Induced pluripotent stem cell-derived dendritic cell vaccine therapy genetically modified on the ubiquitin-proteasome system

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Induced pluripotent stem cell-derived dendritic cell vaccine therapy genetically modified on the ubiquitin-proteasome system

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Short running head: iPS-DC therapy using Ub-PS system

Abstract

We previously reported that dendritic cells (DCs) transduced with the full-length tumor-associated antigen (TAA) gene induced TAA-specific cytotoxic T lymphocytes (CTLs) to elicit antitumor responses. To overcome the issue of quantity and quality of DCs required for DC vaccine therapy, we focused on induced pluripotent stem cells (iPSCs) as a new tool for obtaining DCs and reported efficacy of iPSCs-derived DCs (iPSDCs). However, in clinical application of iPSDC vaccine therapy, further enhancement of the antitumor effect is necessary. In this study, we targeted mesothelin (MSLN) as a potentially useful TAA, and focused on the ubiquitin-proteasome system to enhance antigen-presenting ability of iPSDCs. The CTLs induced by iPSDCs transduced with MSLN gene (iPSDCs-MSLN) from healthy donors showed cytotoxic activity against autologous lymphoblastoid cells (LCLs) expressing MSLN (LCLs-MSLN). The CTLs induced by iPSDCs transduced ubiquitin-MSLN fusion gene exhibited higher cytotoxic activity against LCLs-MSLN than the CTLs induced by iPSDCs-MSLN. The current study was designed that peripheral T-cell tolerance to MSLN could be overcome by the immunization of genetically modified iPSDCs simultaneously expressing ubiquitin and MSLN, leading to a strong cytotoxicity against tumors endogenously expressing MSLN. Therefore, this strategy may be promising for clinical application as an effective

cancer vaccine therapy.

Introduction

Dendritic cells (DCs) are the most potent professional antigen-presenting cells. The presentation of peptides generated by the ubiquitin-proteasome pathway on MHC class I molecules, known as cross-presentation, is essential for the initiation of antigen-specific CD8+ T cells responses [1]. The cross-presentation is an extremely useful feature for antitumor immune responses. We previously reported that DCs transduced with the full-length tumor-associated antigen (TAA) gene induced TAA-specific cytotoxic T lymphocytes (CTLs) to elicit antitumor responses [2-5]. However, the DC vaccine therapy has not reached breakthrough in the cancer treatment, it requires a large quantity of DCs generated from peripheral blood monocytes of patients with cancer. Frequent apheresis must be performed, which is burdensome upon such patients [6]. Moreover, the function of DCs such as maturation, antigen-presenting ability, and migration is diminished in patients with cancer [7]. To overcome these problems of DC vaccine therapy, we focused on induced pluripotent stem cells (iPSCs) as a new tool for obtaining DCs. Several reports have highlighted methods for differentiating DCs from iPSCs (iPSDCs) in mouse and human studies [8-12]. Our previous study reported that

mouse iPSDCs transduced with TAA gene have a capacity for TAA-specific antitumor immunity equal to that of bone marrow-derived DCs [13]. In another study, we reported that iPSDCs transduced with carcinoembryonic antigen (CEA) gene have the capacity for CEA-specific antitumor immunity in our mouse and human models [14]. Furthermore, *in vitro* CTLs obtained by transfecting tumor-derived RNA into iPSDCs from patients with colorectal cancer showed potent tumor-specific killing effect [15]. However, we have not been able to create an iPSDC vaccine that exerts a strong antitumor effect. In clinical application of iPSDC vaccine therapy, further enhancement of the antitumor effect is necessary.

In the current study, we targeted mesothelin (MSLN) as a potentially useful TAA, and focused upon the ubiquitin-proteasome system to enhance antigen-presenting ability of iPSDCs. MSLN is a CA125 (Carbohydrate Antigen 125)-binding protein. CA125 might contribute to the peritoneal metastasis of ovarian cancer by initiating cell attachment to the mesothelial epithelium via binding to MSLN [16]. Co-expression of MSLN and MUC16 (Mutin16, CA125) is related to the invasion process in pancreatic ductal adenocarcinoma [17]. MSLN is a predictive factor for peritoneal recurrence and correlated with poor outcomes in gastric cancer [18]. MSLN is known as an attractive target for cancer immunotherapy, owing to its low expression on normal mesothelial

cells and high expression in several solid tumors including gastrointestinal tumors [19]. For cancer immunotherapy targeting MSLN, there are reports of dendritic cell vaccine [5], a peptide vaccine [20] [21], and a DNA vaccine [22] in basic research. Miyazawa et al. reported that CTLs induced by monocytes-derived DCs (MoDCs) transfected with whole MSLN gene elicited MSLN-specific cytotoxicity against pancreatic cancer cell line[5]. In clinical practice, phase I/II trials of antibody therapy have been conducted and some efficacy has been shown [23].

The ubiquitin was originally identified as the signal to target proteins for proteasomal degradation [24]. The addition of a ubiquitin monomer sequence to an adenoviral transgene as a source of antigen increased both *in vivo* specific CD8 immunogenicity and *in vitro* MHC class I-restricted presentation by DCs of the encoded peptides [25]. In tumor immunity, the use of ubiquitin-fused epitopes reportedly enhanced the antitumor effect in mouse DNA vaccines [26] [27] [28] and in human DC vaccines [29].

We investigated whether genetically modified human iPSDCs expressing MSLN could induce MSLN-specific CTLs. Furthermore, we assessed whether the induction of ubiquitin-MSLN fusion gene into iPSDCs enhanced the antigen-presenting ability through the ubiquitin- proteasome system and whether it enhanced the cytotoxic ability

of CTLs.

Materials and Methods

Cell line

Autologous Epstein-Barr virus (EBV)-transfected B-lymphoblastoid cells (LCLs) were generated from healthy donor peripheral blood mononuclear cells (PBMC), as previously described [3]. LCLs were cultured in RPMI-1640 medium containing 2 mM L-glutamine supplemented with 10% fetal bovine serum, 100U/mL penicillin and 100μg/mL streptomycin.

Generation of human iPSDCs

iPSCs were derived from PBMCs using Cyto Tune-iPS 2.0 (ID Pharma, Tokyo, Japan). PBMCs were obtained from healthy volunteers. The differentiated protocol of the human iPSDCs was performed as previously described [15]. Briefly, in step 1, undifferentiated iPSCs were disseminated onto a 100-mm culture dish coated with growth factor-reduced Matrigel (Corning, NY) in mTeSR Plus-cGMP medium (Stemcell Technologies, Vancouver, BC) supplemented with 80 ng/mL rhBMP4 (R&D Systems, Minneapolis, MN). In step 2, on day 4, mTeSR Plus-cGMP medium was replaced with StemPro-34 serum-free medium (Thermo Fisher Scientific, Waltham,

MA) containing 2 mM L-glutamine supplemented with 80 ng/mL rhVEGF (R&D Systems), 25 ng/mL basic FGF (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), and 100 ng/mL rhSCF (Peprotech, Cranbury, NJ). In step 3, on day 6, the cytokines in StemPro-34 were changed to cytokines mixed with 50 ng/mL rhSCF, 50 ng/mL rhIL-3 (R&D Systems), 5 ng/mL rhTPO (Peprotech), 50 ng/mL rhM-CSF (Peprotech), and 50 ng/mL rhFlt-3 ligand (Peprotech). In step 4, on day 13, the cytokine in StemPro-34 were changed to cytokines mixed with 50 ng/mL rhM-CSF, 25 ng/mL rhGM-CSF (Peprotech), and 50 ng/mL rhFlt3 ligand. CD14 positive monocytic lineage cells were sorted using an autoMACS Pro Separator with CD14 MicroBeads, human (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) on days 16 to 28. In step 5, 1.5 $\times 10^6$ CD14 positive monocytic cells/well in 6-well Ultra-Low Attachment Surface plates (Corning) were cultured in the StemPro-34 medium containing 25 ng/mL rhGM-CSF and 40 ng/mL rhIL-4 (R&D Systems) for five days for differentiation into immature iPSDCs.

Recombinant adenoviral vector construction and gene transfer into DCs

The recombinant adenoviral vector AxCAMSLN, encoding MSLN, was generated by the cosmid-terminal protein complex (COS-TPC) method, as previously described [5]. AxCALacZ, encoding β-gal, was also generated by the COS-TPC method. The

ubiquitin gene was amplified by PCR from PBMC genomic DNA, using the 5' primer (5' AGT CCG CTA GCC GCC ACC ATG CAG ATC TTC GTG AAG ACC 3') and the 3' primer (5' TAG TCC GTC GAC GTA TTT AAA TCG ACC CCC CCT CAA GCG CAG GAC 3'). The 5' primer contained *Nhe I* restriction site, the *Kozak* sequence (CGCCACC), and the ATG start codon. The 3' primer added an arginine to the N terminal extremity of ubiquitin protein and contained *Swa I* restriction site [25] [28]. The monomer ubiquitin cDNA was inserted into the cosmid vector pAxCAwt to yield the ubiquitin-expressing recombinant cosmid. The MSLN gene was inserted into the *Swa I* restriction site to prepare the ubiquitin-MSLN gene expression recombinant cosmid. AxCAubiquitin-MSLN, encoding ubiquitin-MSLN, was generated by the COS-TPC method (Fig. 1). Immature DCs were transfected with each recombinant adenoviral vector using the centrifugal method at 100 multiplicity of infection (MOI) [2] [4]. The genetically modified DCs were placed at 1.5×10^6 cells/well in 6-well Ultra-Low Attachment Surface plate in the presence of 100 ng/mL rhIL-6, 10ng/mL rhIL-1β, 10 ng/mL rhTNF-α, and 1μg/mL PGE2 to induce final maturation for 48 hours [14].

Immunohistochemistry for MSLN

MSLN protein expression was examined by immunohistochemical staining in MSLN cDNA-transduced iPSDCs and ubiquitin-MSLN combined cDNA-transduced iPSDCs.

iPSDCs were collected using Smear Gell (GenoStaff, Tokyo, Japan). The endogenous peroxidase activity was suppressed by a solution of 0.3% hydrogen peroxide in methanol for 30 min. After being rinsed in Tris-buffered saline (TBS), the sections were incubated with a blocking reagent; Protein block (Dako, Kyoto, Japan) for 20 min at room temperature. The sections were incubated for 30 min at room temperature with the primary antibody, a 1:1500 dilution of an anti-human MSLN monoclonal antibody (EPR19025-42, Abcam, Cambridge, UK). After rinsing in TBS, the primary antibody was visualized using anti-rabbit antibody (Dako) for 60 min at room temperature, and DAB kit (Nichirei, Tokyo, Japan).

Induction of MSLN-specific CTLs from PBMCs and MSLN pentamer assay

Autologous PBMCs from healthy donors were used as responder cells. MSLN cDNA-transduced iPSDCs (iPSDCs-MSLN), LacZ cDNA-tranduced iPSDCs (iPSDCs-LacZ), and ubiquitin-MSLN fusion cDNA-transduced iPSDCs (iPSDCs-UbMSLN) were used as stimulator cells. On day 0, a total of 4×10^6 responder cells and 2×10^5 stimulator cells were mixed in AIM-V medium containing 10 ng/mL rhIL-7 and cultured in 24-well Ultra-Low Attachment Surface plate at a total volume of 1mL/well. On day 2, AIM-V medium containing 20U/mL of rhIL-2 was added at total volume of 2 mL/well. On days 7 and 14, the cultures were re-stimulated with stimulator cells at a ratio 20:1.

AIM-V medium containing 20U/mL of rhIL-2 was added every 2-3 days. After three cycles of re-stimulation, CD8+ cells as CTLs were sorted from the stimulated PBMCs on day 21 using an autoMACS Pro Separator. The CTLs were stained with the PE-labelled pentamers of HLA-A2-binding MSLN epitope peptides, SLLFLLFSL ($A2_{(20-28)}$) and VLPLTVAEV ($A2_{(530-538)}$) respectively (ProImmune, Oxford, UK) in combination with FITC-conjugated anti-CD8 antibody (Becton Dickinson), and analyzed using flow cytometry.

Cytotoxicity assay

The cytotoxic activity was analyzed using a 4h ^{51}Cr release assay. The protocol of a 4h ^{51}Cr release assay was modified from a previously established protocol [2]. Briefly, the target cells were labeled with ^{51}Cr for 1 hour. The target cells were plated at a concentration of 1×10^3 cells/well with 4×10^4/well unlabeled K562 cells and CTLs as effector cells at various E/T ratio at 37℃ in a 5% CO2 atmosphere for 4 hours. The supernatant was collected and measured for released ^{51}Cr. MSLN cDNA-transduced LCLs and Lac-Z cDNA-transduced LCLs were used as target cells. MSLN peptides that were HLA-A24 or HLA-A2 restricted CTL epitope, as previously described [20] [30], were synthesized and purified to >95% purity (Sigma-Aldrich, St. Louis, MO). HLA-A24-binding MSLN epitope peptides, AFYPGYLCSL ($A24_{(442-451)}$), FYPGYLCSL

(A24$_{(435-443)}$), LYPKARLAF (A24$_{(475-483)}$), and HLA-A2-binding MSLN epitope peptides, SLLFLLFSL (A2$_{(20-28)}$), VLPLTVAEV (A2$_{(530-538)}$) were synthesized for the experiments. LCLs pulsed with these MSLN epitope peptides were also used as target cells.

Flow cytometric analysis

PE-conjugated anti-human CD11c (Becton Dickinson) was used for monocyte-derived DCs (MoDCs) and iPSDCs staining. Intracellular staining with an anti-human MSLN monoclonal antibody (EPR19025-42, Abcam) and Alexa Fluor 488 conjugated goat anti-rabbit IgG antibody (Abcam) was performed using a Fixation and Permeabilization Solution Kit (Becton Dickinson).

Results

Expression of MSLN in genetically modified DCs

Immature DCs were transduced with recombinant adenoviral vector by centrifugal method at 100 MOI. The genetically modified DCs were analyzed using intracellular MSLN-staining flow cytometry to compare the transfection efficiency of the MSLN gene between MoDCs-MSLN and iPSDCs-MSLN. The percentage of positively stained cells among the MoDCs and iPSDCs was 83% and 84%, respectively (Fig. 2a).

Cytotoxic activity of CTLs induced by MoDCs-MSLN and iPSDCs-MSLN

CTLs induced by MoDCs-MSLN from healthy donors 1, 2, and 3 showed cytotoxic activity against autologous LCL-MSLN. CTLs induced by iPSDCs-MSLN from healthy donor A, B, and C also showed comparable cytotoxic activity against autologous LCL-MSLN (Fig. 2b).

MHC class 1-restricted MSLN-specific CTLs response by iPSDCs-MSLN

To investigate whether CTLs recognize MSLN-derived epitope peptides restricted to the HLA-A types, the cytotoxic activity of CTLs against MSLN epitope peptide-pulsed LCLs was examined. CTLs induced by iPSDCs-MSLN from the HLA-A2/A24-positive donor (donors A and B) showed cytotoxic activity against LCLs pulsed with the HLA-A2 binding MSLN peptides, $A2_{(20-28)}$, $A2_{(530-538)}$, and the HLA-A24 binding MSLN peptides, $A24_{(442-451)}$, $A24_{(435-443)}$, $A24_{(475-483)}$. CTLs induced by iPSDCs-MSLN from the HLA-A2/A2-positive donor (donor C) showed cytotoxic activity against LCLs pulsed with the HLA-A2 binding MSLN peptides, $A2_{(20-28)}$, $A2_{(530-538)}$, and no cytotoxic activity against LCLs pulsed with the HLA-A24 binding MSLN peptides. These CTLs showed no cytotoxic activity against LCLs pulsed with HLA-A2 binding WT1 peptide (Fig. 2c). These findings suggests that these genetically modified iPSDCs-MSLN induced an MHC class 1-restricted MSLN-specific CTLs response.

Effect of DCs transduced ubiquitin and MSLN fusion gene

To investigate whether MSLN protein degradation is enhanced within the DCs transduced ubiquitin-MSLN fusion gene, immunohistochemistry and intracellular FACS were examined 48 hours after gene transfer. Cytoplasm was stained in MoDCs-MSLN, whereas cytoplasm was not stained in MoDCs-UbMSLN. Cytoplasm was stained in MoDCs-UbMSLN with MG132, a proteasome inhibitor. The percentage of positively stained cells among the MoDCs-MSLN, MoDCs-UbMSLN, and MoDCs-UbMSLN with MG132 was 56% , 2%, and 46% respectively in intracellular FACS (Fig. 3a). Cytoplasm was stained in iPSDCs-MSLN, whereas cytoplasm was not stained in iPSDCs-UbMSLN. Cytoplasm was stained in iPSDCs-UbMSLN with MG132. The percentage of positively stained cells among the iPSDCs-MSLN, iPSDCs-UbMSLN, and iPSDCs-UbMSLN with MG132 was 82% , 48%, and 64% respectively in intracellular FACS (Fig. 3b).

Induction of MSLN-specific CTLs from PBMCs by iPSDCs-UbMSLN and MSLN pentamer assay

To assess the possibility of iPSDCs inducing MSLN-specific CTLs, a pentamer assay was performed. Of autologous PBMCs stimulated with iPSDC-LacZ, iPSDC-MSLN, or iPSDC-UbMSLN, 0.07%, 1.17%, and 2.27% were stained with pentamer, respectively

(Fig. 3c). The population of MSLN-specific CTLs increased approximately two-fold with the introduction of the Ub-MSLN fusion gene into iPSDCs compared to MSLN-only transgenic iPSDCs.

Cytotoxic activity of CTLs induced by MoDCs-UbMSLN and iPSDCs-UbMSLN

The CTLs induced by MoDCs-UbMSLN exhibited cytotoxic activity against LCLs-MSLN , 46%, 39%, and 24% in E/T ratio 50, 25, and 12.5 respectively. Whereas the CTLs induced by MoDC-MSLN exhibited cytotoxic activity against LCLs-MSLN, 37%, 27%, and 19% in E/T ratio 50, 25, and 12.5. The cytotoxicity of the CTLs induced by MoDCs-UbMSLN were higher than the cytotoxicity of the CTLs induced by MoDCs-MSLN in all E/T ratio. The CTLs induced by iPSDCs-UbMSLN exhibited cytotoxic activity against LCLs-MSLN , 20%, 16%, and 11% in E/T ratio 50, 25, and 12.5 respectively. Whereas the CTLs induced by iPSDCs-MSLN exhibited cytotoxic activity against LCLs-MSLN, 16%, 11%, and 6% in E/T ratio 50, 25, and 12.5. The cytotoxicity of the CTLs induced by iPSDCs-UbMSLN were also higher than the cytotoxicity of the CTLs induced by iPSDCs-MSLN in all E/T ratio (Fig. 4). Experiments were performed three times to confirm the reproducibility of the results, and similar results were obtained.

Discussion

Our results showed that the CTLs induced by genetically modified DCs expressing MSLN could induce MSLN-specific cytotoxic activity. MSLN is the optimal cancer vaccine target for many gastrointestinal cancers[5] [19] [20] [21] [22] [23]. The strategy of this study using DCs-MSLN are expected to be applied to the treatment of gastrointestinal cancer expressing MSLN.

Not only the genetically modified naïve DCs-MSLN but iPSDCs-MSLN could induce MSLN-specific CTLs with cytotoxic activity against autologous LCL-MSLN. In addition, CTLs induced by iPSDCs-MSLN also showed cytotoxic activity against MSLN-specific epitope peptide pulsed LCL in the HLA-A type restricted manner. CTLs induced by iPSDCs transfected with whole MSLN gene were indicated to recognize multiple MSLN-derived epitope peptides in an HLA-A type restricted manner. We previously reported a gene-based vaccination strategy using iPSDCs transduced with the whole TAA gene[13] [14]. This study also clearly showed that iPSDCs had a capacity equal to that of naïve DCs as antigen-presenting cells.

In the current study, iPSCs-derived DCs were adenovirally transduced simultaneously with ubiquitin gene and TAA gene, and it was examined whether the *in vitro* CTLs immunized with these genetically modified DCs induce a strong cytotoxic

activity against LCLs expressing TAA in healthy volunteers. Our result showed that the *in vitro* immunization of iPSDCs-Ub-MSLN elicited a more potent cytotoxic activity than the *in vitro* immunization of iPSDCs-MSLN. This is the first report of production of the recombinant adenovirus expressing the ubiquitin-MSLN fusion gene. To ensure that ubiquitin functions properly, the primers were provided with *Kozak* sequences (CGCCACC) that are important during eukaryotic translation. In addition, arginine was placed at the N-terminus of ubiquitin. The use of arginine as a linker enhances proteolysis of the fusion gene. This is known as the N-end rule [28]. To investigate whether the ubiquitin-MSLN fusion gene was functioning normally, MSLN protein expression in DCs was assessed by immunohistochemistry and intracellular FACS. In the DCs transduced ubiquitin-MSLN fusion gene, MSLN is forced into the ubiquitin-proteasome system and MSLN proteolysis is enhanced because the MSLN protein is thought to be expressed in a form in which ubiquitin is pre-bound to the MSLN protein. As a result, MSLN protein expression in DCs-UbMSLN is thought to decrease. In contrast, inhibition of proteasome function with MG132, proteasome inhibitor, did not reduce MSLN protein expression in DCs-UbMSLN. The ubiquitin-MSLN fusion gene transfer enhanced MSLN proteolysis in DCs-UbMSLN, suggesting that proteolysis is proteasome-dependent. Pentamer assay showed that increased MSLN proteolysis

enhanced the antigen-presenting capacity of MSLN-derived epitope peptides in DCs-UbMSLN and induced more MSLN-specific CTLs. In addition, it was suggested that ubiquitin-MSLN fusion gene-transfected DCs may also enhance cytotoxic activity. To solve the problem of insufficient cytotoxic activity of iPSDC vaccines, it may be useful to introduce ubiquitin-MSLN fusion gene into iPSDCs.

In conclusion, the current study was designed that peripheral T-cell tolerance to MSLN could be overcome by the immunization of genetically modified iPSDCs simultaneously expressing ubiquitin and MSLN, leading to a strong cytotoxicity against tumors endogenously expressing MSLN. Therefore, this strategy may be promising for clinical application as an effective cancer vaccine therapy.

Data Availability

The data are available upon reasonable request.

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Author Contributions

Study concept and design: Tominaga, Miyazawa and Ojima.

Acquisition of data: Tominaga, Miyazawa, Maruoka, and Kitadani.

Drafting of the manuscript: Tominaga and Ojima.

Critical revision of the manuscript for important intellectual content: Yamaue.

Administrative, technical and material support: Maruoka, Kitadani, and Iwamoto.

Study supervision: Yamaue.

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Ethics approval

This study has been approved by the Safety Committee for Genetic Recombination Experiments of this university (approval number: 2020-5). Only healthy volunteer specimens were used in this study.

Competing Interests

The authors declare no competing interests.

Figure Legends

Figure 1

Construction of recombinant adenovirus transduced ubiquitin and MSLN fusion gene.

Figure 2

a) Expression of MSLN in genetically modified MoDCs and iPSDCs by intracellular FACS.
b) Cytotoxic activity of CTLs generated from MoDCs-MSLN (donor 1-3) and iPSDCs-MSLN (donor A-C) against autologous LCLs-MSLN and LCLs-LacZ as a control.
c) Cytotoxic activity of CTLs generated from iPSDCs-MSLN against LCLs pulsed

MSLN specific epitope peptides and WT1 specific peptide as a control.

Figure 3

a) Expression of MSLN in genetically modified MoDCs by immunohistochemistry and intracellular FACS. MG132 is a proteasome inhibitor.

b) Expression of MSLN in genetically modified iPSDCs by immunohistochemistry and intracellular FACS. MG132 is a proteasome inhibitor.

c) Pentamer assay of MSLN specific CTLs generated from genetically modified iPSDCs.

Figure 4

Cytotoxic activity of CTLs generated from MoDCs-MSLN and MoDCs-UbMSLN against autologous LCLs-MSLN (Donor 1). Cytotoxic activity of CTLs generated from iPSDCs-MSLN and iPSDCs-UbMSLN against autologous LCLs-MSLN (Donor A).

Figures

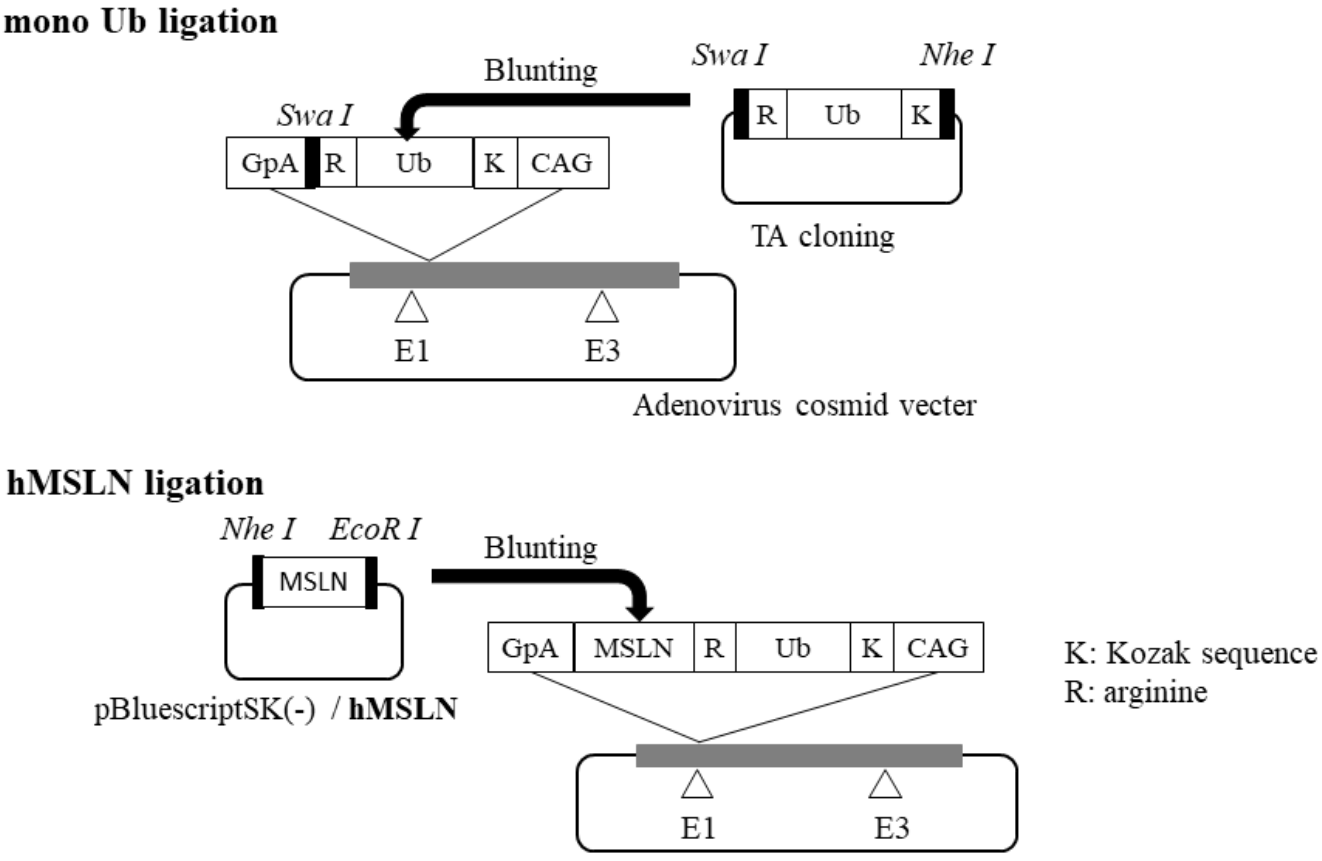


Figure 1

Construction of recombinant adenovirus transduced ubiquitin and MSLN fusion gene.

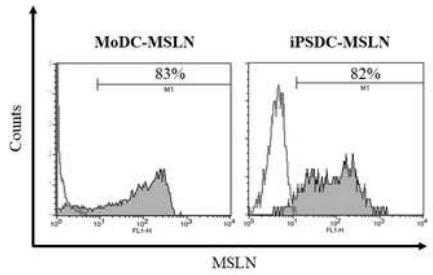
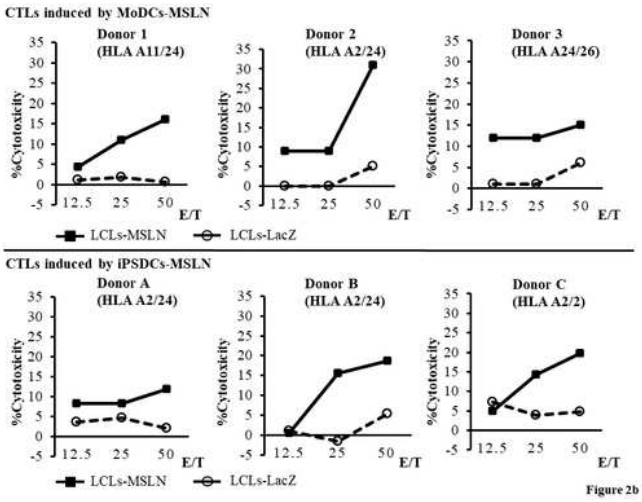


Figure 2a



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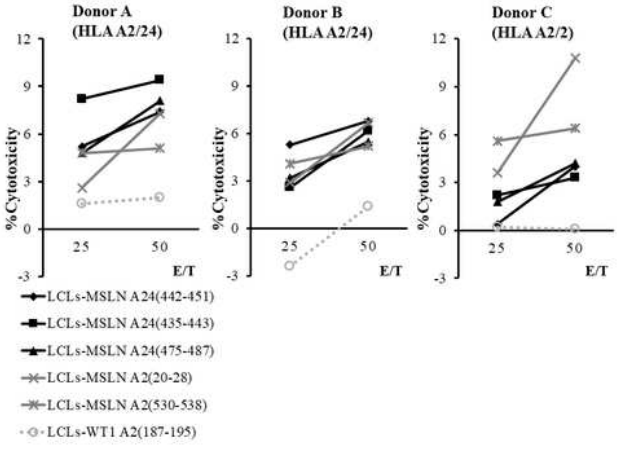

Figure 2c

Figure 2

a) Expression of MSLN in genetically modified MoDCs and iPSDCs by intracellular FACS.

b) Cytotoxic activity of CTLs generated from MoDCs-MSLN (donor 1-3) and

iPSDCs-MSLN (donor A-C) against autologous LCLs-MSLN and LCLs-LacZ as a

control.

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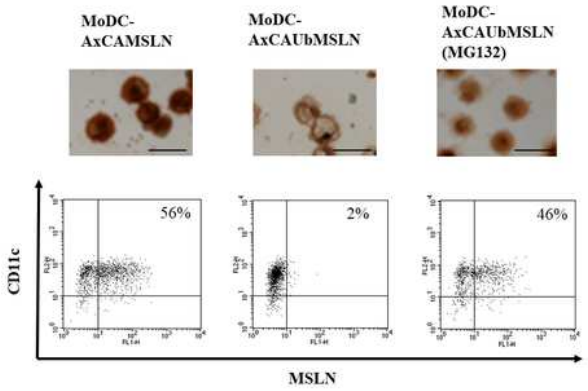


Figure 3a

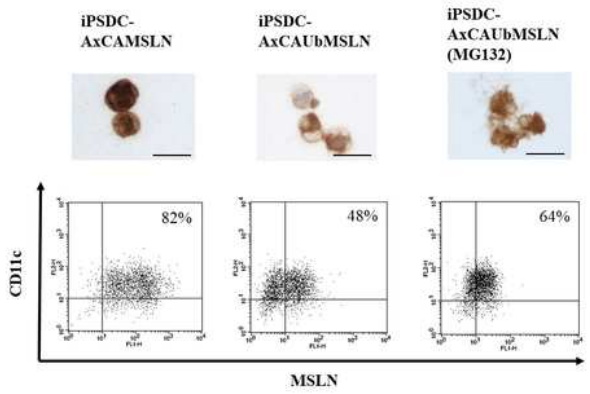
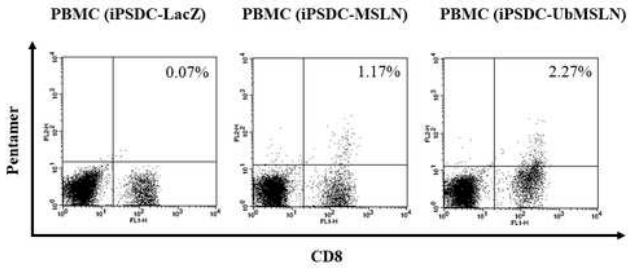


Figure 3b



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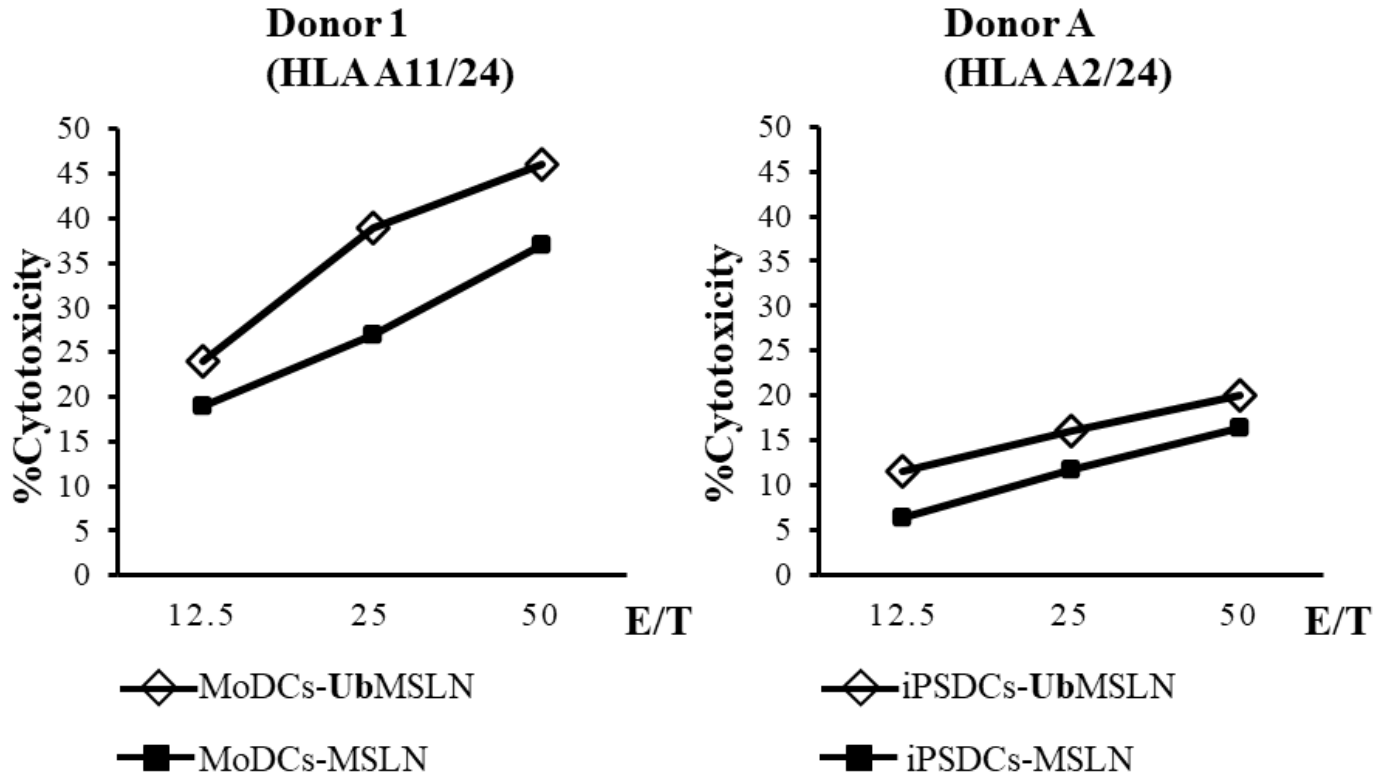

Figure 4

Figure 4

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