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iPSC-derived T cells and macrophages: Manufacturing and next-generation application approaches☆

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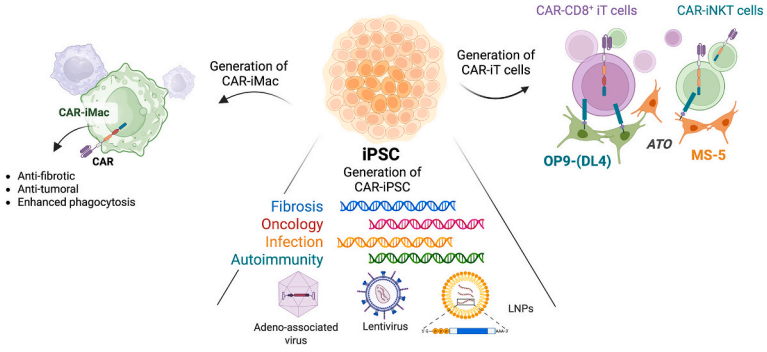
GRAPHICAL ABSTRACT



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ABSTRACT

Chimeric antigen receptor (CAR) technology has transformed the immunotherapy field with significant success in the treatment of hematological diseases. Nonetheless, challenges in scalability, donor variability as well as in the treatment of solid tumors warrants innovative solutions. Induced pluripotent stem cell (iPSC) technology has revolutionized the filed as an emerging renewable source for CAR-based therapies, facilitating the development of off-the-shelf immune cells products. This review focuses on the recent developments of iPSC-derived CAR-T cells and CAR-macrophages, including differentiation protocols, gene engineering strategies and mitigation of Graft-*versus*-Host Disease (GvHD), as well as alternatives for histocompatibility constraints. Additionally, we will

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discuss how iPSC-derivation enhances accessibility of low-frequency immune cell populations including MR1-restricted αβT, γδT, Natural Killer T (NKT) and Microglial cells. Despite great progress achieved, the limited but continuously growing clinical experience and manufacturing challenges, warrant further exploration. Advancements in manufacturing scalability and genetic engineering position iPSC-based therapies at the forefront of clinical strategies to address unmet clinical needs in cancer treatment.

1. Introduction

Chimeric antigen receptor (CAR) technology has revolutionized immunotherapy by enabling immune cells to target and destroy malignancies with precision. Despite the success of autologous and allogeneic CAR-T cell therapies in treating certain hematologic cancers, their limitations, including scalability, donor variability, and toxicity risks, highlight the need for novel approaches. The recent integration of induced pluripotent stem cells (iPSCs) into CAR-based therapy development has opened new horizons; a renewable cell source that can be differentiated and genetically edited for the manufacturing of various immune cell types expressing a CAR. This review focuses on the progress, challenges, and transformative potential of iPSC-derived CAR-T and CAR-macrophage therapies, paving the way towards the next-generation of off-the-shelf immunotherapies.

Differentiation protocols for lymphoid [1–3] and myeloid [4,5], cells have been established from both induced pluripotent stem cells as well as embryonic stem cells (ESCs) [reviewed in [6,7]] REF. Regardless of the stem cell source, these protocols mainly depend on three distinct phases. First, hematopoietic lineage commitment is achieved through the formation of mesoderm and hemogenic endothelium. Second, a hematopoietic induction, where cells are directed into transitioning into hematopoietic stem and progenitor cells, and third, lymphoid or myeloid specification. Lymphoid specification is achieved through Notch stimulation which results in the development of adaptive αβT cells including iPSC-derived Natural Killer T cells (iNKT) [8,9] and mucosal-associated invariant T (MAIT) cells [10] or the innate-like γδT cells [11]. Protocol specificities lie in the manner in which Notch stimulation is provided. Either Notch ligands are expressed on feeder cells (such as OP9 [1,12] or MS5 [3]), which are co-cultured with hematopoietic progenitors in a 2D- or 3D- fashion, or feeder-free protocols using plate- or bead-bound recombinant Notch protein [13–16]. For αβT cell differentiation, all protocols result in the generation of CD4/CD8 double positive T cells, which are then matured to the single positive stage. Contrary to what is found in peripheral blood, *in vitro* protocols mainly generate $CD8^+$ T cells but lack development of substantial $CD4^+$ T cell populations [3,12,17]. Myeloid specification from hematopoietic progenitors is achieved through cytokine stimulation. Different differentiation protocols make use of distinct cytokines combinations which, among others, apply combinations of GM-CSF, M-CSF and/or IL-3, and macrophage maturation/polarization [4,18]. Despite differences in differentiation protocols, all resulting cells possess morphological, phenotypical and functional characteristics associated with macrophages [4,19–22].

The transformational potential of Adoptive Cell Therapy (ACT) with CAR T cells has been firmly established in patients with hematologic malignancies [23]. However, extending ACTs potential into solid tumors has been challenging, due to both the lack of specific tumor associated antigens (TAA), tumor heterogeneity [24] and the inhospitable microenvironment for lymphocyte activation and infiltration [25]. Contrary to T cells, macrophages are known for their ability to infiltrate the tumor microenvironment (TME). Macrophages play crucial roles in host homeostasis with a plethora of functions, which range from phagocytic capabilities and cytokine production to clearance of transformed and dead cells [26]. Their vast potential in clinical applications has for long led to the pursuit of new and more efficient ways of obtaining and engineering this cell population.

Independent of the immune cell type, an additional challenge that wide-spread application of ACT faces is manufacturability costs and accessibility [27,28]. Conventional ACT is autologous, requiring individual generation and validation for each patient. The development of allogeneic sources for ACT would allow for centralization of ACT development for multiple patients, as well as standardization of the resulting cell product through elimination of patient variability [29,30]. Allogeneic ACT can be generated from healthy donor apheresis [31], cord blood [29], or differentiated from induced pluripotent stem cells (iPSC) [32]. In addition to effective tumor targeting and infiltration, allogeneic therapy needs to include strategies to avoid graft-*versus*-host disease (GvHD), graft-rejection as well as establish new processes, analytical methods and release assays and regulation for current good manufacturing (cGMP) compliant production and qualification. This is especially challenging for the development of iPSC-derived ACT due to the unprecedented biological and technical requirements the differentiating and engineering protocols necessitate.

1.1. Harnessing the power of iPSC-derived T cell therapy

T cell-based ACT either utilizes T cells' inherent ability to recognize tumor cells by enriching and expanding T cells carrying a T cell receptor (TCR) responsive to a TAA [33,34] or through re-targeting of the T cells' specificity by means of introducing a CAR [23]. Both TCR-specific and CAR-retargeted T cells have been generated from iPSCs (iT cells) [35–37] TCR-specific iT (TCR-iT) cells can be generated either through the reprogramming and re-differentiation of TCR-selected primary T cells (TiPSC) [35,36] or through introduction of the desired TCR into iPSC from a different parental origin [16,38]. For the generation of CAR-expressing iT cells (CAR-iT), genetic engineering is an inherent requirement and like TCR-iT, this can be applied at different stages during differentiation. However, CAR expression has been shown to affect T cell development and lineage commitment through its antigen-independent signaling, which add complexity in devising strategies to generate CAR-iT cells [12,37,39]. This effect can be mitigated through careful selection of CAR design [12,40,41] as well as its expression pattern during differentiation [12,39,42]. The current state and (biological) challenges of iT differentiation have recently been reviewed in [43]. Several clinical trials investigating the therapeutic potential of CAR-iT cells are currently ongoing or being initiated (NCT04629729, NCT06308978, NCT06241456) [44]. Of note, in the Phase 1 study designed to evaluate the safety, pharmacokinetics, and anti-B-cell activity of FT819 – an off-the-shelf anti-CD19 CAR T-cell therapy- in subjects with systemic lupus erythematosus (SLE) (NCT NCT06308978), rapid and deep peripheral depletion of B cells was observed in 3 patients. No severe adverse events and no cytokine release syndrome, neurotoxicity or dose limiting toxicities were reported and clinical improvements were observed in all patients including a patient who reached 6-month follow-up and who discontinued corticosteroid therapy by month 3 [45]. Based on these findings, these iPSC-derived anti-CD19 CAR T-cell therapy may be investigated in patients with other B-cell mediated autoimmune diseases, including ANCA-associated vasculitis, idiopathic inflammatory myositis and systemic sclerosis.

On the other end, clinical trials assessing the potential of TCR-iT cells are yet to commence. The findings obtained in the clinical trials will elucidate the areas in which the therapeutic capacity of iT cells can be further improved, as well as provide insights into the safety profile and hopefully confirm the lack of tumorigenic risks of using wisely designed and characterized iPSC-derived cells rather than primary cells.

Nonetheless, some anticipated challenges are already being tackled. Firstly, the allogeneic nature of iT cells carry the risk of graft-rejection as well as the induction of GvHD. GvHD can be addressed through elimination of TCR expression in the iT cells [12] but amelioration of graft-rejection requires a more multifaceted approach. The high heterogeneity of the αβTCR repertoire is crucial for T cells' ability to recognize a plethora of foreign and disease-associated peptides in self-HLA. During normal development, 'positive selection' determines the HLA-specificity of the generated TCRs, and 'negative selection' eliminates self-reactive TCRs [46]. However, small fractions of T cells with alloreactive or self-reactive TCRs also mature. In an allogeneic setting, these alloreactive T cells can contribute to GvHD [47,48]. HLA-matching can significantly reduce the risk of GvHD, but does not fully eliminate it [49]. HLA- and peptide-specific, such as Mart-1 [35,50], WT-1 [17] or LMP2 [51] iT cell populations can be generated from TiPSC, reducing the risks associated with unknown target specificity. Alternatively, truncated TCRα and TCRβ chains, termed mini-TCRs, which lack variable domains responsible for recognizing human leukocyte antigen (HLA)-peptide complexes have been developed to mitigate the risk of GvHD associated with αβTCR diversity [52].The potential of CAR-based therapies is also extensively being explored in malignancies of the central nervous system (CNS). This includes primary and secondary CNS lymphoma, as well as glioblastoma (GBM) and diffuse midline glioblastoma (DMG) [53–55]. For GBM and DMG, CARs targeting a wide range of antigen have been explored, including, but not limited to EGFR [56] and its variant EGFRvIII [57], IL-13Rα2 [58] and GD2 [59]. For CNS lymphomas CNS lymphomas, CD19-targeting CAR-T have been applied [53]. Although clinical responses were observed, therapeutic efficacy was challenged by antigen downregulation and limited T cell persistence in the CNS microenvironment [53,60]. The lack of T cell persistence within the CNS could be due to the fact that the CNS is an immune-privileged environment, meaning there is no T cell presence in healthy states, making infiltration and persistence of engineered T cells in that environment challenging [61]. This is where microglia (MG), the CNS-resident immune population, provide an attractive cellular alternative.

Mouse models have shown there is therapeutic potential in engineering MG. The design and expression of anti-Amyloid CARs in mouse microglia resulted in enhanced phagocytosis of Amyloid-beta, and engineered secretion of IL-15 by MG increased their pro-inflammatory phenotype and NK cell recruitment in models for glioblastoma [62].

Microglia can be derived both from monocytes (moMG) and iPSC (iMG). Although monocytes can re-populate the CNS upon loss of MG, and acquire MG-like signatures, they are unable to recapitulate a full, *bona fide*, MG phenotype [63,64]. This is likely due the fact that, although MG are closely related to monocytes and macrophages, they are derived from a distinct hematopoietic wave and progenitor population [65]. *In vitro* differentiation from iPSC is able to recapitulate the Yolk Sac-derived hematopoietic wave and generate MG progenitors [66] as well as mature MG cells [67–69]. iMG are transcriptionally similar to MG but require the CNS microenvironment and associated cellular interactions to more closely recapitulate MG epigenetic landscape and function [70–72] iMG are reported to be more similar to MG compared to moMG [73,74], however due to protocol variabilities in iMG generation, this might not be universally the case. iMG have shown therapeutic potential in mouse models of leukoencephalopathy [75,76] and neuropathic mucopolysaccharidoses [77], but CAR-expression in iMG is yet to be explored. Direct functional comparisons of iMG and moMG within CNS-associated malignancies are yet to be performed, however in a model for HIV infection, differences in viral replication and particle capture were noted [78]. These findings suggest that depending on therapeutic application, MG source could be a valuable consideration.

1.2. Engineering TCR-restricted T cell populations

The development of ACT with T cell subsets which have a more restricted TCR repertoire compared to αβT cells is an appealing alternative for allogeneic application. T cell populations with a specific TCR such as mucosal-associated invariant T (MAIT) cells [73], iNKT cells [74] or a restricted TCR repertoire such as γδT cells [75] can more easily be applied in an allogeneic setting when compared to αβT cells for the above mentioned reasons. Additionally, MAIT, iNKT and γδT cells have the benefit of being activated in a HLA-independent manner, decreasing the stringency of HLA-matching requirements, reducing the risk of GvHD [76] and expanding the ability for dual targeting through TCR-specificity and CAR expression [77–79]. However, their use as primary cells is limited due to low frequencies in peripheral blood making their application as iPSC-derived cells more feasible [80–82]. Nonetheless, all three populations can be obtained from cord blood as well as apheresis products [83–85], have successfully been reprogrammed into iPSC (from apheresis products) and subsequently re-differentiated into T cells, while retaining their original TCR specificity [9–11,86]. Primary MAIT cells are being explored for application in ACT both through their endogenous TCR [87] as well as through CAR expression [79]. MAIT cells express a semi-invariant major histocompatibility complex class I-related (MR1) restricted TCR and recognize metabolites and bacterial antigens [88] and are devoid of alloreactive potential [89]. Wakao et al. showed that human MAIT cells could be isolated from cord blood, reprogrammed into iPSC and redifferentiated into iMAIT cells. The resulting iMAIT cells were responsive to bacterial challenges both *in vitro* and *in vivo* [10]. They also showed that in an immunocompetent lung cancer model, murine-iMAIT cells have anti-tumor potential, thereby providing encouraging proof-of-principle data to explore iMAIT application in a human, clinical, setting. MAIT cells are not the only MHC class I–related molecule MR1-restricted T cell population. MR1-restricted T cells (MR1T) have a higher TCR diversity than MAIT cells, do not recognize bacterial antigens but are activated by MR1-bound self-antigens. It has been shown that specific TCR clones are able to kill an array of tumor cell lines [90]. MR1T are yet to be reprogrammed and differentiated *in vitro*, however their low presence in peripheral blood (<0.1 %) and unique tumor-specificity make them an unexplored but appealing additional candidate for iPSC-derived ACT.

On the other hand, iNKT cells express a semi-invariant, CD1d restricted TCR, along with NK-associated cell surface markers [74,91] but are not activated through HLA-mismatch [92]. Both autologous and allogeneic iNKT cells have achieved clinical results. Autologous CAR-iNKT cells have shown anti-tumor activity in pediatric neuroblastoma [93] and allogeneic, healthy donor derived iNKT cells have safely been applied in a phase 1/2 clinical trial for acute respiratory distress syndrome [94,95]. These studies confirm both efficacy of the cell population as well as safety in their allogeneic application. In spite of these encouraging early clinical results, iNKT cells are present at extremely low frequency (0.01 to 0.1 %) in human peripheral blood, hence the necessity to engineer a more sustainable source of these cells such as iPSC-derived iNKT (i-iNKT). i- iNKT cells are able to control tumors in xenograft models, through either their endogenous TCR [9,86] or CAR-expression [96–98] including potential cytokine release as a result of CAR-stimulation [99]. The first-in-human clinical trial utilizing allogeneic iNKT is currently ongoing in head and neck squamous cell carcinoma (jRCT2033200116) [86].

Whereas MAIT, MR1T and iNKT cells carry a restricted αβTCR, γδT cells express the γδTCR [75]. γδT cells are activated in an HLA-independent manner by nonpeptide antigens such as phosphoantigens [100]. In addition, the presence of γδ T cells in tumors has been demonstrated to be associated with the most favorable prognostic factor for overall survival in a meta-analysis across 39 tumor types examined in 18,000 patients [101]. ACT with autologous γδT cells have shown to be safe and induce progression free survival in Neuroblastoma [102] and show promise in glioblastoma multiforme [103]. Autologous, NKG2D-targeting CAR-γδT also did not induce any dose-limiting toxicities, but did show limited expansion and persistence in a phase 1 trial (NCT02203825) [104]. Prostate stem cell antigen-targeting is currently

Table 1-
Considerations and criteria for the production of Adoptive Cell Therapies from induced Pluripotent Stem Cells.

Process Step	Scientific Methodology	Regulatory Criteria	Items / Compliances
Cell Collection	Isolation of somatic cells (*e.g.*, skin or blood cells) from a donor	Donor health and consent	Informed consent Origin and ethnicity Health screening
		Cell quality	Sterility Genetic stability and viability Purity
		Ethical considerations	Ethical approval
Reprogramming process	Introduction of reprogramming factors (*e.g.* OCT4, SOX2, KLF4, and c-MYC) into the somatic cells to convert them into iPSCs	Compliance	GMP compliance Local and international regulatory compliance
		Reprogramming method	Viral system (adenovirus, Sendai virus) Non-viral system (Episomal, polycistronic minicircle DNA vectors)
		Quality standards	Reprogramming efficiency and quality Self-renewal capacity Clonal purity Genetic stability Pluripotency Viability Absence of reprogramming factors Identity Microbiological sterility
iPSC Cultivation, Expansion, and Banking	Culture the reprogrammed cells in a suitable environment to allow them to proliferate and generate stable iPSC lines	Compliance	GMP compliance Local and international regulatory compliance
		Culture components and cell expansion	GMP-compatible culture media and reagents Controlled cell expansion
		Cell banking	Traceability Reproducibility Cryopreservation SOPs
		Quality standards	Self-renewal capacity Clonal purity Genetic stability Pluripotency Viability Absence of reprogramming factors Identity Microbiological sterility
Differentiation Induction	Expose the iPSCs to specific differentiation signals (*e.g.*, growth factors, small molecules) to guide their differentiation into the desired cell type	Compliance	GMP compliance Local and international regulatory compliance
		Cell characterization	Phenotype Function Genetic stability
		Quality standards	Viability Identity Potency Safety
		Compliance	Standardized differentiation protocol GMP-compatible process GMP-compatible differentiation reagents
Final cell product preparation	Preparation of differentiated cells under conditions that support their growth and maturation upon final cell product	Cell characterization	Phenotype (*e.g.*: HLA, TCR) Function Genetic stability
		Quality standards	Viability Identity Potency Safety
		Compliance	GMP-compatible culture media and reagents GMP-compatible process

Abbreviations: iPSC: induced pluripotent stem cells; DNA: Desoxyribonucleic acid; GMP: good manufacturing practice; SOP: standard operating procedure; HLA: human leukocyte antigens; TCR: T-cell receptor; OCT4: octamer-binding transcription factor 4; SOX2: SRY-box transcription factor 2; KLF4: Krüppel-like factor 4.

being evaluated in a phase 1 trial (NCT06193486) [105] and allogeneic CAR-γδT cells are being tested in phase 1 trials in both solid (NCT05302037) and hematologic malignancies (NCT04735471) [106], and in autoimmune diseases (NCT06375993) such as lupus nephritis. These trials will provide valuable insight into the tolerability and persistence of γδT cells in an allogeneic setting, and thereby, will serve as proof-of-principle for development of iPSC-derived γδT cells (iγδT). Therefore, the development of a sustainable and reliable source of iPSC-derived γδT (iγδT) cells is essential due to the low frequency of endogenous γδT cells in the peripheral blood of patients and healthy donors (0.5 to 10 %). In preclinical studies, iγδT cells have already shown to be effective against a range of tumors *in vitro* [11] both through their TCR or NKG2D. Preliminary reports on CAR-iγδT suggest efficacy *in vivo* against hematologic malignancy [107,108].

The restricted, HLA-independent TCRs from these cell types, along with the potential to utilize both their inherent anti-tumor recognition with CAR-retargeting makes iMAIT, i-iNKT and iγδT appealing αβT cell alternatives considering the reduced risk for GvHD and less stringent histocompatibility requirements. However, the comparatively limited clinical experience utilizing their primary counterparts compared to αβT cells make identification of the best malignancies, engineering strategies and treatment designs more challenging. Additionally, clinical results cannot as easily be benchmarked against autologous primary cells, providing reduced insight into whether clinical attributes should be prescribed to the cell-type itself, or a limitation due to their iPSC-derived origin.

1.3. Early developments in macrophage-based therapy

As mentioned, while clinical success has been achieved with the use of CAR-T cells in hematological tumors, which resulted in Food and Drug Administration (FDA) approval of several cell therapy drug products, and very promising clinical outcome have been demonstrated in patients with autoimmune diseases [109,110], the same cannot be said for solid tumors [25]. In this scenario, the immunosuppressive TME that hampers

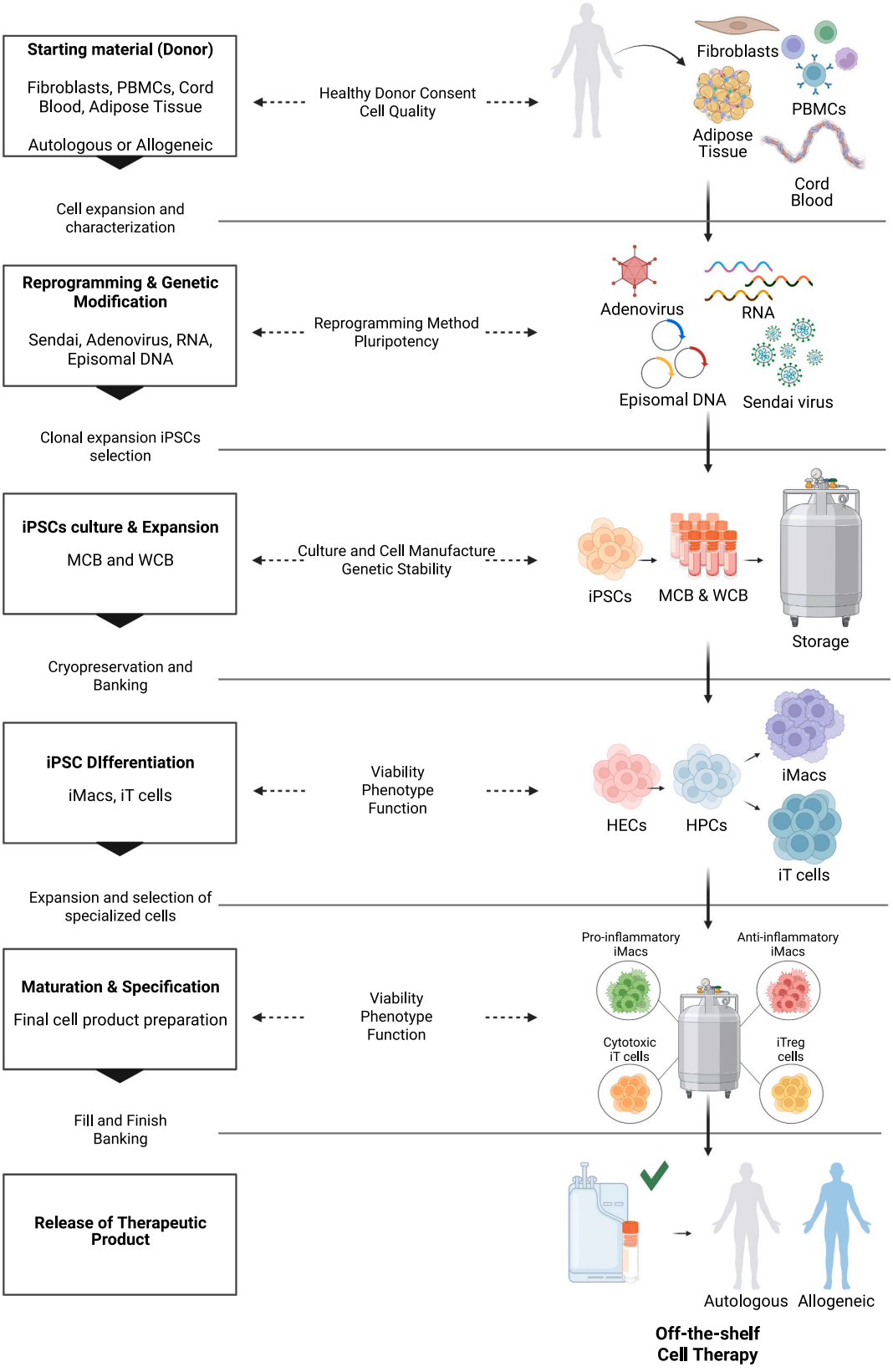

Fig. 1. Overview of key processes in induced Pluripotent Stem Cell-derived therapy production.

immune activation, coupled with poor tumor penetration of T cells, has resulted in less than promising outcomes. This was shown in a meta-analysis study concluding that the response rate to CAR T therapy is only 9 % in solid tumors, with gastrointestinal malignancies proving to be the least responsive to this type of therapy [111]. However, contrary to T cells, macrophages are known for their ability to infiltrate the TME and play crucial roles in host homeostasis. For these reasons, the development of more effective ways of obtaining macrophages has for

long been of interest. Among others, two major approaches are currently worth noting; Monocyte-derived Macrophages and induced iPSC-derived macrophages.

More than 30 years ago, in 1988, Dumont, S. *et. al*, reported on the effectiveness of monocyte-derived autologous macrophages in tumor elimination both *in vitro* and *in vivo*. In this study, co-culture of mononuclear cells in hydrophobic Teflon bags for 7 days (supplemented with autologous serum on day 5) resulted in the differentiation of monocytes into macrophages. Further, the addition of recombinant human Interferon gamma (IFNγ) and muramyldipeptide for 24 h, led to activated macrophages with cytotoxic capacities [112]. Just two years later, in 1990, a clinical trial harnessing the tumoricidal capacities of autologous monocyte-derived macrophages in the context of lung cancer, ovarian cancer and melanoma, among others, was conducted. Here again, mononuclear cells were cultured in hydrophobic Teflon bags in the presence of autologous serum for 7 days and cells were stimulated with IFNγ prior to harvest. Monocyte-derived macrophages were purified from contaminating lymphocytes and thrombocytes by recourse to elutriation systems. This approach resulted in mature, secretory and cytotoxic macrophages *in vitro*, with good toleration of the adoptively transferred cells *in vivo* [113]. In fact, many other early studies demonstrated the safety of adoptive transfer of autologous monocyte-derived macrophages in metastatic solid tumors. However, *in vivo* effectiveness of the cell product was lacking [114–117]. This was believed to be a consequence of the TME and for this reason; efforts to further manipulate the monocyte-derived macrophages were undertaken. Going beyond this historical view, more recent and refined strategies are now being used with the purpose of rendering macrophages more efficient in their cancer fighting capabilities. For instance, Klichinsky et al. reduced the frequency of erythrocytes, platelets, granulocytes and lymphocytes by elutriation followed by $CD14^+$ enrichment of healthy donors' apheresis prior to differentiation. The differentiation is then achieved in culture bags in the presence of GM-CSF for 7 days. Furthermore, a genetic manipulation step of the cell product, for instance by viral transduction, in process can be introduced [118,119].

1.4. iPSC-derived macrophage and new therapeutic applications

Despite the great progress made since the very first steps in macrophage therapy taken in 1988, reliance on continuous leukapheresis from autologous or allogeneic sources, as performed in the aforementioned studies, is a long and strenuous process. The development of iPSC technology may offer a solution for a reliable and standardized source of macrophages. The work by Senju [120], Higaki [121] as well as our group, using iPSC [4], and others, using ESCs [5], highlight the versatility of established protocols for the differentiation of macrophages and the possibility of cell modification (also reviewed in [5] [7,122,123]).

In addition, the ability to genetically modify iPSC-derived macrophages (iMacs) further supports the advantageous use of iPSC as cell source over autologous or healthy donors-derived cells. Yet another advantage of using iPSC as primary material lays in the ease of scaling up, which is necessary in order to reach the high cell numbers usually required for ACT and be harnessed for an unlimited, off-the-shelf, universal product, reducing bottlenecks at the cell procurement level [124,125]. Even though new developments have facilitated the genetic manipulation of myeloid cells thought the use of a lentiviral vector packaging the Vpx accessory protein [126,127] macrophages are known to be resistant to genetic manipulations [128]. In contrast, iPSC are highly permissive to genetic modifications [129], which facilitates engineering at the pluripotent stage that can be retained by these cells progeny. Alternatively, Klichinsky et al., showed that monocyte-derived CAR-macrophages were able to promoted chemotaxis of both resting and activated T cells *in vivo* and retained antigen-presenting capabilities. Moreover, viral transduction skewed these macrophages towards an M1 phenotype, thereby helping to overcome the immunosuppressive tumor microenvironment (TME) [119]. Furthermore, Shah et al. developed CAR-iMacs directed at prostate stem cell antigen, which co-expresses membrane-bound IL-15, capable of promoting immune activation, and a truncated form of epidermal growth factor receptor, used as a suicide gene. The authors also optimized the cryopreservation of CAR-iMacs progenitors, promoting in this way, an off-the-shelf product [124]. Our group was also able to genetically engineer iMacs to express anti-CD19 CAR with increased phagocytosis of $CD19^+$ target cancer cells accompanied by increased secretion of pro-inflammatory cytokines *in vitro*. Furthermore, the scalability of the production of these CAR-iMacs was also achieved [130].

The ability to genetically engineer iMacs with their innate capabilities of tumor infiltration and an unlimited source of progenitor cells, opens up completely new avenues of possible applications in the field of cancer immunotherapy, namely, in the treatment of solid tumors. It is well established that tumor-associated macrophages (TAMS) play a crucial role in tumor-promotion. Therefore, having the limitation in cell source and cell numbers resolved by the use of iPSC, the education of macrophages and T cells prior to adoptive transfer achieved through genetic manipulation, and GMP-compliant conditions established, the next milestone of therapeutic development could focus on the genetic manipulation of cells *in vivo*. This can be a key factor both as a mechanism of prevention of exhaustion/modification by the TME and as a promoter of tumor burden reduction, controlling the number of TAMs and suppressive T cells. In fact, TAMs were initially identified as immune cells capable of eliminating malignancies in the 70's [131], but subsequent studies have demonstrated their involvement in tumor initiation and progression [132]. For this reason, only providing 'educated' macrophages may not produce a definitive solution for cancer treatment. Instead, rational manipulation of these cells once they have been adoptively transferred may be required. Several approaches are being examined in this context. For instance, targeting colony-stimulating factor 1 (CSF1) or its receptor (CSF1R) using monoclonal antibodies (mAbs) in pre-clinical models of glioblastoma multiforme and lung tumor resulted in a decreased density of TAMs and tumor-growth and increased the tumor's sensitivity to chemotherapeutic drugs. This was reported as the result of the reprogramming of macrophages from the tumor-bed towards an anti-tumor phenotype, where pro-tumorigenic genes expression was lowered and upregulation of antigen presentation and lymphocyte activation genes was seen, rather than their depletion [133,134]. The same strategy was applied in diffuse-type tenosynovial giant cells and clinical significance was verified in 71 % of the patients included in the study [135]. In contrast, clinical trials assessing the combinatory effect of CSF1R inhibition coupled with different chemotherapeutic drugs in other types of tumors have shown little to no activity. However, several phase 2 trials are still ongoing (summarized in [136]) and outcomes are still pending at the time of writing.

Many other ongoing studies explore the use of monoclonal antibodies, namely to target checkpoint inhibitors. The role of programmed cell death protein (PD-1) and its ligand interaction is well established in T cells and it has been demonstrated that T cells are activated when this receptor-ligand interaction is disrupted. However, only recently the role of PD-1 has been investigated in TAMs, after its expression on macrophages was described in the context of infections [137–139]. A triple negative breast cancer clinical trial assessing the effect of Cabiralizumab (targeting CSF1R) with Novilumab (targeting PD-1) and neoadjuvant chemotherapy is undergoing (NCT04331067). However, as of an abstract from March 2023 reporting on the interim analysis, severe toxicity was seen in many enrolled patients [140]. Conversely, two other studies following the same approach of targeting both PD-1 and CSF1R reported on its safety and efficacy in 2020 [141] and 2022 [142]. In both cases, the authors reported on the safety of the compounds tested while efficacy/clinical benefit were lacking.

Another focal point of investigation for *in vivo* targeting strategies relates to the blockade of signal-regulatory protein alpha (SIRPα) and CD47 interaction. The interaction between CD47 and SIRPα triggers a

'don't eat me' signal, which results in phagocytic inhibition by macrophages. Therefore, since the first reports demonstrating that this blockade could restore the phagocytosis of tumor cells by macrophages, clinical trials have been undertaken (NCT02216409, NCT02641002, NCT02367196). Once again, conflicting outcomes have been achieved with one study being discontinued due to lack of activity and presence of anti-drug antibodies in patients' blood [143]. On the other hand, patients with advanced solid tumors or lymphoma exhibited a good tolerance to the anti-CD47 antibody in the used dose regimen, no maximum tolerated dose was achieved, good receptor occupancy, mild to moderate toxicities as well as two partial remissions in the case of ovarian/fallopian tube cancer patients [144].

1.5. From bench to bedside: Pre-clinical concepts and CMC standards in iPSC-derived therapies

1.5.1. Clinical translation of iPSC-derived therapies

The next milestone in the production of iPSC-derived ACT relies on the adaptation of established protocols under GMP conditions. Several groups have reached clinical trials in the setting of autologous transplantation for applications ranging from non-acute strokes, which resulted in better neurological recovery of treated patients [145] and in the liver cirrhosis field, where safety and feasibility of the cell transplant was demonstrated [146]. Also, the treatment of solid tumors overexpressing HER2, where autologous macrophages were engineered to express anti-HER2 CAR, with preliminary clinical results demonstrating the safety and feasibility of manufacturing, as well as, trafficking of transplanted cells, TME modulation and potential induction of T cell was demonstrated [118]. However, in many instances, the use of autologous cells is not advised or even possible. Albeit the growing interest, the progress made in the development of iPSC-derived immune and blood cell therapies is still in its earlier stages and GMP-compliant differentiation techniques are still emerging and being transferred into clinical trials (reviewed in [147]). Wilgenburg, B, et al. have been able to genetically modify iPSC, induced their differentiation in high numbers of macrophages in serum-free, fully defined culture conditions [19]. As pharmaceutical companies have been leading the way in clinical application of iPSC-derived T cells, differentiation protocols are therefore not publicly available [147], however, several feeder-free protocols that could eventually be applied for GMP-compliant differentiations have already been described [13–15].

1.5.2. Challenges in allogeneic cell transfer and immune evasion

The next critical step in iPSC-derived ACT relates to the possibility of graft rejection, as allogeneic cell transfer comes with an inherent risk of rejection. Several immune mechanisms contribute to this, requiring a combination of (engineering) strategies often referred to as 'cloaking' or 'stealth' to ensure persistence of allogeneic cells. HLA-matching reduces the risk of T- and NK-cell mediated rejection [148] and anti-HLA antibody responses [149,150]. However, it does not protect against other rejection mechanisms, and still requires 8/8 HLA matching. The utilization of iPSC derived from HLA-homozygous cord blood units [151–154] increases the accessibility of matching donors, but still requires a range of iPSC master cell banks (MCBs) to serve a diverse population. The requirement of HLA-matching can be simplified through elimination of class I and/or class II expression [155–157] thereby eliminating T cell-mediated rejection, but rendering the cells sensitive to NK cell-mediated rejection due to 'missing-self'. Overexpression of HLA-E [157], HLA-G [158] or expression of a novel CD300a agonist ligand [159] have been applied to avoid NK activation. HLA-independent rejection mechanisms also need to be addressed. For example, overexpression of CD47 reduces macrophage activation [157,160] and complement deposition can be avoided through elimination of CD46, CD55 and CD59 expression [155]. Full 'cloaking' of the allogeneic cells requires multiplexed engineering combining these strategies [155,157]. This can more effectively be applied in iPSC compared to primary cells due to the ability to clonally select the iPSC carrying all required edits. However, genetic edits can affect immune cell differentiation and additionally, iPSC are not immune to off-target effects [161] or genomic instability [162]. How to best monitor these risks during prolonged use of MCBs remains yet to be addressed.

1.5.3. Advantages and risks of iPSC-derived ACTs

iPSC-derived immune and blood cell therapies can offer several potential advantages in comparison to conventional auto- and allogeneic therapies. As already highlighted, and unlike autologous cells, iPSCs provide a renewable and scalable source of cells, which allows the generation of (standardized) large batches of off-the-shelf products [37,163]. Their permissiveness towards genetic engineering facilitates the introduction of further improvements such as CAR expression, cloaking strategies and even functional enhancements [37], which may be difficult to implement in primary cells. However, the risks associated with the use of iPSCs are noteworthy. These cells are susceptible to genomic instability and off-target effects during reprograming, expansion or gene editing can lead to increased risk of tumorigenicity, as well as the risk of teratoma formation resulting from infusing undifferentiated iPSCs [164–166]. Furthermore, long-term extensive *in vivo* persistence and characterization of iPSC-derived immune cells is still lacking or incomplete compared to more conventional therapies [37,163]. While iPSC-derived ATCs present transformative opportunities for scalable, standardized and highly engineered therapies, caution and risk-based approaches with thorough and robust pre-clinical validation and long-term patient monitoring is warranted.

1.5.4. HLA-homozygous donor banks and population coverage

As previously mentioned, generation of iPSC from HLA-homozygous donor material can greatly simplify HLA-matching requirements. Stem cell donor registries and cord blood banks are attractive sources for such banks because it allows for identification of units with optimal immune tolerance, both based on blood type and HLA profile. In addition, cord-blood derived cells have less somatic damage compared to adult, healthy-donor derived cells. Worldwide several banks are being established (recently reviewed in [167]).These banks mainly target coverage of the HLA-diversity present in their respective geographic location. The population-wide HLA-diversity dictates the size requirements of such banks. For example, due to the low HLA diversity of the Japanese population, a total of 10 donors could cover 50 %, and 140 donors roughly 90 % [154]. Similarly, Saudi Arabia would be able to serve 30 % of their population with 13 donors, 50 % with 39, and 90 % with 596 [168]. Spain has identified 7 donors, covering 20 % of their population [151] and 559 lines would cover 95 % of the Brazilian population [169]. In more diverse populations, however, significantly more donors are required. For example in the United Kingdom, 150 donors would cover 20 % and 10,000 donors merely 37.7 % of the population [170]. For such diverse populations, further simplification of the HLA profile through, for example, targeted disruption of HLA haplotypes [171] could significantly reduce the amount of donors required.

1.5.5. cGMP-compliant differentiation of iPSC-derived immune cells

The differentiation of iPSC-derived T cells (iTs) and iPSC-derived macrophages (iMacs) under conditions compliant with cGMP standards is challenging due to several factors such as xenogeneic feeder cells (for iTs), culture components, hurdles in standardization and scalability, and regulatory requirements. The use of feeder cells for iTs is driven by the physiological T-cell development requirement, whereby bone marrow-derived hematopoietic progenitors commit to the T-cell lineage in the thymus *via* interaction with the Notch signaling, although alternative differentiation protocols without feeders are being developed [13–15]. The risk-based approach strategy in the context of iPSC production involves using scientific and regulatory knowledge to assess and manage risks associated with the identity, quality, purity, safety, and efficacy of the cells as well as their suitability based on the

regulatory requirements in the intended country where they are produced in addition to those where the clinical trials may be conducted. The regulatory requirements may vary from one country to another which increases the risks especially in the early stages of development, when the investigators are facing resource constraints and cannot accommodate global regulatory requirements nor predict the evolution of such regulatory guidelines. This risk-based approach includes evaluating the origin and characteristics of the cells, the biological properties of vectors used in reprogramming iPSCs, the properties of raw materials, and the manufacturing processes in view of the intended use. Specific controls and mitigations need to be introduced to reduce these risks, and regular reviews and reassessments ensure that the measures remain effective or can be adapted to evolving scientific knowledge and regulatory requirements [172].

1.5.6. Cell banking: seed, master, working and research banks

The tiered banking system involves the use of ethically sourced somatic cells for iPSC derivation to generate Seed Banks, which are then used to create cGMP-compliant Master Cell Banks (MCBs) and Working Cell Banks (WCBs). Research Banks (RBs) can also be derived from MCBs with fewer quality control tests, and testing can be staged to mitigate costs, such as not repeating viral tests on downstream stocks as long as quality and traceability of reagents ensure no further exposure [173].

Cell Banks must be tested at various stages of the cell banking process to ensure the safety, identity, and quality of iPSC and iTs/iMacs cell stages. Testing should be performed by accredited laboratories using phase appropriate qualified or validated tests, following FDA/EMA requirements [172] and International Pharmacopeia methods. Specific recommendations for safety, identity, and genomic testing are reviewed in detail elsewhere [173]. The testing is designed to be comprehensive and includes sterility, mycoplasma, human viruses, bovine and porcine pathogens, murine pathogens, post-thaw viable cell recovery, morphology, identity, blood typing, histocompatibility, undifferentiated marker expression, pluripotency, and genomic stability. To this end, the international stem cell banking initiative [174] and the Global Alliance for iPSC therapies (GAiT) provide general guidance regarding clinical-grade iPSC standard in order to support the implementation and clinical application of iPSC therapies [175].

1.5.7. Cryopreservation: challenges and guidelines

Cryopreservation is one of the crucial steps in therapeutic success of cell-based therapies and requires special attention. Preservation of cellular products allows for the storage, transport and wide spread distribution for clinical use. Typically, the cryopreservation process employs cryoprotectant agents (CPAs) like dimethyl sulfoxide (DMSO in combination with controlled-rate freezing devices, as well as ultra-low storage temperatures in order to guarantee full arrest of molecular motions and therefore ensure preserved cell viability and function at thaw [176]. Knowledge gained form the cryopreservation of CAR-T therapies may be applied to other cell types and immune therapies. For instance, the International Society for Biological and Environmental Repositories (ISBER) and the United States Pharmacopeia (USP) have compiled and published guidelines for the establishment of standardized cryopreservation by outlining key elements in the evaluation of recovery and functionality post-thaw [177] [178]. Also the EMA has published documentation to support the GMP practices in the development of Advanced Therapy medicinal products, which among others, includes guidelines on cryopreservation [179]. As more guidelines and recommendations are made available, the more standardized cell products will become and the more expedite their approval will become by regulatory entities such as the FDA and EMA. The cryopreservation of macrophages has been less extensively studied and poses additional challenges. This is related to the fact that macrophages contain cellular structures rich in water, such as vacuoles and lysosomes, which increase the chances of ice formation during cryopreservation [180].

When considering the cryopreservation of CAR-T cells and CAR-macrophages, several factors need to be taken into account, including cryopreservation equipment and protocols, medium, sample vessels, quality control samples and documentation. These factors are well described in the document "UK Review and Recommendations on Cryopreservation of Starting Materials for ATMPs", released by the Advanced Therapy Treatment Centers in the UK [181]. Some of the considerations to take into account in the development of cell products to be used as ACT are summarized in Table 1 and a schematic representation of the different approaches involved in the development of these products can be found in Fig. 1.

2. Conclusion

iPSC-derived immune cells hold great promise to increase both accessibility as well as the cellular diversity of ACT. The first clinical results with iPSC-derived CAR^+ Natural Killer cells confirm this promise [182]. Here we outlined the unique challenges that clinical translation of allogeneic, iPSC-derived therapy faces. This includes both the inherent biological challenges associated with allogeneic cell transfers, as well as the technical challenges of developing xeno-free, GMP-compliant differentiation protocols and cryopreservation methods. With the establishment of HLA-homozygous iPSC MCBs, development of xeno-free reagents and formulation of guidelines, we should see further progression of therapies from bench to bedside.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [N.L. declares a patent application on the generation of human iPSC-derived macrophages (IP PCT/EP2018/061574, EP24176410.9) and received research funding from Novo Nordisk. I.R. is employed at Takeda, Cell Therapy Science].

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Figure Legend: The workflow begins with the procurement of donor cells, either from autologous or allogeneic sources. These cells are reprogramed into induced pluripotent stem cells that are used to generate cell banks (iPSCs). Following this, iPSCs can be used in the differentiation of specialized cell types, such as iMacs and iT cells, under GMP-compliant conditions, ensuring a high-quality, ready-to-use cell therapy product for clinical application.

Abbreviations: iPSCs: induced pluripotent stem cells; PBMCs: peripheral blood mononuclear cells; RNA: Ribonucleic acid; DNA: Desoxyribonucleic acid; MCB: Master cell bank; WCB: Working cell bank; iMac: iPSC-derived macrophages; iT cells: iPSC-derived T cells; HECs: Hemato-endothelial progenitor cells; HPCs: Hematopoietic progenitor cells; iT Regs: Regulatory iPSC-derived T cells

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Data availability

No data was used for the research described in the article.

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