学位論文

Development of HLA-modified induced pluripotent stem cell-derived dendritic cells for a novel cancer immunotherapy

（HLA 遺伝子改変型 iPS 細胞由来の樹状細胞を用いた新規樹状細胞療法の開発）

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Original article

Title: Development of HLA-modified induced pluripotent stem cell-derived dendritic cells applicable for a novel cancer immunotherapy.

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Abstract

We previously established a method to generate human iPS cell-derived myeloid cell line (iPS-ML) from an human leukocyte antigen (HLA)-A*24:02 donor, which could differentiate into dendritic cells (DCs) and had a high potential in stimulating T cells. However, for clinical applications, the histoincompatibility between iPS cells and cells in cancer patients has remained as a key challenge. One solution is to construct the iPS cell library covering the whole HLA haplotypes, but it is excessively laborious and an impractical solution. Herein, in order to overcome this problem, we generated histocompatible iPS cells by disrupting *HLA-A*, *-B* and *DRA* genes in iPS-MLs by using a CRISPR-Cas9 system. These HLA-deficient iPS-MLs could avoid recognition by alloreactive $CD8^+$ T cells. Next, we introduced HLA-A *02:01 into HLA-deficient iPS-MLs. HLA-deficient iPS-MLs with introduced HLA-A *02:01 differentiated into functional DCs upon stimulation with IL-4, and induced HLA-A2-restricted MART-1-specific $CD8^+$ T cells derived from an allogeneic donor. We also introduced *MART-1* gene into HLA-deficient iPS-MLs with introduced HLA-A *02:01. HLA-deficient iPS-MLs with introduced HLA-A*02:01 and MART-1 could induce HLA-A2-restricted MART-1-specific $CD8^+$ T cells derived from an allogeneic donor. Collectively, we developed a method that can exchange HLA alleles from HLA-A*24:02 to *02:01 in iPS-MLs, and our findings may overcome the obstacle of histoincompatibility in DC vaccination therapy, regardless of HLA allele.

Keywords: iPS-DC, HLA, CRISPR-Cas9, DC vaccine, cancer immunotherapy

Abbreviations: antigen presenting cells, APC; clustered regularly interspaced short palindromic repeats associated protein 9, Cas9; clustered regularly interspaced short palindromic repeats, CRISPR; cytotoxic T lymphocyte, CTL; dendritic cells, DC; granulocyte macrophage colony-stimulating factor, GM-CSF; human leukocyte antigen, HLA; head and neck squamous cell carcinoma, HNSCC; immune checkpoint inhibitor, ICI; interleukin, IL; induced pluripotent stem, iPS; macrophage coloney stimulating factor, M-CSF; major histocompatibility complex, MHC; melanoma antigen recognized by T cells-1, MART-1; myeloid cell line, ML; peripheral blood mononuclear cells, PBMC; programmed death protein-1, PD-1; transporter associated with antigen processing, TAP; Zinc-Finger-Nuclease, ZFN

1. Introduction

Recently, cancer immunotherapy has become an attractive therapeutic modality for cancers [1] being regarded as the fourth cancer treatment modality, besides surgery, radiotherapy, and chemotherapy. Especially, immune-checkpoint inhibitors (ICIs) have been successfully introduced for various malignancies [2]. Anti-programmed death protein-1 (PD-1) antibody, nivolumab, and pembrolizumab are approved for patients with recurrent or metastatic head and neck squamous cell carcinoma (HNSCC) [3]. Although some patients show clinical benefits from ICI therapy, its effect are still insufficient in many cancer patients, including those with HNSCC. Therefore, there is an urgent need to develop more effective and durable cancer immunotherapy in improving clinical outcomes.

Dendric cell (DC) vaccines are promising cancer immunotherapy candidates, because DCs have an important role in eliciting antigen-specific adaptive immune responses to cancer cells [4]. Several forms of DC vaccines have been developed and, to date, many clinical trials using them have been widely conducted for various cancer types [5, 6]. Conventional DC vaccines are generated from a culture of monocytes isolated from the peripheral blood of individual patients. Peripheral monocytes are cultured in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) to differentiate them into immature DCs *ex vivo* [6]. However, it could be difficult to generate DCS with sufficient number and quality from peripheral monocytes, especially when the donor is a patient with advanced cancer, due to the low number of monocytes obtained from their peripheral blood. Furthermore, monocyte isolation and culture differentiation to generate DCs need to be carried out individually for each patient, resulting in time-consuming and expensive procedures.

Induced pluripotent stem (iPS) cells have the potential to resolve the above obstacles of DC vaccine. We have developed a method to generate myeloid cells with proliferation capacity from human iPS cells, and designated them as iPS cell-derived myeloid cell line (iPS-ML). iPS -MLs can proliferate for several months with a doubling time of 2-3 days, be amplified more than 10^{10} times, and differentiate into DCs (iPS-ML-DCs) upon stimulation with IL-4 [7-9]. Thus, we can efficiently generate a large quantity of cells for DC vaccination therapy.

For the clinical application of iPS-derived DCs, there is a big challenge regarding the histoincompatibility between iPS cells and cells in cancer patients. The establsihment of an iPS cell library for covering most HLA haplotypes could resolve this problem, but remains impractical. It has been estimated that >150,000 donors must be screened to establish an iPS cell library that can be used for 90% of the Japanese population [10]. Therefore, we disrupted the *HLA-A*, *-B* and *DRA* genes in iPS-MLs by using CRISPR-Cas9 and subsequently introduced different *HLA-A* gene into *HLA*-defficient iPS-MLs in this study. We confirmed the capacity of these cells as antigen presenting cells (APCs) to induce tumor antigen-specific $CD8^+$ T cells derived from an allogeneic donor. This technology may be applied to any patient, regardless of their HLA alleles, and lead to a novel DC-based cancer vaccination therapy.

2. Materials and Methods

2.1 Cells and donors

Human iPS cells were provided by the iPS cell stock for Regenerative Medicine by the Center for iPS Cell Research and Application (CIRA), Kyoto University. Human iPS cells used in this study were derived from a homozygous donor with HLA-A*24:02-B*52:01 haplotype. Peripheral blood mononuclear cells (PBMC) were collected from healthy donors with a HLA-A*02:01 haplotype and used for experiments. The donors' personal information remained confidential, informed consent was obtained from donors, and experiments were carried out

accroding to the procedures approved by ethics review board of Kumamoto University.

2.2 Plasmid construction and generation of recombinant lentivirus

cDNA fragments for HLA-A*02:01 and the Cas9 nuclease of *S. pyogenes* were obtained by PCR and cloned into a pENTR-TOPO vector (Invitrogen, Carlsbad, CA). cDNAs of MART-1 were obtained from the Kazusa DNA Research Institute (Kisarazu, Japan). Expression constructs for guide RNA driven by the U6 promoter were synthesized by GenScript (Nanjing, China). The DNA fragments were transferred to a lentiviral expression vector, pCSII-EF, using an LR clonase (Invitrogen). pCSII-EF and the plasmids for lentiviral vector packaging, pCMV-VSV-G-RSV-Rev and pCAG-HIVgp, were kindly provided by Dr. H. Miyoshi (RIKEN BioResource Center, Japan) [11]. Plasmid constructs were introduced into 293T cells by lipofection (Lipofectamine 2000, Invitrogen) and 3 days later, the recombinant lentivirus was recovered from the culture supernatant by centrifugation (50,000 g for 2 h) or by using the Lenti-X concentrator (Clontech, Mountain View, CA) [12].

2.3 Generation of iPS-MLs and iPS-ML-DCs

The induction of differentiation of human iPS cells into myeloid cells was conducted as previous described [12]. Briefly, iPS cells were cultured on the feeder layers of mouse OP9 cells for 15-20 days. Subsequently, nonadherent cells were isolated and further cultured in the presence of granulocyto macrophage coloney stimulating factor (GM-CSF) and macrophage coloney stimulating factor (M-CSF). After 7-10 days, cells expressing myeloid markers such as CD11b and CD33 were named as “iPS-MCs (iPS cell-derived myeloid cells)”. iPS-MCs were transduced with lentiviral vectors expressing cMYC, BMI1, and MDM2. After 5-10 days, proliferating cells were named as “iPS-ML”. iPS-ML were cultured in the presence of M-CSF (50 ng/ml), GM-CSF (50 ng/ml) and IL-4 (20 ng/ml) for 2-4 days for the induction of their differentiation into dendritic cells (iPS-ML-DC). To induce the maturation of iPS-ML-DC, cells were stimulated with penicillin-killed Streptococcus pyogenes, OK432 (10 μg/ml, Chugai Pharmaceutical Co., Tokyo, Japan) for 2-3 days.

2.4 Generation of HLA-deficient iPS-MLs by CRISPR-Cas9

iPS-MLs were transduced with a mixture of lentivirus vectors for Cas9 nuclease and guide RNAs for *HLA-A*, *-B* and *DRA*. After 14 days, cells were analyzed by flow cytometry to examine the expression of HLA-A/B/DR. To enrich HLA-deficient iPS-MLs, cells were labeled with a biotinilated anti-HLA-A*24:02/B*52:01 mAb (clone 17-A-10, MBL) and anti-HLA-DR mAb. Cells were treated with antibiotin magnetic beads and enriched using a magnetic column (Miltenyi Biotec, Bergish-Gladbach, Germany). Sorted cells were stained and the lack of expression of HLA was confirmed by flowcytometry. Then, genomic DNA was extracted from them and HLA genes were amplified by PCR and cloned into a plasmid vector. Nucleotide sequences of the plasmid clones were analyzed by using a 3130 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, MA).

2.5 Generation of alloreactive $CD8^+$ T cells

$CD8^+$ T cells obtained from an HLA-mismatched allogenic donor (HLA-A*02:01) were cultured with irradiated iPS-ML-DCs (HLA-A*24:02) for 7 days to generate an alloreactive $CD8^+$ T cells. On day 2, IL-2 (20 U/ml) was added to the culture. On day 7, $CD8^+$ T cells were harvested, washed, and cultured with stimulator cells (5×10^3 cells / well) for 16 h to confirm their recognition by iPS-ML-DCs. The number of IFN-γ producing $CD8^+$ T cells

was counted using ELISPOT assay (BD Bioscience, Bedford, MA).

2.6 Flow cytometry

The following mAbs were used; anti-HLA class I-PE (clone G46-2.6, mouse IgG1), anti-HLA class II-FITC (clone TU39, mouse IgG2a), anti-CD80-FITC (clone L307.4, mouse IgG1), anti-CD83-FITC (clone HB15e, mouse IgG1), anti-CD86-FITC (clone FUN-1, mouse IgG1), anti-CD40-FITC (clone 5C3, mouse IgG1), and anti-MART-1-PE (clone sc-20032, mouse IgG1) from BD Pharmingen (San Diego, CA) or eBioscience (San Diego, CA). Mouse IgG2a-FITC (clone G155-178) and mouse IgG1-FITC/PE (clone MOPC-21) were used as isotype-matched controls. Briefly, cells were treated with an Fc-receptor-blocking reagent (Miltenyi Biotec) for 10 min, stained with the fluorochrome-conjugated mAb for 30 min and washed with PBS/2% FCS. Samples were analyzed on a FACSCalibur (BD Bioscience) flow cytometer.

2.7 Induction of MART-1-specific $CD8^+$ T cells by HLA-deficient iPS-MLs transduced with HLA-A2 or HLA-A2/MART-1

HLA-deficient iPS-MLs transduced with HLA-A2 or HLA-A2/MART-1 were stimulated with OK-432 (0.1 KE/ml) for 2 days. Cells were irradiated (45 Gy) and pulsed with HLA-A*02:01-restricted MART-1 26-35 peptide (AAGIGILTV, 10 μM) for 3 h, and plated into 24-well culture plates (1×10^5 cells/well). PBMC were obtained from an HLA-A*02:01-positive donor and $CD8^+$ T cells were purified by using a $CD8^+$ T cell isolation kit (Milteny Biotec). The $CD8^+$ T cells (1×10^6 cells/well) were co-cultured in AIM-V medium (Life Technologies, Carlsbad, CA) containing 5% human plasma and recombinant human IL-7 (10 ng/ml). IL-2 (20 U/ml) was added on day 2. $CD8^+$ T cells were stimulated on day 7. On day 14, cells were harvested, stained with PE-labeled dextramer of HLA-A*02:01/MART-1 peptide complex (MBL, Nagoya, Japan) in combination with FITC-labeled antihuman CD8 mAb (clone T8, Beckman Coulter, Brea, CA), and analyzed by flow cytometry. The number of IFN-γ-producing T cells upon stimulation with the peptide-pulsed T2A2 cells (1×10^4 cells/well) was counted using ELISPOT assay (BD Bioscience).

2.8 Statistical analysis

Mann-Whitney tests were performed to compare groups. All analysis were two-tailed and considered statistically significant at P values were lower than 0.05. All analysis were performed using Prism 8.4 (GraphPad Software, San Diego, CA).

3. Results

3.1 Generation of HLA-deficient iPS-MLs by CRISPR-Cas9

We previously established a method to generate iPS-MLs [12]. iPS-MLs can proliferate for several months in the presence of M-CSF and GM-CSF, while retaining capacity to differentiate into functional DC. Thus, we can use iPS-MLs as unlimited cell source to generate DCs for cancer vaccination therapy. However, the generation of patient-specific iPS cells and subsequent generation of patient-specific iPS-ML require time, presumably more than 2 months. In addition, individual cell processing, including the generation of patient-specific iPS cells may be too laborious and costly to be applied in clinical medicine.

To solve this problem, we attempted to conquer the problem of histoincompatibility by modifying HLA class I

in iPS-MLs. To this end, we attempted the genetic modification of *HLA-A*, *-B* and *DRA* genes by CRISPR-Cas9 method.

We introduced lentivirus expression vectors for CS9 and gRNA expression vectors directed against HLA-A*24:02, B*52:01, and DRA genes. (Figure 1A, S-Fig 1A and data not shown, respectively) Five days after the CRISPR system, we examined the expression of HLA-A*24:02, B*52:01 and DR. We used the monoclonal antibody clone 17-A-10, which reacted to both HLA-A*24:02 and B*52:01. Among CRISPR-introduced iPS-MLs, 14.8% of them did not express HLA-A*24:02 and B*52:01 and 3.2% of them did not express HLA-DR. (data not shown) To increase the purity, we purified CRISPR-introduced iPS-MLs by bitonilated 17-A10 mAb, biotinilated anti-DR mAb, and antibiotin magnetic microbeads. After this procedure, we confirmed that 100% of the cells were HLA-A/B/DR-deficient.

Collectively, we established a procedure to generate HLA-A/B/DR-deficient iPS-MLs (HLA-deficient iPS-MLs). Sequencing analysed f the *HLA-A*, *-B* and *DRA* genes from HLA-deficient iPS-ML indicated in Figures 1B and C, S-Fig 1B and C and data not shown, respectively.

3.2 Evasion of the alloreactivity of HLA-deficient iPS-ML-DCs against $CD8^+$ T cells

We examined whether HLA-deficient iPS-MLs could avoid the recognition against allogeneic $CD8^+$ T cells. To prepare an alloreactive $CD8^+$ T cells, we stimulated peripheral blood $CD8^+$ T cells obtained from an HLA-mismatched allogeneic donor (HLA-A*02:01) with parental iPS-ML-DCs (HLA-A*24:02) for 7 days. Then, we cultured these alloreactive $CD8^+$ T cells with parental iPS-ML-DCs ($HLA^{+/+}$ iPS-ML-DCs) or HLA-deficient iPS-ML-DCs ($HLA^{-/-}$ iPS-ML-DCs). To evaluate the response of $CD8^+$ T cells, IFN-γ production was measured using ELISPOT assay. As shown in Figures 2A and B, IFN-γ production by alloreactive $CD8^+$ T cells cultured with HLA-deficient iPS-ML-DCs was significantly lower than that in those cultured with parental iPS-ML-DCs. These results indicate that HLA-deficient iPS-ML-DCs can avoid the recognition by alloreactive $CD8^+$ T cells.

3.3 Introduction of HLA-A*02:01 into HLA-deficient iPS-MLs

iPS cells used in the current study were originated from an HLA-A24-homozygous donor. We introduced expression vectors for HLA-A*02:01 into HLA-deficient iPS-MLs using a lentivirus. We successfully introduced an expression vector for HLA-A*02:01 into HLA-deficient iPS-MLs, resulting in the generation of HLA-deficient iPS-MLs with introduced HLA-A*02:01 (HLA-deficient iPS-MLs/A2). Flow cytometric analysis was conducted to confirm the expression of HLA-A2 in HLA-deficient iPS-MLs/A2. (data not shown).

3.4 Characteristics of HLA-deficient iPS-ML-DCs and HLA-deficient iPS-ML-DCs/A2

Next, we assessed the characteristics of these gene-modified cells as APCs to apply for clinical demand. For the induction of differentiation into DC, HLA-deficient iPS-MLs and HLA-deficient iPS-MLs/A2 were cultured in the presence of M-CSF (50 ng/ml), GM-CSF (50 ng/ml), and IL-4 (20 ng/ml) for 2-4 days. They were differentiated into DCs and named HLA-deficient iPS-ML-DCs and HLA-deficient iPS-ML-DCs/A2, respectively. To induce the maturation of iPS-ML-DCs, cells were stimulated with penicillin-killed Streptococcus pyogenes, OK432 (10 μg/ml) for 2-3 days.

We analyzed the expression levels of DC maturation markers such as CD80, CD86, CD83 and CD40 on parental iPS-MLs ($HLA^{+/+}$ iPS-MLs), HLA-deficient iPS-MLs ($HLA^{-/-}$ iPS-MLs), HLA-deficient iPS-ML-DCs ($HLA^{-/-}$ iPS-

ML-DCs) and HLA-deficient iPS-ML-DCs treated with OK-432 ($HLA^{-/-}$ iPS-ML-DCs + OK-432). As shown in Figure 3A, CD40 levels on $HLA^{-/-}$ iPS-MLs slightly increased compared with those in $HLA^{+/+}$ iPS-MLs. This increase was also observed after the induction of differentiation into DC ($HLA^{-/-}$ iPS-ML-DC) and maturation of DC ($HLA^{-/-}$ iPS-ML-DC + OK-432).

After the introduction of HLA-A*02:01 into HLA-deficient iPS-MLs ($HLA^{-/-}$ iPS-MLs/A2), this CD40 expression was diminished (Figure 3B). However, we observed larger increase in its expression after the induction of differentiation into DCs ($HLA^{-/-}$ iPS-ML-DCs/A2) and maturation of DCs ($HLA^{-/-}$ iPS-ML-DCs/A2 + OK-432), compared with difference among HLA-deficient iPS-MLs. Additionally, the expression of CD80 and CD86 increased after maturation of DCs ($HLA^{-/-}$ iPS-ML-DCs/A2 + OK-432). These results suggest that HLA-deficient iPS-ML-DCs and HLA-deficient iPS-ML-DCs/A2 have the characteristics of mature DCs and can be used as a sorce of DC vaccination therapy.

3.5 Induction of tumor antigen-specific $CD8^+$ T cells restricted to the substituted allele of HLA-A

We next examined the feasibility of inducing HLA-A*02:01-restricted and tumor antigen-specific $CD8^+$ T cells by using HLA-deficient iPS-ML-DCs/A2 as stimulator cells. We attempted to establish a MART-1-specific $CD8^+$ T cells from peripheral blood $CD8^+$ T cells obtained from an HLA-A*02:01 donor. The experimental protocol is shown in Figure 4A. Briefly, peripheral blood $CD8^+$ T cells obtained from an HLA-A*02:01-positive individual were stimulated with HLA-deficient iPS-ML-DCs/A2 loaded with MART-1-A2 peptide. On day 7, $CD8^+$ T cells were re-stimulated with HLA-deficient iPS-ML-DCs/A2 loaded with MART-1-A2 peptide. On day 14, $CD8^+$ T cells were harvested and analyzed.

$CD8^+$ T cells producing IFN-γ were counted using ELISPOT assay. Briefly, $CD8^+$ T cells were stimulated with MART-1 peptide-pulsed HLA-A*02:01-positive T2 cells for 16 h. As shown in Figure 4B, induced $CD8^+$ T cells produced IFN-γ significantly in an antigen-specific manner. We also confirmed the presence of MART-1-specific $CD8^+$ T cells after stimulation using dextramer assay (Figure 5C). These results indicate that we successfully induced MART-1-specific $CD8^+$ T cells by using HLA-deficient iPS-ML-DCs/A2 as APC.s

Next, we introduced an expression vector of MART-1 protein into HLA-deficient iPS-ML-DCs/A2, resulting in the generation of HLA-deficient iPS-ML-DCs/A2 with introduced MART-1 (HLA-deficient iPS-ML-DC/A2/MART-1). The expression of MART-1 protein on HLA-deficient iPS-ML-DCs/A2/MART-1 was detected by flow cytometry (Figure 5A). Peripheral blood $CD8^+$ T cells obtained from an HLA-A*02:01-positive individual were stimulated with HLA-deficient iPS-ML-DCs/A2/MART-1 as APCs to induce MART-1-specific $CD8^+$ T cells. On day 7, $CD8^+$ T cells were re-stimulated with HLA-deficient iPS-ML-DCs/A2/MART-1. On day 14, $CD8^+$ T cells were harvested and analyzed by ELISPOT assay. As shown in Figures 5B and C, induced $CD8^+$ T cells produced IFN-γ significantly in an antigen-specific manner. Although we did not observe the efficient induction of MART-1-specific $CD8^+$ T cells compared with HLA-deficient iPS-ML-DCs/A2, MART-1-specific $CD8^+$ T cells were successfully induced by using HLA-deficient iPS-ML-DCs/A2/MART-1 as APCs without adding exogenous antigenic peptides.

4. Discussion

Cancer immunotherapy has emerged as the fourth treatment modality for various cancers, including HNSCC [1, 2]. DC vaccination is a cell-based cancer immunotherapy. Compared to antibody-based immunotherapy like ICI use, there are obstacles to be conquered in order to apply clinical demand. One obstacle of DC vaccination is the limited source of cells. We have generated a human myeloid cell line with proliferation capacity from iPS cells (iPS-MLs). These cells can differentiate into functional DCs by *in vitro* stimulation. Although this technology allows us to generate a large number of DCs to conduct cell-based cancer immunotherapy, there is another obstacle regarding differences in histocompatibility between iPS cells and those of cancer patients. In this study, we have established a method to overcome this obstacle by replacing HLA in iPS-MLs.

In our previous study, the injection of mice with antigenic peptide-loaded semi-allogeneic DCs sharing one haplotype of major histocompatibility complex (MHC) with the recipients resulted in the priming of peptide-specific cytotoxic T lymphocytes (CTLs) restricted by the shared MHC class I [13]. However, the efficiency of introduction was lower than that obtained when the genetic background of the DC and the recipient mice was identical, probably because the stimulation of peptide-specific T cells was diminished by the rapid elimination of the transferred DCs by alloreactive CTLs of the recipients [14]. Loyer and colleagues studied target histocompatibility antigens recognized by the immune system of the recipients upon the *in vivo* transfer of allogeneic APCs [15]. They concluded that the elimination of transferred allogeneic APCs was mainly mediated by CTLs reactive to allogeneic MHC class I, but not MHC class II or minor antigens. The efficiency of immunization using DCs *in vivo* has also reported to be significantly reduced by pre-existing CTLs reactive to DCs [16].

Based on these findings, it is conceivable that, if the expression of the intrinsic MHC class I of DCs is inhibited, DCs inoculated into allogeneic recipients can escape from rapid elimination by alloreactive T cells of the recipients, resulting in prolonged survival timess of transferred DCs and improved immunization efficiencies. In a subsequent study, we evaluated the effect of disruption of the *HLA-A*, *-B* and *DRA* genes to evade recognition by alloreactive $CD8^+$ T cells [17]. We demonstrated that the disruption of either of these genes prevented the recognition of the DCs by alloreactive $CD8^+$ T cells *in vitro*.

In another previous study, we examined the possibility of *in vitro* stimulation of antigen-specific human $CD8^+$ T cells derived from allogeneic donors by TAP-deficient human iPS-ML-DCs [18]. We disrupted the *TAP2* gene in the iPS cells by using Zinc-Finger-Nucleases (ZFN) to prevent HLA transport to the cell surface. We showed TAP2-deficient iPS cells or iPS-ML-DCs avoided recognition by alloreactive $CD8^+$ T cells to some extent.

In the current study, we disrupted the *HLA-A*, *-B*, and *DRA* genes in iPS-MLs by applying CRISPR technology, which enables simultaneous gene disruption at multiple genetic loci. HLA-deficient iPS-MLs evaded recognition by alloreactive $CD8^+$ T cells. Subsequently, we introduced an HLA-A*02:01 expression vector to generate HLA-A*02:01-expressing HLA-deficient iPS-MLs (HLA-deficient iPS-MLs/A2) and HLA-deficient iPS-ML-DCs/A2. Using HLA-deficient iPS-ML-DCs/A2 as stimulator cells, we successfully induced HLA-A*02:01-restricted and antigen-specific $CD8^+$ T cells. These results indicated that we might generate artificial APCs expressing any HLA allele. Because iPS-MLs can be propagated almost indefinitely, it is possible to construct a bank of iPS-MLs expressing various types of HLA. The differentiation from iPS-MLs to iPS-ML-DCs takes only 2-3 days, and DCs can be supplied within a short period according to reqirements.

So far, many clinical trials of anti-cancer vaccination have been conducted. In most of them, synthetic peptides are administrated to patients with Fleund Incomplete Adjuvant [19]. Considerable efforts have been devoted to

identifying cancer antigen-derived CTL epitopes that are restricted to the major alleles of HLA class I, such as HLA-A*02:01 [20-26]. On the other hand, relatively few epitopes have been identified for low-frequency *HLA-A* alleles. Thus, cancer patients who are negative for common types of HLA class I are excluded from epitope peptide vaccination therapies. In addition, HLA-B-restricted epitopes have hardly been identified, probably due to the absence of particularly dominant alleles in the HLA-B locus [27], although should be more useful HLA-B-restricted epitopes. As shown in the current study, HLA-deficient iPS-ML-DCs/A2 introduced MART-1 (HLA-deficient iPS-ML-DCs/A2/MART-1) could prime MART-1-specific $CD8^+$ T cells *in vitro*. Vaccination with iPS-ML-DCs carrying an expression vector can be performed in cancer patients with any HLA allele, and information on the T cell epitopes of the cancer antigens is not necessary.

In vitro artificial differentiation methods to generate various kinds of cells or tissues from human iPS cells have been developed, and iPS cells are regarded as the most ideal cell source for the regenerative medicine. However, the individual generation of patient-specific iPS cells and use of them for the therapy is highly laborious, time-consuming and expensive. To solve this problem, a bank of iPS cells established from donors homozygous for the *HLA-A*/*-B*/*DR* alleles has been constructed in CIRA, Kyoto University, Japan [28]. Use of iPS cells from the iPS cell stock is considered to be more practical as compared with those of patient-specific iPS cells. The problem related to the iPS cell stock is the difficulty in finding homozygous donors of rare HLA haplotypes. An estimation based on the data of HLA haplotype frequency of the Japanese people indicated that 10 to 20 % of them cannot be covered by the iPS cell stock [10]. In order to overcome this problem by modification of HLA genes in human iPS cells or cells generated from iPS cells, targeted gene disruption of *HLA-A*/*-B*/*DR* genes in undifferentiated iPS cells may be very useful. The procedure to generate HLA-deficient iPS-MLs by CRISPR-Cas9 method established in the current study should be applicable to undifferentiated iPS cells as well.

5. Conclusion

We demonstrated a novel method to generate iPS cells that can be used for iPS cell-based DC vaccination therapy in any cancer patients, including those with HNSCC, irrespective of their HLA allele, by disruption of HLA genes in iPS cells and subsequent introducution of an expression vector with the intended HLA allele. Our findings may offer a promising strategy applicable to a personalized DC-based cancer immunotherapy, notably promising for patients with uncommon types of HLA class I, thus contributing to the broad clinical applications of DC vaccines like ICI.

Ethical approval

This study was conducted with the approval of the institutional ethics review board of Kumamoto University, Graduate School of Medical Sciences (Approval numbers: 118 and 499).

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Conflict of interest statement

The authors have no conflict of interest to disclose.

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References

[1] Xin Yu J, Hubbard-Lucey VM, Tang J. Immuno-oncology drug development goes global. Nat Rev Drug Discov. 2019;18:899-900.

[2] Robert C. A decade of immune-checkpoint inhibitors in cancer therapy. Nat Commun. 2020;11:3801.

[3] Kitamura N, Sento S, Yoshizawa Y, Sasabe E, Kudo Y, Yamamoto T. Current Trends and Future Prospects of Molecular Targeted Therapy in Head and Neck Squamous Cell Carcinoma. Int J Mol Sci. 2020;22:240.

[4] Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol. 1991;9:271-96.

[5] Nava S, Lisini D, Frigerio S, Bersano A. Dendritic Cells and Cancer Immunotherapy: The Adjuvant Effect. Int J Mol Sci. 2021;22(22).

[6] Hopewell EL, Cox C. Manufacturing Dendritic Cells for Immunotherapy: Monocyte Enrichment. Mol Ther Methods Clin Dev. 2020;16:155-60.

[7] Sakisaka M, Haruta M, Komohara Y, Umemoto S, Matsumura K, Ikeda T, et al. Therapy of primary and metastatic liver cancer by human iPS cell-derived myeloid cells producing interferon-β. J Hepatobiliary Pancreat Sci. 2017;24(2):109-19.

[8] Imamura Y, Tashiro H, Tsend-Ayush G, Haruta M, Dashdemberel N, Komohara Y, et al. Novel therapeutic strategies for advanced ovarian cancer by using induced pluripotent stem cell-derived myelomonocytic cells producing interferon beta. Cancer science. 2018;109(11):3403-10.

[9] Umemoto S, Haruta M, Sakisaka M, Ikeda T, Tsukamoto H, Komohara Y, et al. Cancer therapy with major histocompatibility complex-deficient and interferon β-producing myeloid cells derived from allogeneic embryonic stem cells. Cancer science. 2019;110(10):3027-37.

[10] Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, et al. A more efficient method to generate integration-free human iPS cells. Nature methods. 2011;8(5):409-12.

[11] Miyoshi H, Blömer U, Takahashi M, Gage FH, Verma IM. Development of a self-inactivating lentivirus vector. Journal of virology. 1998;72(10):8150-7.

[12] Senju S, Haruta M, Matsumura K, Matsunaga Y, Fukushima S, Ikeda T, et al. Generation of dendritic cells and macrophages from human induced pluripotent stem cells aiming at cell therapy. Gene therapy. 2011;18(9):874-83.

[13] Fukuma D, Matsuyoshi H, Hirata S, Kurisaki A, Motomura Y, Yoshitake Y, et al. Cancer prevention with semi-allogeneic ES cell-derived dendritic cells. Biochemical and biophysical research communications. 2005;335(1):5-13.

[14] Wells JW, Cowled CJ, Darling D, Guinn BA, Farzaneh F, Noble A, et al. Semi-allogeneic dendritic cells can induce antigen-specific T-cell activation, which is not enhanced by concurrent alloreactivity. Cancer immunology, immunotherapy : CII. 2007;56(12):1861-73.

[15] Loyer V, Fontaine P, Pion S, Hétu F, Roy DC, Perreault C. The in vivo fate of APCs displaying minor H antigen and/or MHC differences is regulated by CTLs specific for immunodominant class I-associated epitopes. Journal of immunology (Baltimore, Md : 1950). 1999;163(12):6462-7.

[16] Hermans IF, Ritchie DS, Yang J, Roberts JM, Ronchese F. CD8+ T cell-dependent elimination of dendritic cells in vivo limits the induction of antitumor immunity. Journal of immunology (Baltimore, Md : 1950). 2000;164(6):3095-101.
[17] Matsunaga Y, Fukuma D, Hirata S, Fukushima S, Haruta M, Ikeda T, et al. Activation of antigen-specific cytotoxic T lymphocytes by beta 2-microglobulin or TAP1 gene disruption and the introduction of recipient-matched MHC class I gene in allogeneic embryonic stem cell-derived dendritic cells. Journal of immunology (Baltimore, Md : 1950). 2008;181(9):6635-43.
[18] Haruta M, Tomita Y, Yuno A, Matsumura K, Ikeda T, Takamatsu K, et al. TAP-deficient human iPS cell-derived myeloid cell lines as unlimited cell source for dendritic cell-like antigen-presenting cells. Gene therapy. 2013;20(5):504-13.
[19] Yoshitake Y, Fukuma D, Yuno A, Hirayama M, Nakayama H, Tanaka T, et al. Phase II clinical trial of multiple peptide vaccination for advanced head and neck cancer patients revealed induction of immune responses and improved OS. Clinical cancer research : an official journal of the American Association for Cancer Research. 2015;21(2):312-21.
[20] Jäger E, Chen YT, Drijfhout JW, Karbach J, Ringhoffer M, Jäger D, et al. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. The Journal of experimental medicine. 1998;187(2):265-70.
[21] Ohminami H, Yasukawa M, Fujita S. HLA class I-restricted lysis of leukemia cells by a CD8(+) cytotoxic T-lymphocyte clone specific for WT1 peptide. Blood. 2000;95(1):286-93.
[22] Rongcun Y, Salazar-Onfray F, Charo J, Malmberg KJ, Evrin K, Maes H, et al. Identification of new HER2/neu-derived peptide epitopes that can elicit specific CTL against autologous and allogeneic carcinomas and melanomas. Journal of immunology (Baltimore, Md : 1950). 1999;163(2):1037-44.
[23] Harao M, Hirata S, Irie A, Senju S, Nakatsura T, Komori H, et al. HLA-A2-restricted CTL epitopes of a novel lung cancer-associated cancer testis antigen, cell division cycle associated 1, can induce tumor-reactive CTL. International journal of cancer. 2008;123(11):2616-25.
[24] Suda T, Tsunoda T, Daigo Y, Nakamura Y, Tahara H. Identification of human leukocyte antigen-A24-restricted epitope peptides derived from gene products upregulated in lung and esophageal cancers as novel targets for immunotherapy. Cancer science. 2007;98(11):1803-8.
[25] Komori H, Nakatsura T, Senju S, Yoshitake Y, Motomura Y, Ikuta Y, et al. Identification of HLA-A2- or HLA-A24-restricted CTL epitopes possibly useful for glypican-3-specific immunotherapy of hepatocellular carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2006;12(9):2689-97.
[26] Tomita Y, Harao M, Senju S, Imai K, Hirata S, Irie A, et al. Peptides derived from human insulin-like growth factor-II mRNA binding protein 3 can induce human leukocyte antigen-A2-restricted cytotoxic T lymphocytes reactive to cancer cells. Cancer science. 2011;102(1):71-8.
[27] Parham P, Ohta T. Population biology of antigen presentation by MHC class I molecules. Science (New York, NY). 1996;272(5258):67-74.
[28] Azuma K, Yamanaka S. Recent policies that support clinical application of induced

pluripotent stem cell-based regenerative therapies. Regenerative therapy. 2016;4:36-47.

Figure legends

Figure 1. Targeted disruption of HLA-A gene in iPS-ML

(A) Construction of HLA-A gene is shown. Targeted disruption of HLA-A gene was performed in exson one. An example of design of primers is shown in the bold letters with an underline. Targeted sequence, initiation codon and PAM are shown in red, green and yellow, respectively. (B) PCR analysis was conducted from established colony. The name of cell line is shown on top. (C) Sequence of DNA from established cell line is shown. The deletion is shown as dashed line. These bulk cell lines were used for further experiments.

Figure 2. Evasion of allo-reactivity against $CD8^+$ T cells by HLA-deficient iPS-ML-DC

Peripheral blood $CD8^+$ T cells obtained from an HLA-mismatched allogeneic donor (HLA-A*02:01) were stimulated with parental iPS-ML-DC (HLA-A*24:02). IL-2 (20 U/ml) were added at day 2. At day 7, $CD8^+$ T cells were harvested and cultured with parental iPS-ML-DC ($HLA^{+/+}$ iPS-ML-DC) or HLA-deficient iPS-ML-DC ($HLA^{-/-}$ iPS-ML-DC). $CD8^+$ T cells producing IFN-γ were measured by ELISPOT assay. (A) Spots of wells are shown. (B) The number of IFN-γ-producing $CD8^+$ T cells is shown. (left) $CD8^+$ T cells (5×10^3) were cultured with stimulator cells (5×10^3). (right) $CD8^+$ T cells (1×10^4) were cultured with stimulator cells (5×10^3). Representative data is shown from three independent experiments.

Figure 3. Characteristics of HLA-deficient iPS-ML-DC and HLA-deficient iPS-ML-DC/A2

(A,B) HLA-deficient iPS-ML ($HLA^{-/-}$ iPS-ML) and HLA-deficient iPS-ML/A2 ($HLA^{-/-}$ iPS-ML/A2) were cultured in the presence of M-CSF (50 ng/ml), GM-CSF (50 ng/ml) and IL-4 (20 ng/ml) for 2-4 days to induce HLA-deficient iPS-ML-DC ($HLA^{-/-}$ iPS-ML-DC) and HLA-deficient iPS-ML-DC/A2 ($HLA^{-/-}$ iPS-ML-DC/A2), respectively. $HLA^{-/-}$ iPS-ML-DC and $HLA^{-/-}$ iPS-ML-DC/A2 were cultured in the presence of OK432 (10 μg/ml) for 2-3 days ($HLA^{-/-}$ iPS-ML-DC + OK-432 and $HLA^{-/-}$ iPS-ML-DC/A2 + OK-432, respectively). Cell surface expression of CD80, CD86, CD83 and CD40 on these cells were analyzed by flowcytomerty. The staining patterns of each mAb (bold line) and an isotype matched control mAb (gray area) are shown. Representative data is shown from three independent experiments.

Figure 4. Induction of HLA-A2-restricted and MART-1-specific $CD8^+$ T cells by HLA-deficient iPS-ML-DC/A2

Peripheral blood $CD8^+$ T cells obtained from an HLA-A*02:01-positive individual were stimulated with HLA-deficient iPS-ML-DC/A2 loaded with MART-1-A2 peptide. After second stimulation, $CD8^+$ T cells were harvested and analyzed. (A) Experimental design (B) $CD8^+$ T cells (1×10^5) were stimulated with HLA-A*02:01 positive T2 cells (2×10^4) loaded with MART-1-A2 peptide (left bar) or control HIV-A2 peptide (right bar) for 16 h. $CD8^+$ T cells producing IFN-γ were counted by ELISPOT assay. (C) $CD8^+$ T cells were stained with HLA-A2/MART-1 dextramer (right panel) or control HLA-A2/HIV dextramer (middle panel) before and after stimulation. The number shown in upper right quadrant indicates the frequency of MART-1 postive $CD8^+$ T cells among $CD8^+$ T cells. Representative data is shown from three independent experiments.

Figure 5. Induction of HLA-A2-restricted and MART-1-specific $CD8^+$ T cells by HLA-deficient iPS-ML-DC/A2/MART-1

Peripheral blood $CD8^+$ T cells obtained from an HLA-A*02:01-positive individual were stimulated with HLA-deficient iPS-ML-DC/A2/MART-1. At day 7, $CD8^+$ T cells were re-stimulated with HLA-deficient iPS-ML-DC/A2/MART-1. At day 14, $CD8^+$ T cells were harvested and analyzed. (A) Cell surface expression of MART-1 protein on HLA-deficient iPS-ML-DC/A2/MART-1 was analyzed by flowcytomerty. The staining pattern of MART-1 mAb (bold line) and an isotype matched control mAb (gray area) are shown. (B,C) $CD8^+$ T cells (1×10^5) were stimulated with HLA-A*02:01 positive T2 cells (2×10^4) loaded with MART-1-A2 peptide (top, left bar) or control HIV-A2 peptide (bottom, right bar) for 16 h. $CD8^+$ T cells producing IFN-γ were counted by ELISPOT assay. Representative data is shown from three independent experiments.

Figure 6. Novel strategy for DC vaccination therapy by using iPS cell-derived DC substituted HLA

Parental iPS-ML ($HLA^{+/+}$ iPS-ML) can be used as cell sorce of DC vaccination therapy for HLA-matched patients (arrow A). To develop DC based immunotherapy, $HLA^{+/+}$ iPS-ML was engineered HLA-deficient iPS-ML ($HLA^{-/-}$ iPS-ML) by CRISPR-Cas9 method (arrow B). HLA-A2 was introduced into $HLA^{-/-}$ iPS-ML ($HLA^{-/-}$ iPS-ML/A2) to apply for patients who have HLA-A2 allele. This method could be applied for other allele of HLA. $HLA^{-/-}$ iPS-ML/A2 differentiates into functional DC ($HLA^{-/-}$ iPS-ML-DC/A2) and can be used as DC vaccination with antigenic peptide for HLA-A2 patients (arrow 1). MART-1 gene was introduced into $HLA^{-/-}$ iPS-ML/A2, resulting the generation of MART-1 expressing $HLA^{-/-}$ iPS-ML-DC/A2 ($HLA^{-/-}$ iPS-ML-DC/A2/MART-1). $HLA^{-/-}$ iPS-ML-DC/A2/MART-1 can be used as DC vaccination without antigenic peptide for HLA-A2 patients (arrow 2).

Supplymentary Figure legend

S-Figure 1. Targeted disruption of HLA-B gene in iPS-ML

(A) Construction of HLA-B gene is shown. Targeted disruption of HLA-B gene was performed in exson one. An example of design of primers is shown in the bold letters with an underline. Targeted sequence, initiation codon and PAM are shown in red, green and blue, respectively. (B) PCR analysis was conducted from established colony. The name of cell line is shown on top. (C) Sequence of DNA from established cell line is shown. The deletion and insertion are shown as dashed line and red underline, respectively. These bulk cell lines were used for further experiments.