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PROGRESS IN HEMATOLOGY

Advances in immunotherapy for hematological malignancies

Cloning and expansion of antigen-specific T cells using iPS cell technology: development of "off-the-shelf" T cells for the use in allogeneic transfusion settings

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Abstract
Recent advances in adoptive immunotherapy using cytotoxic T lymphocytes (CTLs) have led to moderate therapeutic anti-cancer effects in clinical trials. However, a critical issue, namely that CTLs collected from patients are easily exhausted during expansion culture, has yet to be solved. To address this issue, we have been developing a strategy which utilizes induced pluripotent stem cell (iPSC) technology. This strategy is based on the idea that when iPSCs are produced from antigen-specific CTLs, CTLs regenerated from such iPSCs should show the same antigen specificity as the original CTLs. Pursuing this idea, we previously succeeded in regenerating melanoma antigen MART1-specific CTLs, and more recently in producing potent CTLs expressing CD8αβ heterodimer. We are now developing a novel method by which non-T derived iPSCs are transduced with exogenous T cell receptor genes. If this method is applied to Human Leukocyte Antigen (HLA) haplotype-homozygous iPSC stock, it will be possible to prepare "off-the-shelf" T cells. As a first-in-human trial, we are planning to apply our strategy to relapsed acute myeloid leukemia patients by targeting the WT1 antigen.

Keywords Adoptive immunotherapy · iPS cells · CD8αβ T cells · WT1 antigen

Cancer immunotherapy; current status and recent issues

Cancer immunotherapy has evolved remarkably during the past several years. Immune checkpoint blockade drugs, such as Ipilimumab (anti-CTLA-4 monoclonal antibody (mAb)) and pembrolizumab (anti-PD-1 mAb), have shown therapeutic effects leading to their subsequent approval for the treatment of some types of cancers [1–3]. However, their therapeutic efficacy reaches only 20–30%, and side effects such as autoimmune reactions and other immune-related adverse events are frequently observed [4].

As another strategy, the therapeutic delivery of CTLs directly into patients has also shown good results. For example, Rosenberg and colleagues have demonstrated that administration of ex vivo expanded tumor-infiltrating lymphocytes (TILs) was effective in melanoma patients [5]. Transfer of T cell receptor (TCR) genes into patients' peripheral T cells has achieved good clinical outcomes as well. For example, NY-ESO1 antigen-specific TCR gene therapy has been shown to be effective in melanoma, synovial sarcoma, and multiple myeloma [6–8], indicating that targeting a single antigen can be effective for some types of cancer. In addition, T cell-enforced expression of chimeric antigen receptor (CAR), an engineered receptor molecule which combines an antibody recognition domain and cytoplasmic signaling domains, has shown enormous efficacy in the treatment of B cell leukemia [9].

In both TCR and CAR engineering, peripheral T cells are transduced by a retrovirus, bringing about the risk of tumorigenicity due to the random integration of a transfected

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gene into the genome. Moreover, in the autologous setting it would be costly to produce T cells, and in some cases it will be difficult to obtain potent CTLs from patients.

Cloning and expansion of T cells using reprogramming technology

To solve the problems mentioned above, we have thought of a method by which T cells can be expanded. Our concept is the following. When embryonic stem cells (ESCs) or iPSCs are generated from T cells, the genomic structure of rearranged T cell receptor (TCR) genes should be passed on to the ESCs/iPSCs, and T cells regenerated from these ESCs/iPSCs should express the same TCR as the original T cells (Fig. 1a). Considering that ESCs/iPSCs can be expanded almost unlimitedly, it will be possible to produce large numbers of "fresh" T cells of a given specificity.

Based on this concept, several groups at Riken Research Center for Allergy and Immunology including ours launched an initial project in 2004, prior to the invention of iPSC technology. At that time, we utilized the nuclear transfer technology for the reprogramming of T cells, and the "clone mouse" and ESCs were successfully produced from murine NKT cells [10, 11].

After the invention of iPSC technology, we came to use it since it is much easier and more efficient than nuclear transfer technology. Groups around H. Koseki and S.I. Fujii in Riken continued to focus on NKT cells, while our group's effort turned towards the regeneration of CTLs with the aim to target tumor antigens.

Regeneration of CTLs utilizing iPSC technology

At first, we produced iPSCs from human CTLs specific for melanoma antigen MART1 [12]. As a cell source, we obtained long-term cultured MART1-specific CTLs established from a melanoma patient by the National Institute of Health (NIH) (Fig. 1b, c). We then regenerated CTLs from MART1-T-iPSCs and found that almost all regenerated T cells expressed TCR specific for MART1 antigen (Fig. 1d).

Concurrently, H. Nakauchi's group introduced the Riken method and reported their success in regenerating CTLs specific for viral antigen [13] in the same issue as ours. Later that year, M. Sadelain's group reported that CAR-expressing T cells were regenerated from T cell-derived iPSCs transduced with a CAR gene [14], and H. Wakao's group succeeded in regenerating human MAIT cells from MAIT-derived iPSCs [15]. Recently, two groups, S. Kaneko's group and S. I. Fujii's group, reported the regeneration of human NKT cells [16, 17].

Development of a method to induce potent CTLs expressing CD8αα heterodimer

As we reported in 2013, regenerated CTLs from MART1-T-iPSCs expressed TCR specific for MART1 antigen. However, regenerated CTLs expressed CD8αα homodimer. While conventional CTLs express CD8αβ, CD8αα is expressed in a subset of γδT cells and intestinal CD8-positive intraepithelial lymphocytes. It is known that CD8αβ efficiently strengthens the TCR signal by binding to the HLA molecule, while CD8αα does not (Fig. 2a). M. Sadelain's group also reported that T cells regenerated from iPSCs resemble γδT cells [14]. Therefore, we improved our culture procedure and recently succeeded in inducing CD8αβ CTLs [18] (Fig. 2b). While in our previous method whole cells were stimulated by anti-CD3 mAb when CD4/CD8 double-positive (DP) cells were generated in the culture, in our newly developed method DP cells are first isolated and then stimulated by anti-CD3 mAb, making it possible to efficiently produce CD8αβ CTLs. To answer the question why DP cells failed to generate CD8αβ CTLs when stimulated under non-separated conditions, we revealed that TCR-positive mature T cells present in the CD4/CD8 double-negative cell population killed DP cells upon stimulation.

Regeneration of CTLs specific for WT1 antigen

We then used the improved method to regenerate WT1 antigen-specific CTLs. WT1 antigen is known to be expressed in acute myeloid leukemia cells and in various types of solid tumors [19]. We expanded WT1 antigen-specific CTLs from a healthy volunteer and established iPSCs (WT1-T-iPSCs). CTLs regenerated from WT1-T-iPSCs by the new method exhibited antigen-specific cytotoxic activity comparable to the original CTLs (Fig. 3a).

Regenerated CTLs were able to kill leukemia cells expressing endogenous WT1 antigen (data not shown). Regenerated CTLs were also able to prolong the survival in a xenograft leukemia mouse model where regenerated WT1-CTLs were infused after the inoculation of HL60 human leukemia cells (Fig. 3b).

We also investigated the safety of regenerated CTLs. It is known that when human peripheral T cells are infused

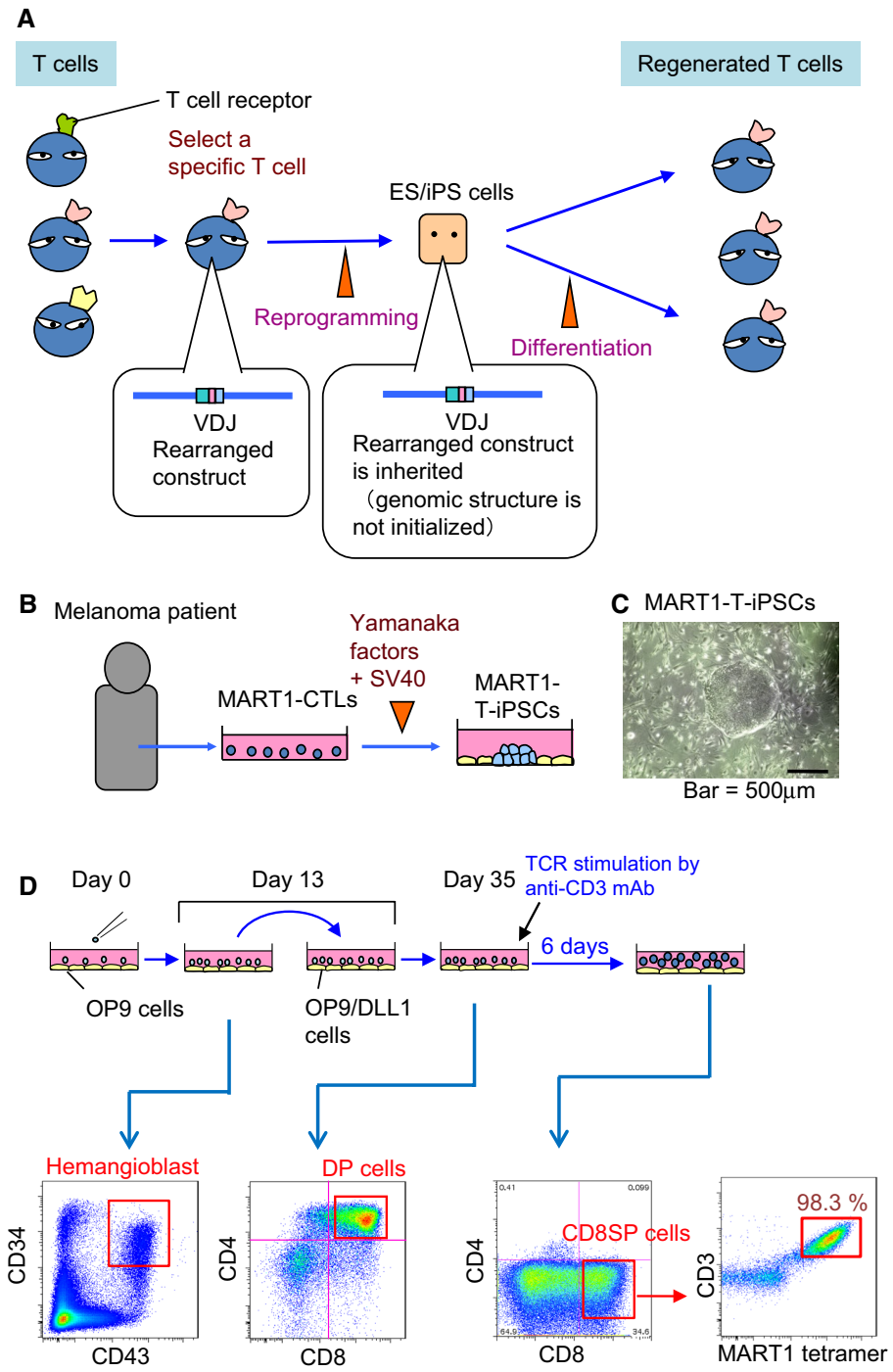

Fig. 1 Regeneration of T cells using iPSC technology. **a** The concept of T-iPSC therapy. When T-iPSCs are established from tumor antigen-specific T cells, the rearranged configuration of TCR genes is passed on to iPSCs. T cells regenerated from these T-iPSCs express the same tumor antigen-specific TCRs. **b** We established MART1-specific T-iPSCs by transducing MART1-specific T cells derived from a melanoma patient with Yamanaka factors and SV40 using Sendai virus vector system. **c** Colony of MART1-T-iPSCs. **d** MART1-iPSCs were sequentially cultured with two types of feeder cells, OP9 and OP9/DLL1 cells. On Day 13, $CD34^{+}CD43^{+}$ hemangioblasts were generated, and CD4/CD8 double-positive (DP) cells were generated on Day 35, when anti-CD3 mAb was added to induce the generation of mature T cells. CD8 single-positive cells were generated 6 days after anti-CD3 mAb stimulation. Virtually all CD8 single-positive cells expressed a TCR specific for MART1 antigen

into immunodeficient mice, mice will soon die due to graft-versus-host disease caused by xeno-reactive T cells present in the polyclonal T cell population. In contrast, in our approach regenerated T cells are monoclonal and thus thought to be basically safe. Indeed, when we infused CTLs into immunodeficient mice, no tissue damage was observed during a follow-up period of 6 months. We also checked the risk of tumorigenicity of infused cells; no leukemic change was observed in the infused mice during 6 months of follow-up (data not shown).

In the following, we describe the cell yield of our culture system. Using the new method shown in Fig. 2b, a total of 2×10^6 CD8αβ T cells are generated in one dish on week 7 (Fig. 4). Subsequently, these T cells are co-cultured with antigen presenting cells loaded with specific peptide. During the 8-week expansion culture period, cells

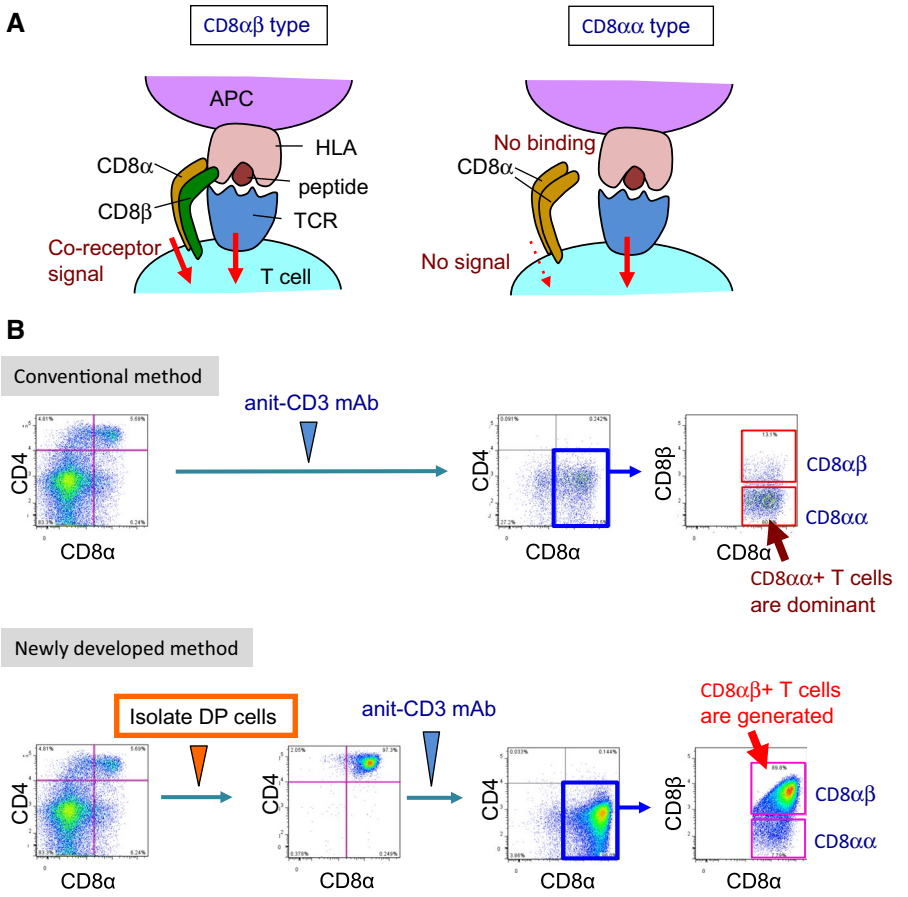

Fig. 2 A new method to induce CD8αβ CTLs. **a** CD8αβ heterodimer can bind to HLA molecules expressed on antigen presenting cells (APC), resulting in the enhancement of TCR signal, while CD8αα homodimer cannot bind to HLA, being unable to enhance TCR signal. **b** In the conventional method shown in Fig. 1, the bulk population containing DP cells is stimulated by anti-CD3 mAb, and regenerated CD8 T cells express CD8αα homodimer. In the newly developed method, DP cells are first isolated and subsequently stimulated by anti-CD3 mAb. With this method, regenerated CD8 T cells dominantly express CD8αβ heterodimer

can be expanded by 10^4-fold. Thus, in total 10^{10} CTLs can be produced in one round. Considering that 10^8–10^9 cells are usually transfused at one time in antigen-specific T cell therapy, the amount of cells generated by our method could be sufficient for several times of cell therapy.

The TCR-iPSC method; another strategy toward the allogeneic transfusion setting

We have thus far described the T-iPSC method in which iPSCs are produced from T cells (Fig. 5a). However, there are several issues with the T-iPSC method: it is rather difficult to produce T-iPSCs of high quality; iPSC clones are heterogeneous in terms of T cell-generating potential, and moreover, while the quality of CTLs depends on TCR affinity, the TCR affinity among TCR-iPSC clones varies a lot. Considering these issues, production of multiple clones and strict selection among them will be required, which would be time-consuming and costly. To overcome these shortcomings, we thought of an alternative approach: to transduce iPSCs with an exogenous TCR gene (TCR-iPSC method) (Fig. 5b). By the TCR-iPSC approach, it would be much easier and faster to produce high-quality TCR-iPSC clones, since it will be possible to use iPSCs and TCR genes of guaranteed quality.

To examine whether this idea works in practice, we cloned WT1-specific TCR genes from CTLs regenerated from WT1-T-iPSCs and transduced them into iPSCs that had been originally derived from monocytes of an HLA-haplotype homozygous healthy volunteer (WT1-TCR-iPSCs). Using these WT1-TCR-iPSCs, we succeeded in regenerating CD8αβ CTLs which exhibited a growth potential and antigen-specific cytotoxic activity comparable to CD8αβ CTLs regenerated from T-iPSCs (data not shown).

We think that applying this method will dramatically expand the versatility of our strategy. We plan to utilize TCR genes whose efficacy and safety have been clinically tested and ensured. As a source of iPSCs, we plan to use those established by the iPSC stock project, which has been initiated in 2013 by the Center for iPS Cell Research and Application in Japan. The iPSC stock project has been preparing HLA-haplotype homozygous iPSCs. Thus, we are envisioning to use this system for preparing “off-the-shelf”

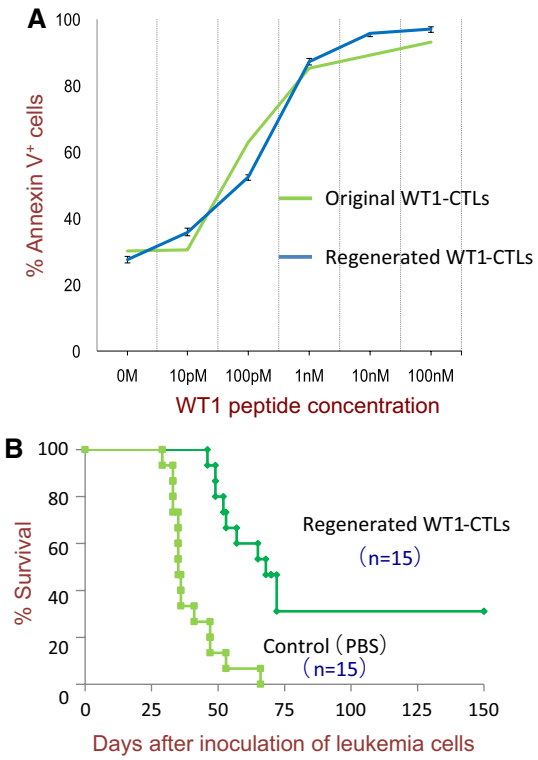

Fig. 3 Regenerated WT1-specific CTLs were highly potent in killing target cells in an antigen-specific manner. **a** WT1-specific regenerated T cells exhibited antigen-specific cytotoxic activity comparable to the original CTLs. WT1 specific CTLs were reprogrammed using the method shown in Fig. 1b, and WT1-CTLs then regenerated using the new method shown in Fig. 2b. As effector cells, regenerated WT1-CTLs and original WT1-CTLs (cryopreserved and thawed) were used. Peptide-loaded B-lymphoblastoid cells were used as target cells. A range of WT1 peptide doses was loaded onto target cells, while the effector:target cell ratio was fixed at 3:1, and dead cells were measured as annexin V-positive cells after 6 h of co-culture. **b** Regenerated WT1-CTLs showed a therapeutic effect in a leukemia mouse model. NOG mice were injected intraperitoneally with 2×10^4 HL60 human leukemia cells on day 0. Beginning on day 1, PBS or 5×10^6 regenerated WT1-CTLs were injected intraperitoneally into tumor-bearing mice once a week for 4 weeks. Human Interleukin (hIL)-2 and hIL-7 were injected intraperitoneally three times a week for 4 weeks

T cells by mass production and cryopreservation of regenerated CTLs. Although this idea seems ideal, it presents a combination of regenerative medicine and gene therapy and is as such expected to face some difficulties with regard to regulatory approval.

At present, we are working to set up a clinical trial targeting WT1-antigen in acute myeloid leukemia (AML) patients in collaboration with the Department of Hematology and Oncology and the Department of Transfusion Medicine in Kyoto University Hospital (Fig. 6). When aged AML patients, who may have no option of stem cell transplantation, undergo relapse after initial chemotherapy, so far no effective therapy is available. Such patients would be appropriate candidates for our trial. As TCR genes, we plan to use WT1-TCR genes that have been cloned and clinically tested by M. Yasukawa's group [20].

Perspective

In the present article, we have proposed a possible strategy to attack cancer by regenerative medicine. Since WT1 antigen is known to be expressed in various types of cancer, the very same CTLs used for the first clinical trial could be used for other types of cancer. We are also thinking of a strategy to target neo-antigens, where we will incorporate personalized medicine into our allogeneic T cell therapy.

We demonstrated that with the technology described in this article it is possible to produce potent CTLs that can kill tumor cells both in vitro and in vivo. However, we are aware that there are several limitations to the current culture procedures which need to be addressed. One point is that the regenerated CTLs are close to effector cells, as shown in our previous report [15], while it is preferable to use naïve or memory-type cells in adoptive T cell therapy. Another point is that, at present, we are using mouse-derived feeder cells (OP9 and OP9/DLL1 cells) for the production of T cells from iPSCs. Although the use of xenogeneic material during the early phase of the culture period might be acceptable for a small-scale clinical trial, it will be essential to perform all culture procedures under xeno-free conditions before this strategy can be implemented in routine clinical settings.

If this "off-the-shelf" T cell strategy can be realized, it will bring about a phenomenal breakthrough in cancer immunotherapy. Furthermore, we are expecting that this

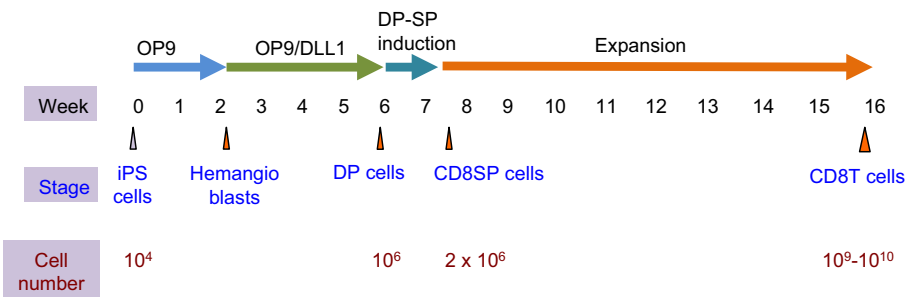

Fig. 4 One round of culture yields 10^{10} CTLs. By using the newly developed method shown in Fig. 2b, 10^6 CD8αβ CTLs are generated by week 7. By co-culturing these CTLs with antigen presenting cells (B-lymphoblastoid cell line) loaded with specific peptide, cells can be expanded 10^4-fold

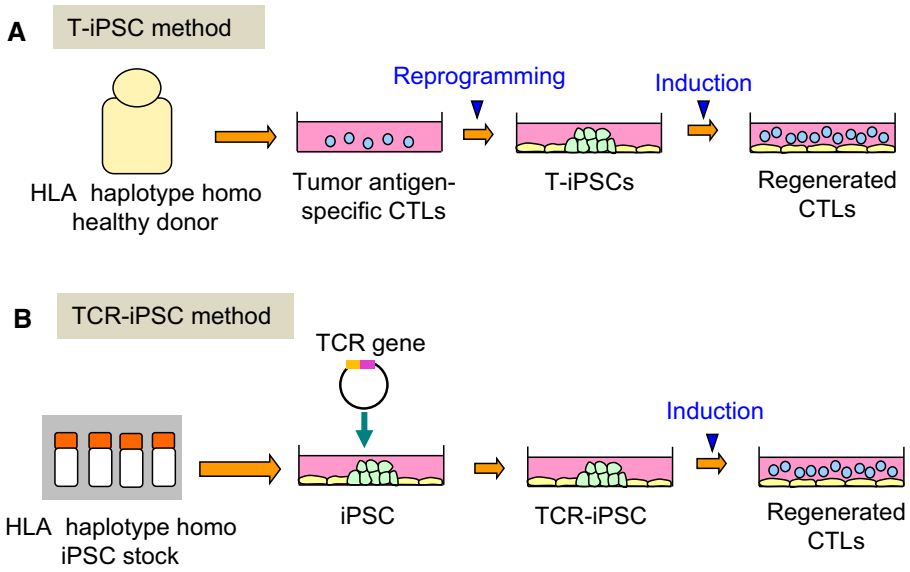

Fig. 5 Two possible methods to regenerate CTLs for the allogeneic transfusion setting. **a** T-iPSC method. While it is possible to produce T-iPSCs from healthy volunteers, this method is costly and time-consuming due to the selection process to determine the best among the T-iPSC clones in terms of quality of iPSC and TCR. **b** TCR-iPSC method. On the other hand, if iPSCs established in the iPSC stock project are transduced with a TCR gene that has been clinically tested, it would be much easier to obtain high quality TCR-iPSC clones, making it possible to prepare "off-the-self" T cells. However, this method will certainly face regulatory approval issues, since it is a gene therapy administered to stem cells

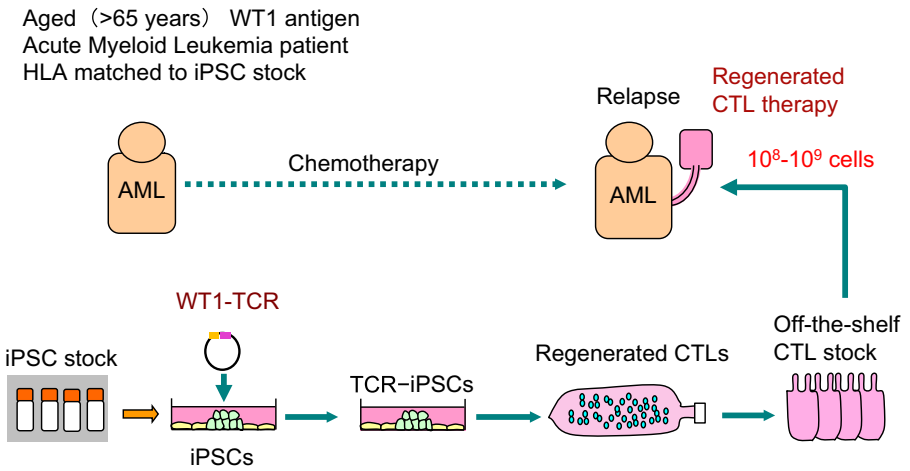

Fig. 6 A scheme for the clinical application of the TCR-iPSC method. The scheme shows how we plan to apply our TCR-iPSC method in the allogenic transfusion setting to acute myeloid leukemia cases. Aged patients bearing WT1-expressing leukemia cells would be subjected to adoptive immune cell therapy with CTLs regenerated from WT1-TCR-iPSCs when they undergo relapse of leukemia after initial chemotherapy. As TCR genes, we plan to use WT1-TCR genes that have been cloned and clinically tested by M. Yasukawa's group (Ehime University)

approach could be utilized not only for cancer but also for the treatment of other diseases related to immunity, such as infectious diseases, autoimmune diseases or allergies.

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