytotoxic antibodies [121]. Additionally, chemical inhibitors can be utilized to eradicate any remaining undifferentiated pluripotent cells [122, 123]. While these strategies have shown promise in reducing the risk, it is important to note that long-term culture for reprogramming and redifferentiation may still give rise to unexpected events that contribute to tumorigenicity. Consequently, caution must be exercised during the first-in-human clinical studies to anticipate and address potential issues. To enhance safety in iPSC-based cell therapies, suicide systems can be implemented as a precautionary measure. These systems are designed to induce apoptosis in transduced cells, thereby potentiating therapy without increasing toxicity or evoking cross-resistance to conventional agents. One example is the HSV-TK (herpes simplex virus thymidine kinase) gene,

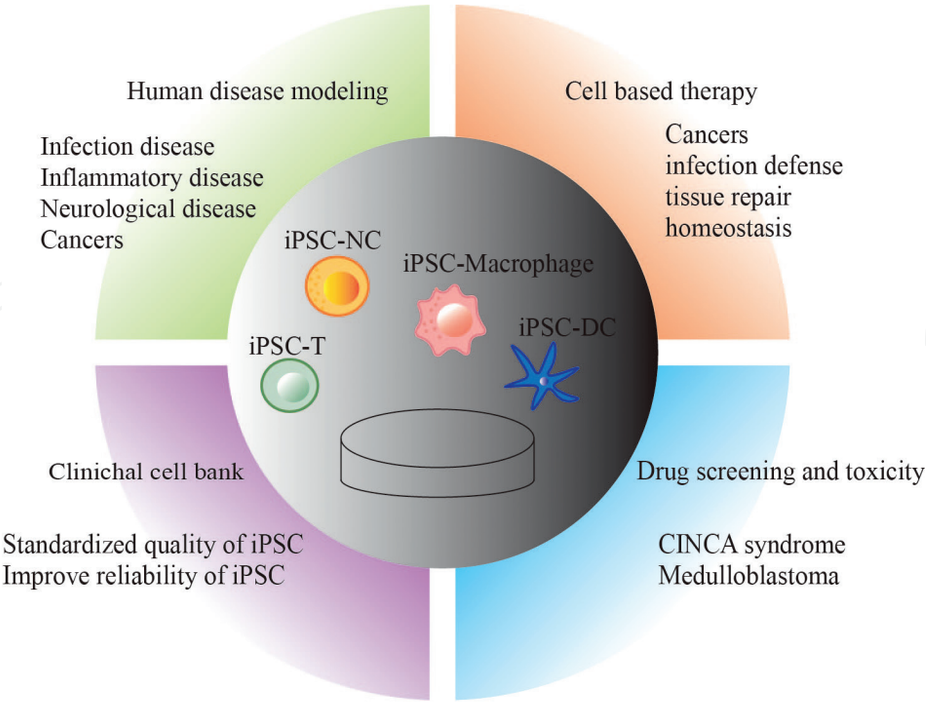

Figure 1.
Application of iPSC immune cells.

which can be combined with the administration of ganciclovir (GCV) as a safety switch in adoptive T-cell therapy or cancer treatment. However, it is important to note that certain suicide gene systems have shown limitations and may not be as clinically effective as desired [124, 125]. To safeguard against potential risks during clinical and translational investigations of iPSC-based cell therapy, Miki Ando et al. utilized the inducible caspase 9 (iC9) system as a safety mechanism. This system, consisting of an inducible caspase 9 (iC9) gene, can be activated to induce apoptosis in iPSCs if any unexpected issues arise [126].

In addition to their applications in cancer therapy, iPSC immune cells also play significant roles in establishing human disease models [91, 127, 128], drug screening, toxicity assessment [91, 97, 129], and clinical cell banking for "off-the-shelf" therapy (**Figure 1**). The versatility of iPSC immune cells enables their potential use in treating various pathological conditions beyond cancer through genetic modifications [94, 130–133]. This opens up exciting possibilities for utilizing genetically engineered iPSC immune cells as regenerative medical products in clinical practice. With further advancements and research, these cells could offer new avenues for personalized and targeted therapies in the future.

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