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Mini Review

Cloning and expansion of antigen-specific T cells using iPSC technology: A novel strategy for cancer immunotherapy

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Cytotoxic T lymphocytes (CTLs) represent the most promising therapeutic avenue in cancer immunotherapy, yet most of the currently ongoing trials utilizing CTLs are still not effective enough to cure patients. To overcome this problem, we came up with the idea to apply induced pluripotent stem cell (iPSC) technology to the cloning and expansion of CTLs. When iPSCs are established from antigen-specific T cells (T-iPSCs), these T-iPSCs should inherit rearranged genomic structures of T cell receptor genes, and thus all T cells regenerated from T-iPSCs should express the same T cell receptor (TCR). Since iPSCs can be expanded almost unlimitedly, it is possible to obtain as many fresh CTLs as needed. Pursuing this idea, we have recently succeeded in regenerating melanoma antigen MART1-specific CTLs from T-iPSCs originally derived from a melanoma patient. Our study illustrates an approach for the cloning and expansion of functional antigen-specific CTLs that might be applicable in cell-based cancer therapy.

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Introduction

Immunotherapy has long been considered the fourth type of cancer treatment in addition to surgery, chemotherapy, and irradiation therapy. However, most of the cancer immunotherapy strategies to date have failed to show sufficient efficacy in curing patients. Although a certain number of cytotoxic T lymphocytes (CTLs) specific for cancer antigen are formed in cancer patients, most of these CTLs remain inactive due to various suppressive mechanisms, such as anergy induction by improper DC activation or suppression

by regulatory T cells.

During the past several years, however, cancer immunotherapy has remarkably progressed. One of the leading achievements is the so called "immune checkpoint blockade", a strategy that uses drugs to block inhibitory signals in the immune system. Another effective method in cancer immunotherapy is the direct use of CTLs in patients. Adoptive transfer of CTLs has been very effective when patients are given an aggressive pretreatment such as a total body irradiation. Alternatively, transfer of T cell receptor (TCR) genes specific for certain tumor antigens to peripheral T cells of the patient has been clinically effective in some types of cancer.

These findings indicate that CTLs present in cancer patients possess a curative potential. If so, questions may arise why aggressive pretreatments or gene therapy approaches are necessary, and why antigen-specific CTLs are not simply expanded *in vitro* or *in vivo*. This is mainly because of difficulties in getting sufficient numbers of antigen-specific CTLs both *in vivo* and *in vitro*, which is attributable to the short life span of activated CTLs. In this article, we will introduce a novel approach able to overcome the above-mentioned problems by using induced pluripotent stem cell (iPSC) technology.

Immune checkpoint blockade is effective in cancer immunotherapy

CTLA-4 and PD-1 are inhibitory receptors expressed on activated T cells and important for the tuning of an immune reaction. By blocking these receptors with specific monoclonal antibodies (mAb), it is possible to augment an immune response. Indeed, anti-PD-1 mAb and anti-CTLA-4 mAb alone or in combination have been shown to be effective for some types of cancers including melanoma, lung cancer, and kidney cancer, even at the advanced stage[1-3], having been already approved to use in certain types of cancers. These mAbs are thought to indirectly activate latent CTLs reactive to cancer cells present in patients.

Immune checkpoint blockade represents an epoch-making breakthrough in cancer therapy, since the approach is straightforward and has a broad applicability in a variety of cancer types. Nevertheless, some problems remain in that i) an autoimmune reaction takes place at a certain frequency, as this strategy activates the immune system in a non-antigen-specific manner, and ii) in most cases, the effect is limited to ～ 20% of patients.

Adoptive transfer of T cells

S. A. Rosenberg's group has been taking the strategy in which tumor-infiltrating lymphocytes (TILs) are collected from the patient, activated *in vitro* and then returned to the patient[4]. Recently, the group is taking a more aggressive strategy in the clinical trial in which the patient first undergoes chemotherapy and lethal irradiation to deplete lymphocytes followed by autologous hematopoietic stem cell (HSC) transplantation and transfer of TILs[5]. Although this method is very invasive, it ensures the efficient survival of TILs in the recipient. The results have been remarkable in that an obvious effect was seen in 70% of metastatic melanoma patients, 40% of who survived more than 5 years .

Another approach has been TCR gene transfer, where peripheral T cells of a patient are transduced with a defined TCR gene using a retrovirus or a lentivirus vector system. While these TCR gene transfer therapies are still under clinical trials, some have been shown to be clearly effective. For example, MART1-specific TCR gene has been used in a clinical trial against melanoma, and 10-30% of patients showed tumor regression[6, 7]. In another trial, T cells transduced with NY-ESO1-specific TCR have been shown to be effective in 60% of synovial cell sarcoma patients and 40% of melanoma patients[8].

A novel method for the cloning of T cells

Hereafter, we introduce our novel strategy, which is currently under development.

Methods to activate the immune system in a non-antigen-specific manner using immune checkpoint-blocking monoclonal antibodies have been shown to be effective in various types of cancer. However, as mentioned earlier an autoimmune reaction inevitably occurs at a certain frequency. Direct use of CTLs has been also shown to be effective, but invasive pretreatment or gene therapy is required for the method to work well. The question may arise why one does not simply expand antigen-specific T cells *in vitro* by using tumor antigens and antigen presenting cells. Antigen-specific CTLs can indeed be expanded *in vitro*, but it has been extremely difficult to get a sufficient number of cells, since *in vitro* expanded CTLs tend to become easily exhausted after a certain period of culture.

To overcome this problem, we considered the idea to utilize iPSC technology for the cloning and *in vitro* expansion of CTLs. Essentially iPSCs are established from mature antigen-specific T cells. Since TCRs are formed from rearranged TCR genes, iPSCs derived from

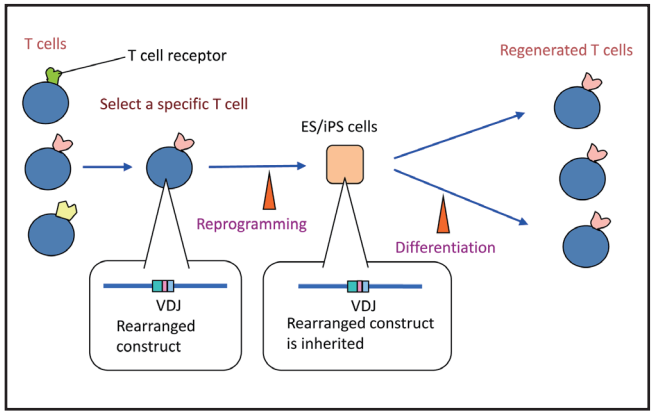

Fig. 1 Reprogramming of T cells with a defined antigen specificity into iPSCs serves as a method of cloning

iPSCs produced by reprogramming a T cell having a defined antigen specificity inherit rearranged genomic constructs of TCR genes of the original T cell. All T cells regenerated from such iPSCs are expected to express the same TCR as the original T cell.

a T cell (T-iPSCs) should inherit those rearranged genomic structures (Fig. 1). All T cells regenerated from T-iPSCs are thus expected to express the same TCR on their surface. Since iPSCs can be expanded almost unlimitedly, it is possible to obtain as many fresh CTLs as needed. While the life span of regenerated T cells is also limited like the original CTLs, the patient will be given with sufficient number of T cells by massive culture of regenerated CTLs or by repeated transfusions of them.

Production of iPSCs from tumor antigen-specific CTLs

Along with this idea, we have recently succeeded in establishing iPSCs from melanoma antigen MART1-specific CTLs originally derived from a melanoma patient, and in regenerating MART1-specific CTLs from these iPSCs[9].

As a cell source we used JKF6 cells[10], long-term cultured MART1-specific TILs that were originally derived from a melanoma patient (Fig. 2A). Although it has been previously shown that iPSCs can be produced from human peripheral T cells by using Yamanaka factors[11, 12], we also used SV40 large T antigen[13] to increase reprogramming efficiency. For transduction, we used the Sendai virus system[14]. We thus succeeded in making iPSCs from MART1-specific CTLs (MART1-T-iPSCs) (Fig. 2A,B).

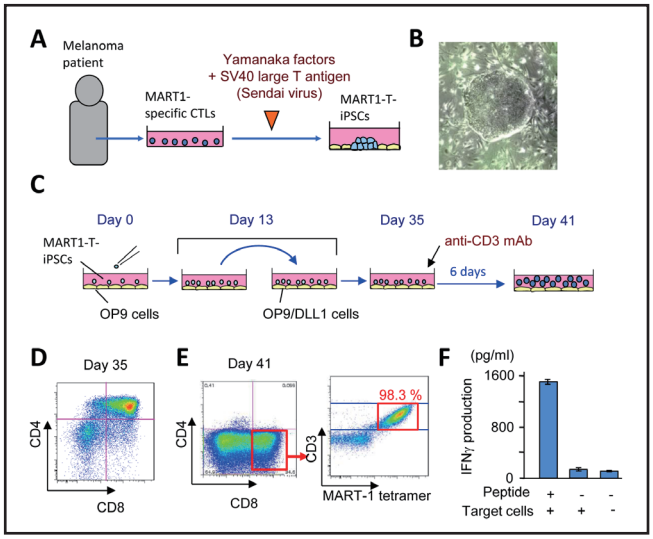

Fig. 2 Regeneration of MART1-specific CTLs from MART1-iPSCs

(A)Generation of iPSCs from MART1-specific CTLs. Long-term cultured MART1-specific CTLs (JKF cells) were transduced with Yamanaka factors and SV40 large T antigen, and iPSCs were established.

(B)A photomicrograph of MART1-T-iPSCs.

(C)MART1-T-iPSCs were sequentially cultured with two types of feeder cells, OP9 and OP9/DLL1 cells. On day 35 of cultivation, anti-CD3 mAb was added to induce the generation of mature T cells.

(D)CD4/CD8 double positive cells were generated on day 35 of cultivation.

(E)A large number of CD8 single positive cells were generated 6 days after the stimulation with anti-CD3 mAb. Virtually all of them expressed a TCR specific for the MART-1 antigen.

(F)Production of IFNγ by regenerated $CD8^+$ T cells upon antigen-specific stimulation. IFNγ secretion by $CD8^+$ T cells was measured by ELISA in cell culture supernatant after co-culturing $1x10^5$ regenerated $CD8^+$ T cells for 24 hours with $1x10^4$ HLA-A*02:01-positive EBV-lymphoblastoid cells (CIRA0201) pulsed or not pulsed with MART-1 peptide (EAAGIGILTV). Mean ± SE of triplicates is shown.

Regeneration of tumor antigen-specific CTLs from iPSCs

We then induced differentiation of T cells from MART1-T-iPSCs (Fig. 2C) by modification of a previously published method[15]. On the 35th day of cultivation, $CD4^+CD8^+$ double positive (DP) cells were generated (Fig. 2D). However, these *in vitro* culture systems have been unable to support the efficient generation of CD8 single positive (SP) cells beyond the DP stage.

To induce further differentiation, we simply added anti-CD3 mAb to the culture on day 35. The proportion of $CD8^+$ T cells clearly increased during a period of 6 days following TCR stimulation (Fig. 2E, left panel). Importantly, thus induced $CD8^+$ T cells almost exclusively expressed a TCR

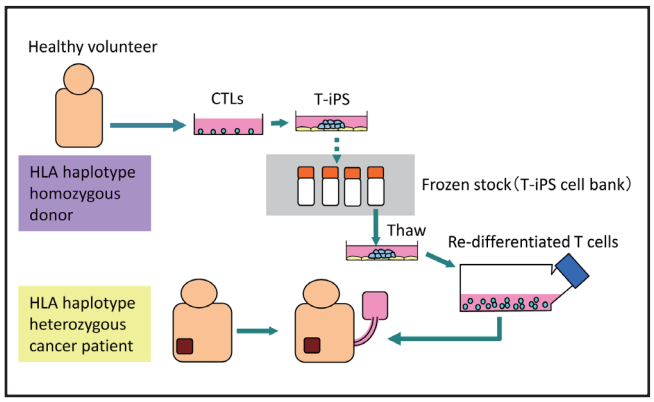

Fig. 3 Application of the method to the allogeneic transplantation setting

T-iPSCs are produced from tumor antigen-specific CTLs collected from healthy HLA haplotype homozygous donors. Regenerated CTLs will be tested with regard to TCR affinity, and if they are sufficient in quality, T-iPSCs will be stored as frozen stocks in the T-iPSC bank. Frozen and pooled regenerated CTLs will then be available for immediate use in HLA-matched patients bearing cancer expressing the same tumor antigen.

specific for the MART-1 antigen (Fig. 2E, right panel).

To examine whether these $CD8^+$ T cells can be activated in an antigen-specific manner, regenerated $CD8^+$ T cells were co-cultured with target cells (human EBV-lympho-blastoid cell line) with or without MART-1 peptide. $CD8^+$ T cells produced a substantial amount of IFNγ only in the presence of specific peptide (Fig. 2F). Collectively, this T-iPSC approach appears to be effective in regenerating functional antigen-specific CTLs.

Application of this method to the allogeneic transplantation setting

The above method, which is based on the autologous transplantation setting, is bound to face some problems. Not only will production of iPSCs for individual patients be costly, it will also be difficult to ensure TCR affinity and iPSC quality, and it will need a substantial amount of time (more than 6 months) to prepare regenerated CTLs.

We propose that an allogeneic transplantation strategy will solve these problems. In order to achieve such a strategy, it will be required to establish a “T-iPSC bank”. In this scheme, T-iPSCs are produced from tumor antigen-specific CTLs collected from healthy donors (Fig. 3). Regenerated CTLs will be tested with regard to TCR affinity, and if they are sufficient in quality, such T-iPSCs will be stored as frozen stocks in the bank. Regenerated CTLs themselves will also be frozen and pooled, such that they will be immediately available when a human leukocyte antigen (HLA)-matched patient develops cancer expressing the same tumor antigen. Preferentially, before the use in patients, it should be tested *in vitro* whether regenerated CTLs happen to exhibit alloreactivity to recipient cells. HLA haplotype-homozygous donors are desired, since regenerated cells can be given to a patient bearing the same HLA haplotype allele as a heterozygote. It is also possible to make iPSCs equivalent to T-iPSCs by transducing non-T derived iPSCs with a defined TCR gene.

By application of allogeneic transplantation strategies, above-mentioned problems of the autologous setting can be solved. Moreover, there will be another big advantage: patients receiving regenerated CTLs will be free from malignant transformation of transferred CTLs. This is because allogeneic grafts will be eventually rejected based on mismatch of minor histocompatibility antigens even in the HLA-matched case. The question may arise here whether such allo-CTLs can do sufficient job before they are rejected. However, it is likely that transferred T cells can work well before rejection, since rejection based on minor histocompatibility mismatch usually occurs slowly in chronic phase. Another question may be that once transferred cells are rejected, then secondly transferred cells will be immediately rejected based on immunological memory of the recipient. This may be avoided by transferring CTLs regenerated from HLA-homo T-iPSCs derived from a different donor, which has different set of minor histocompatibility antigens.

Target antigens and cancer types

Apart from the example of regenerated CTLs specific for the melanoma antigen MART-1 we have shown here, what other candidate antigens and cancer types would potentially benefit from this approach? In general, cancers that are responsive to immune blockade reagents will be good targets. In this respect, lung cancers and kidney cancers, against which anti-PD-1 mAb was reportedly effective, have been listed as candidates[1].

Regarding candidate tumor antigens, TCR-gene therapy trials against several tumor antigens have been already conducted, and some tumor antigens have been reported to be effective and also safe. Those antigens will be good candidates, and in this regard, WT1 (Wilm's tumor 1), NY-ESO-1, or LMP-2 might be suitable targets[16].

It is also possible to target “mutated epitopes”. Since cancer is usually monoclonal, mutations present in a

cancer-initiating cell that bring about alterations of amino acids in proteins are inherited in all progeny cells. Indeed, many types of cancer bear hundreds of such genomic mutations[17]. A recent study showed that cancers against which anti-CTLA-4 mAb had been effective were found to bear more mutations[18], suggesting that at least some of the latent CTLs in cancer patients are targeting mutated epitopes.

Possible side effects

We have mentioned earlier that an autoimmune reaction is inevitable when using a non-antigen-specific strategy. In principle, antigen-specific strategies are able to avoid this type of side effect. However, some risks remain even in the case of antigen-specific strategies. Most tumor antigens are also expressed in normal tissues, although expression levels are usually low. For example, MART-1 is expressed in normal melanocytes, and TCR-gene therapy using MART1-specific TCR often results in vitiligo or vision disorder[7].

In the case of gene therapy using T cells expressing an affinity-enhanced TCR against MAGE-A3, in which amino acids were altered by genetic engineering, the transferred cells led to two patients' deaths, caused by an unexpected TCR cross-reactivity of TCR[19]. Generation of a TCR with such unexpected specificity is attributed to artificial genetic engineering. On the other hand, in case of allogeneic transplantation, it is also possible that transferred T cells just happen to have specificity against allo-HLA or minor antigens. Therefore, the possibility of such risks should be carefully considered.

As to the reproducibility of antigen specificity, some risk presents the generation of a harmful TCR. The TCRα chain gene formed after rearrangement still retains remaining V and J segments upstream and downstream of the rearranged V-J construct, leaving the possibility of further rearrangement to replace the original V-J construct. Although this possibility can be excluded by inactivating Rag genes at the T-iPSC stage using a genome edition system such as CRISPR/Cas9, this in turn may increase the chance that the edition process itself might impair the genome of T-iPSCs.

Another possible approach to reduce side effects caused by unexpected TCR alloreactivity is to use T cell progenitors (induced from antigen-specific iPSCs) as a cell source. In this case, it is expected that such T cell progenitors when transferred to patients migrate to the thymus and produce a large number of naïve CTLs specific for the tumor antigen in thymus. If regenerated T cells then happen to express an autoreactive TCR they may be eliminated by negative selection in the thymus.

Cancer as a target in regenerative medicine

In this article, we have introduced a strategy to regenerate CTLs using iPSC technology as a novel method in cancer immunotherapy. This strategy can also be categorized as one of the therapeutic approaches in regenerative medicine.

Application of iPSC technology has been primarily directed to cases in which compensation of lost organs is required, while it could also be used for drug screening or for drug toxicity testing. However, to date the number of target patients seems not so large. Our approach could make it possible for cancer patients to become targets of regenerative medicine. Just as we have exemplified here the case of malignant melanoma, a large number of peptide antigens recognized by CTLs has been identified comprising various types of solid tumors, including gastric cancer, colon cancer, lung cancer and breast cancer, to name a few[16]. This means that the T-iPSC strategy has the potential to target a broad range of cancer cases.

Perspective: toward clinical application

At present, we are endeavouring to establish T-iPSCs from CTLs specific for a number of cancer antigens from healthy donors. We have succeeded in establishing T-iPSCs from CTLs specific for tumor antigen WT1, which is known to be expressed in various types of cancer cells. We have optimized *in vitro* culture conditions and succeeded in regenerating CTLs expressing CD8 alpha-beta heterodimers. These regenerated CTLs exhibited very high antigen-specific killing activity comparable to that of original CTLs. Regenerated CTLs were able to kill some leukemia cell lines which express endogenous WT1 protein. The cells can be expanded more than ten thousand times by repeated TCR stimulation without reducing their killing activity during an additional several weeks.

Based on these results, we are planning to apply this method to leukemia patients. Aged acute myeloid leukemia patients to whom stem cell transplantation cannot be applied would be good candidates for this new strategy. We will collect WT1-specific CTLs from patients and generate WT1-T-iPS cells. It is also possible to prepare WT1-T-iPS cells from healthy volunteers to be used in the allogeneic setting. Which approach should be taken first will depend on regulatory issues.

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Conflict of interests

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