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Induced pluripotency as a potential path towards *i*NKT cell-mediated cancer immunotherapy

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Abstract Invariant natural killer T (*i*NKT) cells are characterized by the expression of an invariant Vα14–Jα18 paired with Vβ8/7/2 in mice, and Vα24–Jα18 with Vβ11 in humans, that recognizes glycolipids, such as α-galactosylceramide (α-GalCer), presented on the MHC class I-like molecule, CD1d. *i*NKT cells act as innate T lymphocytes and serve as a bridge between the innate and acquired immune systems. *i*NKT cells augment anti-tumor responses by producing IFN-γ, which acts on NK cells to eliminate MHC-non-restricted (MHC^-) target tumor cells, and on $CD8^+$ cytotoxic T lymphocytes to directly kill MHC-restricted (MHC^+) tumor cells. Thus, when *i*NKT cells are activated by α-GalCer-pulsed dendritic cells, both MHC^- and MHC^+ tumor cells can be effectively eliminated. Both of these tumor cell types are simultaneously present in cancer patients, and at present *i*NKT cells are only the cell type capable of eliminating them. Based on these findings, we have developed *i*NKT cell-targeted adjuvant immunotherapies with strong anti-tumor activity in humans. However, two-thirds of patients were ineligible for this therapy due to the limited numbers of *i*NKT cells in their bodies. In order to overcome the problem in cancer patients, we successfully established a method to generate *i*NKT cells with adjuvant activity from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). In this review, we would like to outline the clinical potential for *i*NKT cells derived from ESCs and iPSCs for cancer immunotherapy, and the technical hurdles that must be overcome if we achieve effective ESC/iPSC-mediated cancer therapies.

Keywords Invariant natural killer T (*i*NKT) cells · Embryonic stem (ES) cells · Induced pluripotent stem (iPS) cells · Cellular immunotherapy

Introduction

Invariant natural killer T (*i*NKT) cells are characterized by the expression of a unique single invariant antigen receptor encoded by Vα14–Jα18, mainly associated with Vβ8.2 in mice [1] and Vα24–Jα18 with Vβ11 in humans [2]. In addition, the limited repertoire of *i*NKT cells appears to be autoreactive and, as a result, these cells are persistently activated by endogenous glycolipid antigens in association with the monomorphic MHC class I-like molecule, CD1d [3–5]. These characteristics are quite distinct from conventional T cells, which have a highly diverse repertoire and mainly recognize peptide antigens with polymorphic MHC molecules. The invariant Vα14–Jα18 TCRα is used only by *i*NKT cells, and not by conventional T cells. Thus, when a pre-rearranged Vα14–Jα18/Vβ8.2 gene is introduced into recombination activating gene (RAG)-deficient mice, only *i*NKT cells (and not conventional T cells or NK cells) develop, defining *i*NKT as a unique lymphocyte subset [6]. Conversely, Jα18 knockout mice specifically lack *i*NKT cells, but have normal numbers of conventional T cells [7].

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Here, we describe the properties of *i*NKT cells and their adjuvant activity, features that are now being manipulated therapeutically to treat human cancer. We also describe a method for the generation of large numbers of *i*NKT cells from ES cells or iPS cells in vitro, which is an important advance in overcoming the problem of the limited number of *i*NKT cells in advanced cancer patients.

Mechanisms of *i*NKT cell-mediated adjuvant activity augmenting anti-tumor responses

The promiscuous production of T helper cell (Th)1, Th2, and Th17 cytokines is a characteristic feature of *i*NKT cells [8–10]. Their recognition of self-ligands leads to the accumulation of cytokine mRNAs and up-regulation of activation markers, indicating that *i*NKT cells in the steady state are already primed and ready to quickly mediate their effector functions, serving as a bridge between innate and acquired immunity [1]. However, their recognition of endogenous ligands does not elicit cytokine production, only transcript accumulation, as *i*NKT cells require additional signals to produce cytokines mediating their functions.

The nature of these additional signals is the key event that determines *i*NKT cell function. Immature dendritic cells (DCs) in the steady state can capture antigens, but are unable to activate conventional T cells. However, *i*NKT cells can be activated by immature DCs and then reciprocally induce the maturation of DCs. A single in vivo injection of α-galactosylceramide (α-GalCer), a synthetic exogenous glycolipid ligand for *i*NKT cells presented by CD1d [6], induces a burst of interleukin (IL)-12 production (at 6 h) by DCs followed by Th1 cytokine interferon (IFN)-γ production by *i*NKT cells (at 24 h) [11–18]. This response is triggered by interaction between CD40 on DCs and CD40 ligand (CD40L) on *i*NKT cells, which occurs within 2–6 h after α-GalCer injection [19]. The *i*NKT cells are necessary for this DC maturation, as it does not occur in $J\alpha18^{-/-}$ *i*NKT cell-deficient mice. The maturation of DCs is blocked in tumor patients, due to their production of immunosuppressive cytokines, such as TGF-β and IL-10 [20, 21]. Thus, the in vivo maturation of DCs by activated *i*NKT cells is an important strategy for the augmentation of protective immunity in cancer patients.

Because of their self-reactivity and ability to quickly release large amounts of cytokines, *i*NKT cells link the two immune systems, serving as a bridge between the innate and acquired systems, and thus augment protective immune responses, including anti-tumor immune responses, through the activation of NK and $CD8^+$ CTLs [9]. This augmentation of protective immunity is known as the *i*NKT cell-mediated adjuvant effect, and is critical for tumor eradication. Since tumor cells do not provide any adjuvant effects or "danger signals" to activate the immune system, they fail to induce tumor-specific immune responses sufficiently potent to eradicate tumor cells even when tumor-specific T cells are present. Moreover, there are two general types of tumor cells in a tumor mass: one is MHC^+ and the other is MHC^- [20, 21], and effective tumor immunity requires that both types of tumor cells be eliminated simultaneously. *i*NKT cells are the only cell type that is able both to interact with immature DCs, inducing their maturation, and to augment the function of both NK cells and $CD8^+$ CTLs.

IFN-γ produced by activated *i*NKT cells is a key cytokine for mediating adjuvant activity. By this mechanism, *i*NKT cells can induce the maturation of DCs, which thereby acquire the ability to present tumor antigens to CTLs. The activated CTLs can then eliminate MHC^+ tumor cells. IFN-γ can also activate NK cells, which kill MHC^- tumor targets. The efficacy of this approach has been demonstrated in several studies in which treatment of tumor-bearing mice with α-GalCer-pulsed DCs to activate endogenous *i*NKT cells leads to the eradication of established metastatic tumors [22]. Thus, the activation of endogenous *i*NKT cells is a promising strategy for the treatment of cancer by selectively triggering protective anti-tumor immunity.

*i*NKT cell-targeted adjuvant cell therapy for patients with advanced lung cancer

Based on the above translational studies in tumor-bearing mice and the highly conserved nature of the *i*NKT/CD1d system, α-GalCer was approved for use as a drug for clinical applications. Several clinical trials involving the injection of α-GalCer-pulsed DCs have been carried out in patients with cancer, including colon cancer, multiple myeloma, anal cancer, and renal cell cancer [23, 24]. Although no clear tumor reduction was detected, tumor markers were significantly decreased.

We and colleagues have completed a phase I/IIa clinical trial of *i*NKT cell-targeted adjuvant therapy using α-GalCer-pulsed DCs (total 4×10^9 cells per patient in four consecutive injections at 1 week intervals) on 17 patients with advanced lung cancer, including stage IV, IIIB primary cancer, and recurrent tumor after surgery [25–28]. The patients' peripheral blood mononuclear cells were cultured with GMP grade GM-CSF and IL-2 for 2 weeks to increase the number of DCs, pulsed with α-GalCer for 24 h, and then administered intravenously into the donor. Significant increases in the number of IFN-γ-producing cells were detected in 60 % of enrolled patients (10 of 17) and this correlated with a prolonged median survival time (MST) of

31.9 months without tumor progression and metastasis, with only a primary treatment and no further additional treatment. Interestingly, none of the 10 patients with longer survival times showed tumor regression [28]. By contrast, the patient group with low IFN-γ production had a MST of 9.7 months, which is equivalent to the MST of 10 months after treatment with commercially available molecular target drugs such as anti-VEGFR antibody (Avastin: 13.1 months), anti-EGFR (Erbitux: 10.1 months), Folic acid inhibitor (Alimta: 8.3 months), EGFR inhibitor (Iressa/Tarceva: 6.7 months). Thus, IFN-γ appears to be a good biological marker predictive of a favorable clinical course.

However, despite the clear anti-tumor activity of the *i*NKT cell-targeted therapy, two-thirds of patients were not eligible, as they no longer had sufficient *i*NKT cells (<10 cells/ml of blood).

Generation of ES cells and iPS cells from mature *i*NKT cells

In order to overcome the problem of the limited number of *i*NKT cells in cancer patients, we attempted to establish methods to supply *i*NKT cells. Embryonic stem (ES) cells are a powerful model system to study in vitro lymphocyte differentiation, addressing the questions on the cells with *i*NKT precursor potential and the ability of *i*NKT cells to produce both Th1 and Th2 cytokines during their development. For example, embryoid bodies generated from ES cells contain $CD34^+$ cells that develop into lymphocytes when co-cultured with OP9 stromal cells plus appropriate cytokines [29]. However, in most cases, ES cells generate B and NK cells, but not T or *i*NKT cells on OP9 co-culture [30, 31]. In contrast, OP9 stromal cells transduced with Notch ligand delta-like1 (OP9/Dll-1) are used for the directed differentiation of ES cells to T cell lineages. Over-expression of active Notch1 directs stem cells to differentiate into immature DP T cells and inhibits B cell development, indicating that Notch signaling is required as a proximal event in T cell commitment from progenitors [32, 33].

Another approach is to use cloned ES (*i*NKT–ES) cells established by nuclear transfer of a cell with a rearranged T cell receptor (*Tcr*) gene [34, 35]. Interestingly, *i*NKT cells retain high genome re-programmability (71 % for *i*NKT vs. 12 % for T) [35]. The *i*NKT cell nucleus may therefore provide important clues for analysis of differentiation pathways from ES cells to mature *i*NKT cells. Based on these findings, we have established *i*NKT–ES cells generated by nuclear transfer of C57BL/6 (B6) liver *i*NKT cells, into B6D2F1 unfertilized eggs followed by re-transfer of 2-cell stage nuclei into the 2-cell stage cytoplasm of (B6D2F1xICR)F1 in vivo fertilized embryos. In total, six blastocytes were obtained from the 147 initial 2-cell stage embryos. This efficiency (4 %) is similar to that reported for clones from cumulus cells (2.3–6.9 %) or adult fibroblasts (1.1–3.8 %) [35]. Four of six cell lines established were analyzed by genomic PCR using primers that detect the non-rearranged *Vα14* gene and primers that specifically detect the *Vα14–Jα18* gene rearrangement. This analysis demonstrated that all established *i*NKT–ES clones contained the rearranged genomic *Vα14–Jα18* on one chromosome and the germline configuration on the other [36].

Since iPS cells are functionally equivalent to ES cells in many respects [37–39], these results suggested the feasibility of using iPS cells for *i*NKT cell-targeted adjuvant therapy. It is important to note that iPS cells rather than ES cells are more feasible in the clinical setting, as embryos or donor oocytes are unnecessary for the generation of iPS cells. In addition, iPS cells are fully syngeneic, while ES cells, even if introduced with patient cell nucleus, carrying oocyte-derived mitochondrial maternal antigens may trigger allogeneic immune responses.

Although several attempts have been made to generate iPS cells from mature B and T cells by the introduction of *Oct3/4*, *Sox2*, *Klf4* and *c-Myc*, the efficiency is very low [40]. Since *i*NKT cells have a higher reprogramming activity compared to conventional T cells, we thought that *i*NKT cells would be suitable for generating iPS cells. In fact, we successfully generated iPS cells from mature *i*NKT cells and were then able to generate functional *i*NKT cells from the iPS cells (*i*NKT–iPS) in vitro. *i*NKT cells (10^6) isolated from spleen cells of normal B6 or B6 *i*NKT clone mice were first activated by anti-CD3/CD28 together with IL-12 and IL-2 for a week, followed by reprogramming according to the conventional protocol [37]. We established three independent iPS cell lines, which were *i*NKT cell-derived, based on the presence of rearranged *Vα14Jα18* sequences. The expression of SSEA1, Oct3/4 and Nanog, and also that of endogenous *Oct3/4*, *Sox2*, *Klf4*, *Nanog*, *Ecat1*, *Gdf3*, *Rex1* and *Zfp296* mRNA in the iPS cells were detected at similar levels to those observed in ES cells. Genome-wide gene expression profiles of the iPS cell lines showed a higher correlation coefficient to ES cells than to splenic *i*NKT cells [41].

Generation of functional *i*NKT cells from ES cells and iPS cells

Using *i*NKT–ES cells, we established an *i*NKT–ES culture system using OP9/Dll-1 as an adherent cell layer in the presence of IL-7 and Flt-3 ligand (Flt3L). In the T cell development, pre-TCR signaling is essential for differentiation of $CD4^-CD8^-$ double negative (DN) cells to the DP stage. The DN cells are divided into DN1–DN4 subsets,

according to the expression of CD44 and CD25. The earliest $CD44^{+}CD25^{-}$ (DN1) cells provide the $CD44^{+}CD25^{+}$ (DN2) population that progress to the $CD44^{-}CD25^{+}$ (DN3) stage, followed by the $CD44^{-}CD25^{-}$ (DN4) stage. *i*NKT–ES cells were sustained in culture as the DN1 population from days 11 to 14. On the following days of culture, cells displayed the phenotypes similar to thymic DN2/3 population ($CD44^{lo/-}CD25^{hi}$) by day 16, subsequently into DN3/4 cells ($CD44^{-}$ $CD25^{-/int}$) on day 17. Finally, DP cells appeared on day 20 of culture, most of which α-GalCer/CD1d $dimer^{+}/TCR\beta^{+}$ *i*NKT cells. Therefore, it seems that the *i*NKT–ES culture system populated *i*NKT cells in vitro, tracing the similar developmental progression detected in the un-manipulated ES culture system as well as thymocyte in vivo [36].

Although there are no reports of cytokine production of DP *i*NKT cells, ES-derived *i*NKT cells produce IL-4, but only low levels of IFN-γ. The results indicate that they are functionally equivalent to IL-4-producing *i*NKT cells of the $NK1.1^{-}CD4^{+}$ or $CD4^{-}CD8^{-}$ *i*NKT cells in the thymus that mainly produce IL-4 [42, 43]. Although there is no report on cytokine production of DP *i*NKT cells, it is likely that IL-4-producing *i*NKT cells developed from *i*NKT–ES cells are similar to $CD24^{hi}$ DP *i*NKT cells in the thymus, which give rise to $NK1.1^{-}CD4^{+}$ or $CD4^{-}CD8^{-}$ *i*NKT cells. Moreover, in keeping with their immaturity, these IL-4-producing *i*NKT cells showed potent proliferative activity [36].

Even though there are no reports of the successful regeneration of functional B or T cells from lymphocyte-derived iPS cells [44–48], the potential of the *i*NKT–iPS cell lines to re-differentiate into mature functional *i*NKT cells in vitro was investigated using a modified above protocol [36, 49] of a 20- to 25-day culture system containing IL-7 and Flt3L as supportive cytokines and OP9/Dll-1 as stromal cells [50].

We first attempted to investigate the potential of *i*NKT cell development from *i*NKT–iPS cells using the 20-day culture system used for analysis of ES cells described above. Similar to ES-derived *i*NKT cells, iPS-derived *i*NKT cells mainly produced IL-4 on OP9/Dll-1 culture for 20 days, indicating that *i*NKT–iPS cells are potentially similar to *i*NKT–ES cells.

It is important to obtain high yield of *i*NKT cells with desired function for the establishment of *i*NKT cell therapy. For this purpose, we attempted to develop a new 25-day culture system efficiently to generate functional *i*NKT cells from the *i*NKT–iPS cells in vitro. The addition of IL-7 at the culture day 20 for 5 days yielded the highest numbers of *i*NKT cells, and also the highest amounts of IFN-γ production, equivalent to those of splenic *i*NKT cells. Under these conditions, 1×10^{5} iPS cells gave rise to 3×10^{7} iPS-derived *i*NKT cells at the culture day 25 that expressed TCRβ on their surface and bound α-GalCer-loaded soluble CD1d dimer, which are specifically recognized by the *i*NKT-specific TCR. The iPS-derived *i*NKT cells did not express NK1.1. Moreover, a significant fraction of the iPS-derived *i*NKT cells were doubly positive for CD4 and CD8. Therefore, the iPS-derived *i*NKT cells generated in this 25-day culture system were similar to immature DP thymocytes than mature liver *i*NKT cells. Moreover, they were $CD24^{hi}$, $CD44^{lo/int}$, $CD62L^{hi}$, $CD69^{+}$, $CD122^{lo/-}$ and $NKG2D^{-}$, which are phenotypically similar to the earliest thymic *i*NKT cells, so-called stage 0 *i*NKT cells.

In vivo function of iPS-derived *i*NKT cells

The in vivo survival and function of iPS-derived *i*NKT cells were investigated after transfer into *i*NKT cell-deficient ($J\alpha18^{-/-}$) mice [50]. Similar to the ability of normal wild type mature *i*NKT cells to repopulate the $J\alpha18^{-/-}$ mice, a considerable number of iPS-derived *i*NKT cells could repopulate in the recipient liver 2 weeks after adoptive transfer. Although the in vitro-generated iPS-derived *i*NKT cells that we injected had an immature phenotype (see previous section), the *i*NKT cells recovered from the liver were $CD24^{lo}$, $CD44^{hi}$, $CD62L^{lo}$, $CD69^{+}$, $CD122^{+}$, and $NKG2D^{+}$, phenotypically similar to mature liver *i*NKT cells in wild type mice, indicating that their maturation occurs in vivo. Similar to the in vitro activity of iPS-derived *i*NKT cells obtained in the 25-day cultures, the iPS-derived *i*NKT cells transferred in vivo produced large amounts of IFN-γ upon antigen stimulation with α-GalCer intravenously, and expanded significantly in number (~10-fold increase: 0.3–0.4 % to 4.9–5.7 %). These iPS-derived *i*NKT cells also showed down-modulated TCRβ expression, consistent with their in vivo activation. Similar results were observed after transferring ES-derived *i*NKT cells, indicating both ES- and iPS-derived *i*NKT cells survive and mature after transfer in vivo.

Adjuvant activity of ES and iPS-derived *i*NKT cells

As the iPS-derived *i*NKT cells produced IFN-γ upon stimulation with α-GalCer (see "Generation of functional *i*NKT cells from ES cells and iPS cells"), we investigated their adjuvant effects on anti-tumor responses in mice. The $J\alpha18^{-/-}$ mice that had received iPS-derived *i*NKT cells (4×10^{6}) were immunized with cell-associated ovalbumin (OVA) (spleen cells from $Tap^{-/-}$ mice that were treated with hypertonic medium in the presence of OVA) together with α-GalCer (TOG). A week later, the mice were challenged with $OVA_{257–264}$ peptide and analyzed for IFN-γ

production by OVA-specific CTLs and by NK cells [50]. We observed significant production of IFN-γ by the NK cells and OVA-specific CTLs, as well as an increase in the number of these CTLs (>70 times higher than the control group). Thus, iPS-derived *i*NKT cells were shown to function as a cellular adjuvant for both innate and adaptive immune responses.

Under these conditions, iPS-derived *i*NKT cells significantly augmented antigen-specific anti-tumor CTL responses against EL-4-derived OVA-bearing EG7 tumor cells (OVA serves as tumor antigen in this system) but not against EL4 in TOG-immunized B6 mice. The growth of OVA-bearing EG7 tumor cells in $J\alpha18^{-/-}$ mice was significantly suppressed by adoptive transfer of iPS-derived *i*NKT cells, whereas the growth of EL4 was not. Therefore, the iPS-derived *i*NKT cells are functionally competent to mediate adjuvant activity in vivo.

Towards generation of human iPSCs that give rise to *i*NKT cells in vitro

These studies using mouse ESCs and iPSCs and the functional similarity of human *i*NKT cells with those in mice point to the clinical potential of human ESCs or iPSCs that harbor rearranged *Tcra* and *Tcrb* genes specific for *i*NKT cells for cancer treatment. To test this possibility, we need to establish human ESCs or iPSCs able to give rise *i*NKT cells in vitro and also optimize induction assays for functionally maturated T cells from human ESCs or iPSCs. Since generation of human ESCs with rearranged *Tcr* genes needs nuclear transfer of T cell nuclei into human unfertilized eggs, use of iPSCs is more realistic option at this point. We, however, found it is not simple to induce iPSCs from human *i*NKT cells circulating in the peripheral blood due to two main technical hurdles: the low frequency of *i*NKT cells in the peripheral blood, and the low induction efficacy of iPSCs from adult mature T cells. In our pilot experiments, we observed that *i*NKT cells represent 0.0001–1 % of mononuclear cells in peripheral blood, and that this frequency is quite variable in human population. This suggests we can collect approximately 10^2–10^6 *i*NKT cells from 200 ml of adult peripheral blood and this number theoretically warrants us to generate iPSCs from peripheral blood lymphocytes (PBL) since previous studies reported reprogramming frequency of peripheral blood T cells was approximately 0.1 % (50 hESC-like colonies were observed from 5×10^4 peripheral blood T cells) by Sendai virus vector (SeV)-mediated expression of OCT3/4, SOX2, KLF4 and c-MYC (OSKM) [51]. Moreover, we found co-expression of SV40 T-antigen with OSKM further increased reprogramming rate of cord blood T cells to approximately 0.5 % (474 hESC-like colonies were observed from 1×10^5 cord blood T cells). In fact, we have successfully induced ample iPSCs, which were shown to possess productively rearranged *Tcra* and *Tcrb* genes (unpublished), from $CD4^+$ and $CD8^+$ cord blood T lymphocytes. We, however, found the reprogramming rates of adult PBL T cells were more than 10^2 lower than those of cord blood T cells. This number is insufficient to induce *i*NKT–iPSCs from PBL containing average number of *i*NKT cells using currently available technology. We clearly need further technical advances to improve reprogramming frequency of adult T cells.

An alternative strategy to generate human T-iPSCs harboring rearranged *Tcra* and *Tcrb* gene configurations specific for *i*NKT cells may involve induced replacement of rearranged variable regions in T-iPSCs with those in *i*NKTs using zinc finger nuclease (ZFN) or transcription activator-like effector nuclease (TALEN) technology. Recently, several groups have reported gene targeting in human iPSCs by homologous recombination using these technologies. We need two consecutive steps for this replacement at both *Tcra* and *Tcrb* genes, as the targeting reaction by either ZFNs or TALEN does not allow efficient direct replacement. Therefore, first steps should be deletion of genomic regions encompassing rearranged variable region of *Tcra* or *Tcrb* loci by ZFNs or TALEN. Indeed, ZFNs have been shown to efficiently produce targeted insertion of small genetic elements concomitant with genomic deletions [52, 53]. We may thus induce deletion of *Tcra* and *Tcrb* genomic regions and concurrently introduce some known ZFN- or TALEN-target sequence, which is not present on the human genome, to enable insertion of DNA fragment encoding other rearranged *Tcra* or *Tcrb* region by secondary targeting reaction (Fig. 1a). These T-iPSCs with deletion of variable regions at *Tcra* and *Tcrb* genes could be versatile recipient for exogenous variable region genes in the second step. Flanking genomic sequences of this ZFN- or TALEN-target sequence are used to generate donor vectors containing drug selection markers to knock-in NKT cell-specific variable regions. Donor vectors are transfected into versatile recipient iPSCs, together with ZFN or TALEN. After cloning of homologous recombinants, we may delete selection marker genes using PiggyBac or Cre-loxP system (Fig. 1b). Moreover, this technology is theoretically applicable to express antigen-specific T cell receptors once we could know their primary structures.

Although human iPSCs that can give rise to *i*NKT cells may be an attractive device to realize *i*NKT cell-mediated immunotherapy for cancer treatment, above considerations indicate that we still need technical development even at the first step. Moreover, functional properties of iPSCs are

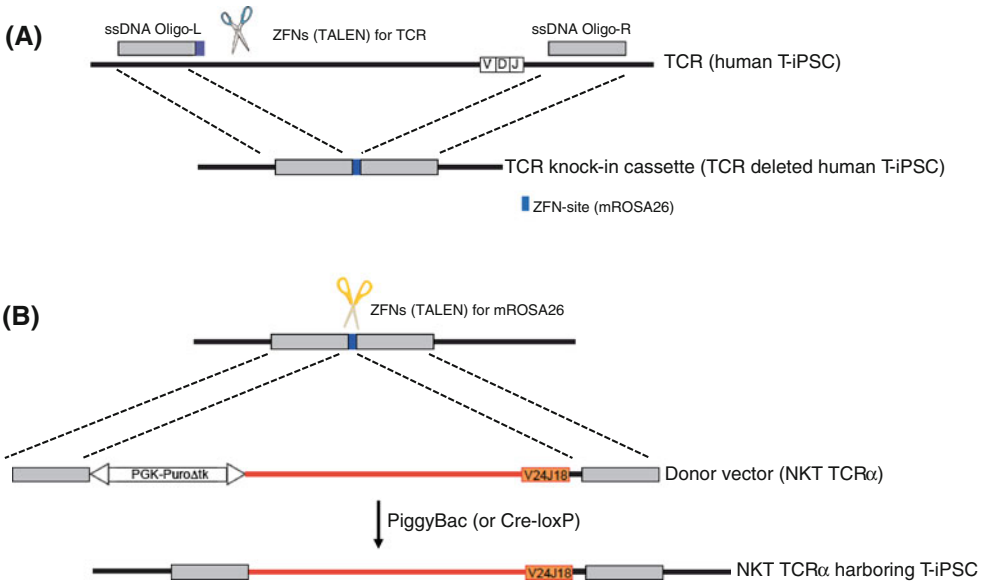

Fig. 1 Strategy for *i*NKT-specific TCR genome editing in human T-iPSC. **a** Generation of T-iPSC harboring TCR knock-in cassette (mROSA26) using ssDNA oligos together with ZFNs or TALEN introduction. **b** Generation of T-iPSC harboring NKT cell-specific variable regions using TCR knock-in cassette-specific ZFNs (TALEN). After cloning, selection marker is deleted by PiggyBac or Cre-lox. By way of example, *i*NKT TCRα is shown in this figure

known to be quite variable, but we still do not have legitimate quality and safety standards for human T-iPSCs. Although differentiation protocols for the induction of T cells from ESCs or iPSCs are not fully optimized, we can check differentiation ability of these cells in vitro and tumorigenicity of induced cells in immunocompromised mice. As a first step to develop iPSC-mediated immunotherapy, we should perform small-scale banking of human T-iPSCs, in which we describe nucleotide sequences of *Tcr* genes, HLA haplotype, differentiation ability in vitro, tumorigenicity in mice and possibly other parameters as a pilot trial. On one hand, this bank should be publically shared and enabled for access of researchers harboring various ideas. On the other hand, we need to select well-qualified T-iPSCs to generate versatile recipient iPSCs for further modifications of *Tcr* loci.

Future perspectives

Our previous studies in mice have clearly provided a proof of concept for the use of iPSC-derived *i*NKT cells for adjuvant cell therapy against cancer, which is composed of four segments: (1) collection of *i*NKT cells, (2) reprogramming of *i*NKT cells into iPSCs, (3) re-differentiation of *i*NKT cell-derived iPSCs into *i*NKT cells and their expansion in vitro, and (4) injection of iPSC-derived *i*NKT cells into tumor-bearing animals (Fig. 2). Since the functions and α-GalCer reactivity of *i*NKT cells are highly conserved between mice and humans, and human CD1 is monomorphic as well as murine CD1d, it is conceptually possible to tailor *i*NKT cell-targeted adjuvant cell therapy to human cancer treatment beyond extensive heterogeneity

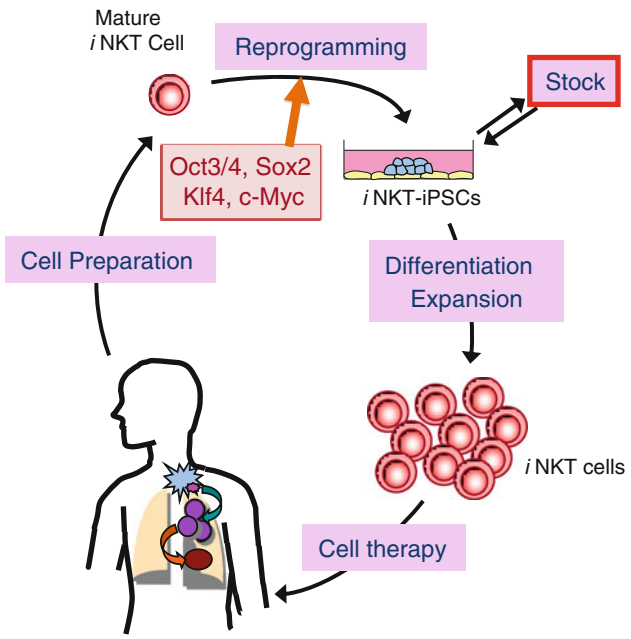

Fig. 2 Schematic representation of *i*NKT cell supplemental cellular immunotherapy. iPS cells that differentiate into *i*NKT cells can be developed from peripheral mature *i*NKT cells by the introduction of Oct3/4, Sox2, Klf4 and c-Myc (OSKM) or *i*NKT-specific TCR genome editing shown in Fig. 1. The iPSCs can be saved for later use, and differentiated into *i*NKT cells with desired properties using this reliable culture system. *i*NKT cell-mediated cellular immunotherapy shows promising anti-tumor activity, and it will be crucial to establish methods for delivering *i*NKT cells to the body

of human HLA. Establishment of iPSC lines that can differentiate into functionally competent *i*NKT cells should be addressed to test the clinical reality for iPSC-derived *i*NKT cell-mediated immunotherapy.

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