Immunotherapy against metastatic melanoma with human iPS cell–derived myeloid cell lines producing type I interferons

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Abstract

In recent years, immunotherapy for advanced melanoma has been gaining increased attention. The efficacy of anti-cytotoxic T-lymphocyte antigen 4 antibodies, anti-programmed cell death 1 antibodies and the BRAFV600E kinase inhibitor has been proven in metastatic melanoma. At the same time, adoptive cell transfer has significant effects against metastatic melanoma, however, it is difficult to apply on a broad scale because of the problems related to cell preparation. To overcome these problems, we developed immune cell therapy using induced pluripotent stem (iPS) cells. The benefit
of our method is that a large number of cells can be readily obtained. We focused on macrophages for immune cell therapy because macrophage infiltration is frequently observed in solids cancers. In this study, the efficacy of human iPS cell–derived myeloid cell lines (iPS-ML) genetically modified to express type I IFNs against human melanoma cells was examined. The morphology, phagocytic ability and surface markers of iPS-ML were similar to those of macrophages. The iPS-ML that express type I IFNs (iPS-ML-IFNs) showed significant effects in inhibiting the growth of disseminated human melanoma cells in SCID mice. The infiltration of iPS-ML into the tumor nests was confirmed immunohistologically. The iPS-ML-IFNs increased the expression of CD169, a marker of M1 macrophages that can activate antitumor immunity. The iPS-ML-IFNs could infiltrate into tumor tissue and exert anticancer effects in the local tumor tissue. In conclusion, this method will provide a new therapeutic modality for metastatic melanoma.

**Introduction**

In recent years, immunotherapy for malignant melanoma (MM) has garnered much attention. The efficacy of antibodies to CTLA-4 and programmed cell death 1 (PD-1) against metastatic melanoma have been demonstrated in large clinical trials (1, 2).
the other hand, immune cell therapy has a more durable effect for metastatic melanoma (3, 4). Cell therapies, including adoptive cell transfer (ACT), that use genetically engineered T cells (TCR/CAR), are therefore considered to be crucial to the effective treatment of melanoma patients. However, the TCR/CAR technique is difficult to apply in the clinical setting for several reasons: 1) an ideal membrane-bound melanoma-associated antigen that can be recognized by CAR is yet to be found; 2) ‘neoantigens’ in tumors, which can be strongly recognized by T cells, are important for a strong CTL response (5). The neoantigens vary by individuals, necessitating TCR-transduced T cells be prepared on an individual basis; 3) TCR/CAR cells are made from autologous lymphocytes, which are isolated from the patient’s peripheral blood by leukapheresis. However, this technique is sometimes difficult to apply in advanced cancer patients with bone marrow failure.

To overcome these problems associated with ACT, we have conducted a study of immune cell therapy with induced pluripotent stem (iPS) cells. The benefit of our method is that a large number of cells can be readily obtained, and these can exert additional immune functions when genetically modified.

We have established a method to differentiate iPS cell cultures to generate dendritic cells and macrophages via CD43⁺CD11b⁺ myeloid precursor cells (iPS-MC) (6). We
induce the proliferation of iPS-MC by lentivirus-mediated transduction of genes that can promote cell proliferation or inhibit cell senescence, such as cMYC plus BMI1, MDM2, or EZH2, to generate iPS cell–derived myeloid cell lines (iPS-ML) (7). The iPS-ML can proliferate for at least three months in a M-CSF–dependent manner. These iPS-ML possess the capacity to differentiate into macrophages that exhibit the morphology, molecule expression, and phagocytic activity of physiological macrophages upon treatment with M-CSF and GM-CSF (8). Expression vectors for anticancer molecules known to induce death or inhibit the growth of cancer cells, including IFNα, IFNβ, IFNγ, TNFα, FAS-ligand, and TRAIL, can be introduced into iPS-ML via lentivirus vectors to make them efficiently attack cancer cells and overcome the influence of the tumor environment (8). However, only the iPS-ML expressing IFNβ effectively inhibited the growth of peritoneally disseminated gastric and pancreatic cancers in xenograft models (8).

Melanoma is one of the most aggressive cancers, responsible for most skin cancer deaths (9). Peritoneal dissemination is often seen in MM, and is difficult to treat because surgical resection and radiation therapy are ineffective. Therefore, more potent immune therapy is urgently needed.

The efficacy of IFNs against melanoma has been known for quite some time (10, 11).
However, the efficacy of IFNs for metastatic melanoma has not been studied. In contrast, local injection of IFNβ for skin metastasis is effective (10). In addition, IFNα has been used as an adjuvant therapy after the surgical removal of primary melanoma. High-dose IFNα adjuvant therapy showed a benefit in terms of the disease-free survival (DFS) in high-risk resected primary melanoma patients, however, grade 3/4 adverse effects occurred in 45% of patients who received long-term high-dose IFNα, and 31% of patients stopped treatment due to toxicity (12, 13). Practitioners should be aware that elderly patients (age 65 years and older) were under-represented in the high-dose interferon trials (12). Given the toxicities of interferon, particularly in the presence of other significant comorbidities, caution is advised (12). It follows that although the local injection of IFN is effective and safe, and systemically-administered IFN can also be effective, systemic application is associated with potentially severe adverse effects.

Therefore, we evaluated the potential of iPS-ML as anticancer effector cells with the use of genetically modified iPS-ML that expressed type I IFNs. Macrophage infiltration is frequently observed in clinical samples of cancer tissues (14). It was expected that the iPS-ML expressing type I IFNs would be able to infiltrate into the tumor tissue and exert anticancer effects in the local tumor tissue. In this paper, we report that iPS-ML–expressing type I IFNs could exert therapeutic effects against peritoneally
disseminated MM in xenograft models.

Materials and Methods

Cells and reagents

This study was approved by the ethics review board of Faculty of Life Sciences Kumamoto University. The human malignant melanoma cell line, SK-MEL28, was provided by ATCC. Cell line authentication was performed using short tandem repeat profiling and comparison with known cell line DNA profiles. SK-MEL28 cells were transduced with a lentivirus vector encoding the firefly luciferase gene as described previously (7) for the analysis based on luciferase activity.

The methods used for the generation and maintenance of human iPS cells have been described (6).

Generation of proliferating myeloid cells from human iPS cells

The methods used for the differentiation of human iPS cells into dendritic cells (iPS-DC) and macrophages (iPS-MP) have been described (6). The differentiation of iPS-DC and iPS-MP occurred through CD43\(^+\)CD11b\(^+\) myeloid precursor cells
(iPS-MC). These iPS-MC, which possess the capacity to differentiate into DC upon treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL) 4, and the capacity to differentiate into MP in the presence of M-CSF and GM-CSF, could proliferate for seven to 14 days in the presence of GM-CSF and M-CSF, but then ceased to grow. Therefore, we introduced several genes with the potential to induce cell proliferation into the iPS-MC using lentiviruses to confer the iPS-MC with long-term proliferative capacity (7). The cDNA fragments were inserted into the lentiviral vector CSII-EF (15) (a gift from Dr. H. Miyoshi together with the packaging constructs, RIKEN, Tsukuba, Japan), with or without IRES-puroR, to generate lentiviral expression constructs. As a consequence, we found that simultaneous introduction of cMYC, together with BMI1, MDM2 or EZH2, resulted in the continuous proliferation of iPS-MC for at least three months, and their proliferation was dependent on the addition of M-CSF. We named the iPS-MC–derived long-term proliferating cells iPS-ML (iPS cell–derived myeloid/macrophage cells) (7).

**Generation of genetically modified iPS-ML–expressing type I IFNs**

iPS-ML were transduced with a lentivirus vector encoding human IFNα or IFNβ as described (7, 8). To select cells stably expressing the transgenes, the cells were cultured
in medium containing hygromycin (0.5-2 mg/mL). To quantify the production of transgene-derived cytokines, the transfected iPS-ML were cultured (1×10^5 cells/well in 200 μl) in 96-well culture plates for 24 hours, and the concentration of type I IFNs in the culture supernatant was measured using ELISA kits (Endogen or R&D Systems).

**Zymosan phagocytosis assay**

The phagocytic ability of the iPS-ML was analyzed using FITC-labeled zymosan A particles (Molecular Probes). The cultured iPS-ML were treated with FITC-labeled zymosan particles. After an 80-min incubation, they were observed under a microscope.

**Flow cytometric analysis**

We analyzed the cell surface molecule expression of iPS-ML by a flow cytometric analysis. The following monoclonal antibodies (mAbs) to human or mouse mAbs conjugated with FITC or PE were purchased from BD Pharmingen (San Diego, CA), Beckman Coulter (Brea, CA), Miltenyi Biotec (Bergish-Gladbach, Germany), Sigma (St. Louis, MO) or eBioscience (San Diego, CA): anti-human CD36 (FA6.152, mouse IgG1), anti-human CD11b (ICRF44, mouse IgG1), anti-human CD14 (61D3, mouse IgG1), anti-human TLR4 (HTA125, mouse IgG2a), anti-human CD163 (GHI/61, mouse IgG1),
IgG1), anti-human CD169 (7-239, mouse IgG1), anti-human CD206 (19.2, mouse IgG1) and anti-mouse CD169 (MOMA-1, rat IgG2a). Isotype-matched mouse IgG2a (G155-178), mouse IgG1 (MOPC-21) and rat IgG2a (RTK2758) were used as controls. Polyclonal antibody to mouse CD163 was purchased from Cosmo Bio Co. and goat anti-rabbit IgG-PE secondary antibody was purchased from Santa Cruz Biotechnology. Rabbit primary antibody isotype control was purchased from Invitrogen. Cell samples were treated with a human or mouse FcR-blocking reagent (Miltenyi Biotec) for 10 min, stained with the fluorochrome-conjugated mAbs for 30 min and washed three times with PBS/FCS (2%). Stained cell samples were analyzed using a FACScan (Becton Dickinson) flow cytometer.

**Flow cytometry after culture with tumor-derived molecules.** We examined whether the phenotype of the iPS-ML was affected by the cancer microenvironment, tumor-derived molecules like IL10 and TGFβ. The iPS-ML were cultured (2×10⁶ cells/well in 4000 μL) in 6-well culture plates in the presence or absence of 10 ng/mL of recombinant human IL10 (Cell Signaling Technology, Danvers, USA) and recombinant human TGFβ (R&D System, Minneapolis, USA). After 48 hours, the expression of cell surface molecules by iPS-ML was analyzed by flow cytometry.
Cytokine production ELISA

We evaluated the type I IFNs expression by iPS-ML with or without type I IFNs using an ELISA kit (PBL InterferonSource). The iPS-ML were cultured (2×10^4 cells/well in 100 μL) in 96-well culture plates for 72 hours, and the concentration of type I IFNs in the culture supernatant was measured.

Luciferase cell death assays

Type I IFNs. Luciferase-expressing SK-MEL28 cells were cultured (1×10^4 cells/well in 100 μL) in 96-well culture plates in the presence or absence of 0.5 - 20 ng/mL recombinant human IFNα and IFNβ. Three days later, luciferase substrate solution (SteadyLite Plus, Perkin-Elmer) was added (100 μL/well), and the luminescence was measured on a microplate reader (TriStar, BertholdTech, Bad Wildbad, Germany) to determine the number of live SK-MEL28 cells based on the luciferase activity.

iPS-ML and iPS-ML/IFNs. Luciferase-expressing SK-MEL28 cells were cultured alone or were cocultured in 96-well culture plates (1×10^4 cells/well) with iPS-ML (2×10^4 cells/well), iPS-ML–expressing type I IFNs or recombinant type I IFNs. The number of live SK-MEL28 cells was measured by assessing the luciferase activity after a three-day culture.
In vivo antitumor activity of iPS-ML

SCID mice were intraperitoneally (i.p.) injected with luciferase-expressing SK-MEL28 cells (10×10⁶ cells/mouse). On day 3, the mice underwent luminescence image analysis for the establishment of tumors. Mice with established tumors were randomly divided into control (n = 3) and treatment (n = 3 per cell type) groups. The mice in the treatment group were injected with iPS-ML, iPS-ML-FNα, iPS-ML-IFNβ or iPS-ML-IFNα plus iPS-ML-IFNβ (total 1×10⁷ cells/mouse for each injection) on days 4-8 and 11-15. All mice underwent a bioluminescence analysis on days 10 and 17 to evaluate the effects of the treatment.

Tumor infiltration in SCID mice

SK-MEL28 cells (5×10⁶) were injected i.p. into SCID mice. After 15 days, the mice were subjected to a luminescence imaging analysis. Mice engrafted with melanoma cells were injected i.p. with iPS-ML labeled with the red fluorescent dye, PKH26. The mice were sacrificed the following day and dissected to determine the location of the injected tumor cells and iPS-ML by a fluorescence analysis. The tumor tissues in the greater omentum of the mice were isolated, and 4-μm thick frozen sections were made.
Tumor cells were stained with Alexa Fluor 488–conjugated antibody to human MART-1 and the sections were analyzed using a fluorescence microscope.

**Phenotyping endogenous mice macrophages.**

SCID mice were i.p. injected with luciferase-expressing SK-MEL28 cells (5×10^6 cells/mouse). After 15 days, a luminescence imaging analysis was performed. Mice engrafted with melanoma cells were randomly divided into treated (n = 3) and non-treated (n = 3) groups. The mice in the treatment group were intraperitoneally injected with human iPS-ML-IFNα cells (1×10^7 cells/mouse) labeled with the red fluorescent dye, PKH26. The mice were dissected on the following day and the greater omentum and spleen were isolated. We then analyzed the cell surface molecule expression of endogenous macrophages by a flow cytometric analysis using antibodies to mouse CD169 and CD163. We simultaneously examined control mice, which had not been intraperitoneally injected with human melanoma cells.

**Colony forming cell assays**

Colony forming cells (CFC) assays examined the ability of iPS-ML to proliferate and differentiate into colonies in a semi-solid media in response to cytokine stimulation.
Human methylcellulose complete media without epo, specially formulated and optimized for CFC assays with colony-forming myeloid progenitors of human origin, (R&D systems). Malignant tumor development from human iPS-ML-IFN cells in SCID mice was examined by injecting human iPS-ML-IFNα and human iPS-ML-IFNβ cells (1×10^7 cells/mouse for each injection) into SCID mice without tumor cells. Twelve weeks later the mice were sacrificed, and examined under a stereomicroscope.

**Statistical analysis**

The statistical analyses were carried out using the Bonferroni’s test. A $P$-value < 0.05 was considered to be statistically significant.

**Results**

**Characterization of human iPS cell–derived myeloid cells**

We analyzed the morphology and the phagocytic ability of iPS-ML. The iPS-ML had round nuclei and abundant cytoplasm (Fig. 1A). The phagocytic ability of the iPS-ML was assessed by their ingestion of FITC-labeled zymosan A particles. The iPS-ML were cultured after adding FITC-labeled zymosan particles. After an 80-min incubation, we
microscopically observed that fluorescence signals were detected in most cells, indicating that the majority of the iPS-ML ingested the zymosan particles (Fig. 1B).

We then generated iPS-ML expressing IFNα or IFNβ (iPS-ML-IFNα, iPS-ML-IFNβ) as described (7, 8). First, we analyzed the cell surface expression of the iPS-ML by flow cytometric analysis. The iPS-ML expressing type I IFNs expressed several macrophage markers, including CD11b, CD14, CD36, CD68, CD169, TLR-4, and CD206 (Fig. 1C). The iPS-ML expressing type I IFNs had increased expression of CD169, a marker of inflammatory macrophages that can activate antitumor immunity. In contrast, iPS-ML without type I IFNs had increased expression of CD206, a marker of macrophages that display a pro-tumor activity (Fig. 1C). This indicated that the type I IFNs produced by the cells themselves could change their expression of cell surface molecules.

Macrophages are thought to be affected by the local cytokine environment and are polarized to an M1 phenotype (positive for CD169), which exerts an antitumor effect, or the M2 phenotype (positive for CD206 and/or CD163), which support tumor survival (16 - 18). The macrophages observed in clinical samples of solid cancer, called TAM (tumor-associated macrophages) (14) were affected by the cancer microenvironment and were polarized to an M2 phenotype in most cases. However, the iPS-ML expressing type I IFNs started to express CD169, indicating that the iPS-ML were polarized to an
M1 phenotype and could exert anticancer effects. This phenotype of the iPS-ML expressing type I IFNs was durable and not affected by tumor-derived cytokines found in the cancer microenvironment, like IL10 and TGFβ in vitro (data not shown.).

**Anticancer activity of iPS-ML expressing type I IFNs in vitro**

First, we evaluated whether a melanoma cell line, SK-MEL28, was sensitive to type I IFNs. SK-MEL28 cells were transduced with a lentivirus vector encoding the firefly luciferase gene for an analysis of cell viability based on the luciferase activity. The luciferase-expressing SK-MEL28 cells were cultured in the presence or absence of recombinant IFNα and IFNβ. Three days later, the number of live SK-MEL28 cells was measured by the luciferase activity. Both IFNα and IFNβ had decreased the number of live SK-MEL28 cells in a concentration-dependent manner (Fig. 2). Recombinant IFNβ (Fig. 2B) exhibited a more potent effect at a lower concentration than IFNα (Fig. 2A).

We next evaluated the expression of type I IFNs by the iPS-ML with or without type I IFNs using an ELISA. The iPS-ML were cultured for 72 hours, and the concentration of type I IFNs in the culture supernatant was measured (Fig. 3A). The iPS-ML-IFNα and β had the ability to produce IFNα and β, respectively, whereas the iPS-ML hardly produced type I IFNs.
To examine the anti-tumor effects of iPS-ML expressing type I IFNs, the luciferase-expressing SK-MEL28 cells were cultured alone, with iPS-ML, with iPS-ML expressing type I IFNs, or with recombinant type I IFNs. The concentration of recombinant type I IFNs was decided based on the results of the ELISA of the iPS cells (Fig. 3A). The number of live SK-MEL28 cells was again assessed by the luciferase activity after three days in culture. We observed that all treatments, including treatment with the iPS-ML without type I IFNs, significantly reduced the number of live SK-MEL28 cells compared to the untreated cells ($p < 0.05$) (Fig. 3B and C). The expression of IFNβ in the iPS-ML enhanced the inhibitory effects against the growth of SK-MEL28 cells (Fig. 3C), but the iPS-ML-IFNα did not have these effects. One possible reason for these results may be the differences in the sensitivity of the SK-MEL28 cells to IFNα and IFNβ (Fig. 2).

**Anticancer activity in SCID mice**

We then examined the *in vivo* anticancer effects of iPS-ML expressing either IFNα or IFNβ. SCID mice were intraperitoneally (i.p.) injected with luciferase-expressing SK-MEL28 cells (Fig. 4). On day 3, the mice underwent a luminescence image analysis to assess the establishment of tumors. Mice with established tumors were randomly
divided into control \((n = 3)\) and treatment \((n = 3\) per treatment\) groups. The mice in the treatment group were injected with iPS-ML, iPS-ML-IFN\(\alpha\), iPS-ML-IFN\(\beta\), or iPS-ML-IFN\(\alpha\) plus iPS-ML-IFN\(\beta\) (total \(1 \times 10^7\) cells/mouse for each injection) on days 4-8 and 11-15. All mice underwent another bioluminescence analysis on days 10 and 17 to evaluate the effects of the treatments (Fig. 4A). Cancer cell growth \textit{in vivo} was not inhibited by treatment with iPS-ML. In contrast, iPS-ML-IFN\(\alpha\), iPS-ML-IFN\(\beta\), and iPS-ML-IFN\(\alpha\) plus iPS-ML-IFN\(\beta\) significantly inhibited the growth of the tumors \((P < 0.05)\) (Fig. 4B). These results indicated that iPS-ML did not exhibit significant anticancer effects \textit{in vivo}; however, genetic modification to produce type I IFNs conferred anticancer activity on the iPS-ML.

Additionally, we noted differences in cytokine expression between the iPS-ML and the iPS-ML expressing type I IFNs, with the use of an ELISA of the culture supernatants. Cytokines that can enhance the activity of macrophages, including IL6, IL8, MCP-1, RANTES, and IP-10, were increased in the culture supernatants of iPS-ML expressing type I IFNs compared to the iPS-ML (Fig. 5). These results suggested one possible reason why the genetic modification to produce type I IFNs conferred significant anticancer activity on iPS-ML.
Accumulation and infiltration of iPS-ML in established tumor tissue

Macrophage infiltration is frequently observed in clinical samples of cancer tissues\textsuperscript{14}. We evaluated whether or not the i.p. administered iPS-ML infiltrated cancer tissue that had been established in the peritoneal cavity of mice. In this experiment, SK-MEL28 cells were injected i.p. into SCID mice. After 15 days, the mice were subjected to a luminescence imaging analysis (Fig. 6A). Mice engrafted with melanoma cells were injected i.p. with iPS-ML labeled with a red fluorescent dye, PKH26. The mice were sacrificed on the following day, and were dissected to determine the location of the injected tumor cells and iPS-ML by a fluorescence analysis.

The tumor tissues in the greater omentum of the mice were isolated, and 4-μm thick frozen sections were made. Tumor cells were stained with Alexa Fluor 488-conjugated antibody to human MART-1. The sections were analyzed using a fluorescence microscope (Figs. 6B and C). We observed that the PKH-labeled iPS-ML infiltrated into the tumor nests of Alexa Fluor 488-conjugated anti-human MART-1 antibody-stained SK-ME28 cells. iPS-ML accumulated and infiltrated into the tumor tissues upon i.p. injection into SCID mice bearing peritoneally implanted cancer, and exerted anti-cancer effects by expressing type I IFNs.
Human iPS-ML-IFNα cells decrease endogenous mouse M2 macrophages

Next, we examined whether or not the injection of human iPS-ML cells producing type I IFNs could change the phenotype of mouse endogenous M2 macrophages that had already infiltrated the solid tumor tissues into M1-type macrophages. SCID mice were intraperitoneally injected with luciferase-expressing SK-MEL28 cells. After 15 days, a luminescence imaging analysis was performed. Mice engrafted with melanoma cells were randomly divided into treated ($n = 3$) and non-treated ($n = 3$) groups. The mice in the treatment group were intraperitoneally injected with human iPS-ML-IFNα cells labeled with the red fluorescent dye, PKH26. The mice were dissected on the following day and the greater omentum and spleen were isolated. Cell surface expression of mouse CD169 and CD163 on endogenous macrophages was then measured by flow cytometry. We simultaneously examined control mice, which had not been intraperitoneally injected with human melanoma cells. That is to say, we analyzed three groups: the non-melanoma bearing (control) group, the non-treated melanoma bearing group and the iPS-ML-IFNα–treated melanoma bearing group. We conducted these experiments using iPS-ML-IFNα cells, because IFNβ has high species specificity. It is known that human IFNβ does not demonstrate its functional activity in mouse macrophages. In contrast, human IFNα has high homology with mouse IFNα,
and has functional activity in mouse macrophages. As a result, we found that the
endogenous M2 macrophages (positive for CD163) were increased in non-treated
melanoma bearing mice in comparison to the control mice. On the other hand, M2
macrophages were decreased in the mice treated with iPS-ML-IFNα cells in comparison
to the non-treated group. (Fig. 7) However, the number of M1-type macrophages
(positive for CD169) did not increase after the injection of iPS-ML-IFNα cells (Fig. 7).
These results showed that the melanoma cells engrafted in mice induced an increase in
the number of endogenous M2 macrophages, and that the intraperitoneal administration
of iPS-ML-IFNα cells could decrease the number of endogenous M2 macrophages. We
have recently reported that melanoma-bearing patients, especially those with recurrence,
have few M1-type macrophages in their regional lymph nodes (19). It therefore makes
sense to supply melanoma patients with iPS-ML cells that produce type I IFNs (M1
macrophage-like cells).

No evidence of development of malignancy from human iPS-ML in SCID mice.

We found that human iPS-ML-IFN cells had colony forming capacity in vitro.

(Supplemental Fig. S1) The myeloid series of cells have differentiation capability
(proliferation ability), whether or not they are in non-tumor cells; it is therefore possible
that iPS-ML-IFN cells have a colony forming capacity. We evaluated malignant tumor
development from human iPS-ML-IFN cells in SCID mice for up to 12 weeks. During
the observation period, the mice were healthy, and no significant difference was
observed in the body weight of human iPS-ML-IFN–injected mice and control mice
(Supplemental Fig. S2A). We found no evidence of tumor formation in the organs—including lung, spleen, liver, and greater omentum—in pathological specimens
from mice that were injected with human iPS-ML-IFN cells, when compared to
non-injected mice. (Supplemental Fig. S2B) Thus IPS-ML cells were rejected for at
least 12 weeks after injection, allowing no malignant tumors to develop in SCID mice
that were injected with human iPS-ML-IFN cells. Haga et al. reported that allogeneic
recipients of TAP-deficient mouse ES cell-derived ML cells do not develop leukemia
(20). In our experiments, even in immune-deficient SCID mice, the injection of iPS-ML
cells did not lead to the development of leukemia or other malignancies. We are
therefore of the opinion that allogeneic cell therapy is safe, because the cells will be
eliminated from the host immune system. It is therefore reasonable to use allogeneic cells
if the allogeneic cell therapy is able to induce antitumor activity.
Discussion

We herein demonstrated that iPS-ML expressing type I IFNs inhibited the growth of SK-MEL28 melanoma in xenograft models. We also found that genetic modification of the iPS-ML to produce type I IFNs changed their characteristics from exerting pro-tumor into anticancer activity. Macrophage infiltration is frequently observed in clinical samples of solid cancers, and these macrophages are called TAM, which are involved in tumor cell proliferation and tumor development (21, 22). TAM are affected by the cancer microenvironment, including tumor-derived molecules, such as IL10 and TGFβ, and become polarized to the M2 phenotype, which reduces the adaptive Th1 response by producing anti-inflammatory factors, including IL10 and TGFβ, and promoting angiogenesis (14, 18, 23).

To overcome the influence of the tumor environment and to make them exert anticancer effects, we introduced expression vectors for anticancer molecules into iPS-ML. The iPS-ML expressing type I IFNs were polarized to the M1 phenotype by the type I IFNs they produced, enabling them to maintain their M1 phenotype in the cancer microenvironment, even in the presence of IL10 and TGF-β in vitro. M1 macrophages have attracted attention as antigen-presenting cells (APCs). Recent studies have shown that the M1 macrophages act as APCs that control CD8+ T cells and natural
killer T cell activation (24, 25). They also could infiltrate into the tumor tissue and exert anticancer effects in local tumor tissue in vivo. In this study, iPS-ML expressing IFNβ inhibited SK-MEL28 cell growth more potently than did the iPS-ML expressing IFNα in vitro and in vivo. One reason for these results may be the differences in sensitivity of the SK-MEL28 cells to IFNα and IFNβ (Fig. 3). Another possible reason may be the differences in tissue affinity between IFNα and IFNβ. IFNβ is more lipophilic than IFNα, and therefore, may possess greater tissue affinity (8, 26).

IFNα has been used as an adjuvant therapy after the resection of primary melanoma in many countries. High-dose IFNα adjuvant therapy is beneficial in terms of disease-free survival (DFS) in high-risk resected primary melanoma patients. In Japan, the local injection of IFNβ for the treatment of skin metastasis has been approved. Type I IFNs, thus, can be effective in the treatment of melanoma. However, systemic IFN treatment for metastatic melanoma has not been efficacious. To treat metastatic melanoma, patients would require an enormous amount of systemic IFNs. Even adjuvant therapy after the surgical removal of primary melanoma requires a high dose of IFNα and serious adverse events are a concern in most patients. Grade 3/4 adverse effects occurred in 45% of the patients who received adjuvant therapy with long-term high-dose IFNα, and 31% of patients stopped treatment due to toxic effects, which
included fatigue, liver toxicity, depression, appetite loss, and dyspnea (12, 13). Our data show that human iPS-ML-IFN cells can infiltrate the tumor tissues via an intraperitoneal injection into SCID mice with peritoneally-implanted melanoma. These cells then exert local anticancer effects by expressing type I IFNs. Thus, treatments using iPS-ML-producing IFNs can deliver a high dose of IFNs to the tumor microenvironment better than the systemic administration of IFNs, and this can be achieved without systemic side effects.

Additionally, we previously demonstrated that the iPS-ML can proliferate in an M-CSF-dependent manner for at least three months following the lentivirus-mediated transduction of genes that can promote cell proliferation or inhibit cell senescence, such as cMYC plus BMI1, MDM2 or EZH2. This makes it possible to repeatedly administer a large number of cells to achieve clinical effects during the treatment of cancer patients.

However, in clinical applications, the proliferative capacity of autologous iPS-ML transduced with these genes may be cause for concern, because of the possibility of leukemia development. To avoid the risk of leukemia, we plan to use HLA-matched/minor antigen mismatched allogeneic iPS-ML in future clinical applications. We predict that the injected HLA-matched allogeneic iPS-ML will survive in the recipients for
several days, providing anticancer effects, but then be completely eliminated by the recipient’s immune system’s response to the mismatched minor antigens. Thus, therapy with allogeneic iPS-ML would spare patients the risk of developing leukemia. Haga et al. reported that allogeneic recipients of TAP-deficient mouse ES cell-derived ML cells did not develop leukemia (20). In our experiments, even in immune deficient SCID mice, the injection of iPS-ML cells did not lead to the development of leukemia or other malignancies. We are therefore of the opinion that allogeneic cell therapy is safe, because the cells will be eliminated from the host immune system.

In conclusion, iPS-ML expressing type I IFNs were durably inhibited the growth of SK-MEL28 melanoma in xenograft models. Although further preclinical studies to establish the safety of these cells are necessary; this method may eventually provide new hope for metastatic melanoma patients. Furthermore, this method is antigen-nonspecific immunotherapy and therefore, widely applicable to a variety of cancers.

References


Figure Legends

Figure 1: Characteristics of generated iPS-ML.

(A) A phase-contrast image of iPS-ML in a culture plate (left) and an image of iPS-ML stained with May-Giemsa in a slide glass (right). (B) The iPS-ML in culture plates were added to FITC-labeled zymosan particles. A phase-contrast image (left), fluorescence image (middle) and merged image (right) after 80 minutes of incubation are shown. (C) Flow cytometry analysis of the cell-surface expression of macrophage marker molecules CD11b, CD14, CD36, CD68, CD169, CD163, TLR4, and CD206 on the iPS-ML and iPS-ML expressing type I IFNs. The staining profiles of the specific mAb (grey area) and an isotype-matched control mAb (thick lines) are shown. The experiments were conducted two times.

Figure 2: Sensitivity of SK-MEL28 cells to type I IFNs.
Luciferase-expressing SK-MEL28 cells were cultured (1×10^4 cells/well in 100 mL) in 96-well culture plates in the presence or absence of 0.5 - 20 ng/mL of recombinant IFNα and IFNβ. Three days later, the number of live SK-MEL28 cells was measured according to the luciferase activity. (A) Sensitivity of SK-MEL28 cells to IFNα. (B) Sensitivity of SK-MEL28 cells to IFNβ. The experiments were conducted three times. The data are presented as the mean ± SD of triplicate assays. The P-values were determined using the Bonferroni’s t test. * P < 0.05.

Figure 3: Effects of iPS-ML expressing type I IFNs against SK-MEL28 cells in vitro.

(A) Type I IFN expression in the iPS-ML with or without type I IFNs were analyzed using ELISA. The iPS-ML were cultured (2×10^4 cells/well in 100 μL) in 96-well culture plates for 72 hours, and the concentration of type I IFNs in the culture supernatant was measured. (B) Luciferase-expressing SK-MEL28 cells were cultured alone or co-cultured in a 96-well culture plate (1×10^4 cells/well) with iPS-ML (2×10^4 cells/well), iPS-ML/IFNα and IFNα. The number of live SK-MEL28 cells was measured according to the luciferase activity after three days of culture. (C) Luciferase-expressing SK-MEL28 cells were cultured alone or co-cultured in a 96-well culture plate (1×10^4}}
cells/well) with iPS-ML (2×10⁴ cells/well), iPS-ML/IFNβ and IFNβ. The number of live SK-MEL28 cells was measured according to the luciferase activity after three days of culture. The changes in the luminescence signals were calculated as relative values, where the photon count at no treatment was defined as 1. The experiments were conducted three times. The data are presented as the mean ± SD of triplicate assays. The $P$-values were determined using the Bonferroni’s $t$ test. * $p < 0.05$.

**Figure 4: Effects of iPS-ML producing type I IFNs against SK-MEL28 in vivo.**

(A) Luciferase–expressing SK-MEL28 cells were injected i.p. into SCID mice (5×10⁶ cells/mouse). On day 3, the mice underwent a luminescence imaging analysis. The mice engrafted with melanoma cells were randomly divided into control ($n = 3$) and treatment ($n = 3$) groups. The mice in the treatment group were injected with iPS-ML, iPS-ML/IFNα, iPS-ML/IFNβ and iPS-ML/IFNα and β (1×10⁷ cells/mouse for each injection) on days 4, 5, 6, 7, 8, 11, 12, 13, 14 and 15. All mice underwent a bioluminescence analysis on days 10 and 17. Luminescence images are shown. (B) For each mouse, the change in the luminescence signal was calculated as a relative value, where the photon count on day 3 was defined as 1. The experiments were conducted two times. The $p$-values were determined using the Bonferroni’s $t$ test. * $p < 0.05$. 
**Figure 5: Production of cytokines from iPS-ML.**

iPS-ML were cultured \((1 \times 10^4 \text{ cells/well in 100 mL})\) in 96-well culture plates for 72 hours, and the concentrations of cytokines, including IL1β, IL2, IL6, IL8, IL12, IFNγ, TNFα, MCP-1, RANTES, IP-10, and MIG, in the culture supernatant were measured using a multi-analyte ELISAArray kit. The expression of cytokines produced by iPS-ML was calculated as relative values, where the concentration of the cytokine standard was defined as 1. The experiments were conducted two times.

**Figure 6: Accumulation and infiltration of iPS-ML in established tumor tissues in the mouse peritoneal cavity.**

SK-MEL28 cells \((5 \times 10^6)\) were injected i.p. into SCID mice. After 15 days, the mice were subjected to a luminescence imaging analysis (A). Mice engrafted with melanoma cells were injected i.p. with iPS-ML labeled with red fluorescent dye PKH26. The mice were sacrificed the following day and subsequently dissected in order to determine the location of the injected tumor cells and iPS-ML using a fluorescence analysis. Tumor tissues in the greater omentum of the mice were isolated, and 4-μm-thick frozen sections were made. The tumor cells were stained with Alexa Fluor 488-conjugated
anti-human MART-1 antibodies, and the sections were analyzed on a fluorescence microscope. A merged image with green fluorescence indicating SK-MEL28 cells and red fluorescence indicating PKH26-stained iPS-ML (indicated by arrows) is shown (B, C).

Figure 7: The intraperitoneal administration of human iPS-ML-IFNα cells could decrease the number of mouse endogenous M2 macrophages

We analyzed the cell surface molecule expression of endogenous macrophages from the greater omentum and spleen by a flow cytometric analysis using anti-mouse CD169- and CD163-specific antibodies. We analyzed three groups: the non-melanoma bearing (control) group, the non-treated melanoma bearing group and the iPS-ML-IFNα-treated melanoma bearing group.
Figure 1

(A) Image 1

(B) Image 2

(C) Table and graphs

<table>
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<th>CD11b</th>
<th>CD14</th>
<th>CD36</th>
<th>CD68</th>
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<td>Image 1</td>
<td>Image 2</td>
<td>Image 3</td>
<td>Image 4</td>
<td>Image 5</td>
<td>Image 6</td>
<td>Image 7</td>
<td>Image 8</td>
</tr>
</tbody>
</table>

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Figure 3

(A) Concentration of Type I IFNs (ng/ml)

- iPS-ML
- iPS-ML/IFNα
- iPS-ML/IFNβ

(B) Relative luminescence activity

- NT
- iPS-ML 2×10^4
- iPS-ML/IFNα 2×10^4
- IFNα 25 ng/ml
- IFNα 50 ng/ml
- iPS-ML 2×10^4+IFNα 25 ng/ml

(C) Relative luminescence activity

- NT
- iPS-ML 2×10^4
- iPS-ML/IFNβ 2×10^4
- IFNβ 30 ng/ml
- IFNβ 60 ng/ml
- iPS-ML 2×10^4+IFNβ 30 ng/ml

* p < 0.05
Figure 4

(A) No therapy

Day 3  Day 17

iPS-ML  iPS-ML/IFNα

Day 3  Day 17

iPS-ML/IFNα+β  iPS-ML/IFNβ

Day 3  Day 17

(B) Fold change in tumor-associated luminescence (from day 3)

Day 3  Day 17

Legend:
- □ No therapy
- ○ iPS-ML
- ▲ iPS-ML/IFNα+β
- ■ iPS-ML/IFNα
- ● iPS-ML/IFNβ

* p < 0.05
Figure 6
Figure 7

Control (non-melanoma bearing) vs melanoma bearing non-treated vs treated

**Spleen**

- CD163
- CD169

- 19.1%
- 60.5%
- 18.3%

**Greater Omentum**

- CD163
- CD169

- 17.9%
- 36.0%
- 18.6%
Cancer Immunology Research

Immunotherapy against metastatic melanoma with human iPS cell-derived myeloid cell lines producing type I interferons

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