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Anti-tumor effects of interferon-beta cell therapy in murine model of melanoma

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Original Article

Abstract

Purpose: Recombinant interferon beta (IFN-β) has been used for a treatment of cancers. However, the efficacy of recombinant IFN-β is limited because of its short half-life and side effects. To overcome these problems, we focused on the efficacy of cell-based therapy (cell therapy) using IFN-β-producing cells in the treatment of melanoma. **Methods:** IFN-β-producing therapeutic cells were constructed by gene transduction using retrovirus vector. Anti-tumor effects of the cell therapy were investigated by a murine melanoma model. **Results:** IFN-β cell therapy significantly suppressed the proliferation of B16 melanoma *in vitro* and the growth of B16-derived tumor *in vivo*, accompanied with the activation of natural killer (NK) cells. IFN-β cell therapy did not show any systemic side-effects concerning hepatic dysfunction and bone marrow suppression. **Conclusion:** IFN-β cell therapy could be a candidate as a novel cancer treatment.

Keywords: Cell therapy, Interferon-beta, Melanoma

1. Introduction

Interferon-beta (IFN-β) is a member of the type I family of interferons, which has various activities such as inhibition of viral replication, activation of immune cells and regulation of cell cycle.[1] Since 1980's, it has been reported that interferon has anti-tumor effects, then applied to the treatment of cancer.[2]

Type I interferon is known to exert anti-tumor effects by both direct and indirect pathways.[3, 4] In the direct pathway, type I interferon binds to the specific interferon hetero dimer receptor (IFNAR1 and R2) expressed on the surface of tumor cells, and activates complicated signal transduction such as JAK-STAT, MAPK/p38 and PI3 kinase cascade for various cellular functions including anti-tumor effects.[5] In the indirect pathway, type I interferon activates many kinds of immune cells such as macrophages and natural killer (NK) cells,[6, 7] which also leads to the anti-tumor effects.

IFN-β has been clinically used for the treatment of several types of cancers including melanoma and brain tumor.[2] In the treatment of melanoma, recombinant IFN-β is subcutaneously administrated into the tumors. However, the repetitive injection of recombinant IFN-β is required to get clinically enough concentration of IFN-β in patients, because of the short half-life of interferon in the tissues. Systemic administration of recombinant IFN-β is also performed in the treatment of brain tumor[8] and hepatitis C virus infection.[9] In these cases, systemic adverse effects such as fever, hepatic dysfunction and bone marrow suppression have previously reported and remained serious problems for the treatment.[10]

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Cell therapy is one of the unique treatments against several diseases, where cultured-cells such as embryonic fibroblasts are used as therapeutic agents.[11, 12] In clinical use, inoculation of bone marrow cells has been performed as an angiogenic therapy for arteriosclerosis obliterans.[13] We have successfully demonstrated the efficacy of the cell therapy using IL-10 producing mouse embryonic cells in murine acute inflammation models such as gout and asthma models, followed by local and continuous high concentrated IL-10 production without side effects.[14, 15] Thus, the cell therapy could be a quite possible strategy for the treatment of various disorders, such as cancer.

In this study, we investigated anti-tumor effects of IFN-β cell therapy in the treatment of melanoma *in vitro* and *in vivo*. As a result, strong anti-tumor effects such as inhibition of cell growth, suppression of tumor development and activation of immune cells including NK cells and macrophages were observed in both *in vitro* and *in vivo*. Our results strongly sugessted that IFN-β cell therapy might be a novel candidate of cancer treatment *via* both direct suppression of tumor growth (direct pathway) and activation of immune cells (indirect pathway).

2. Methods and Materials

2.1. Cells

C57BL/6J-emb (C57) fibroblasts derived from C57BL/6J murine embryo and murine melanoma cells (B16) were purchased from RIKEN Cell Bank (Tsukuba, Japan) and DS Pharma Biomedical (Osaka, Japan), respectively. Cells were cultured with Dulbecco's modified Eagle's medium (DMEM; SIGMA-ALDRICH, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; GIBCO by Life Technologies, Grand Island, NY), and 100 units/ml penicillin/100 μg/ml streptomycin (GIBCO) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

2.2. Mice

Six-week-old male mice on the C57BL/6NJcl background were purchased from CLEA-Japan (Tokyo, Japan) and were fed with standard chow pellets and water ad libitum. All animal handlings and procedures were approved by the Animal Experimentation and Ethics Committee of Kitasato University (Approval no. AHS-R 14-32, 15-28).

2.3. Construction of therapeutic and control cells for the therapy

2.3.1. IFN-β gene transduction into murine fibroblasts using retroviras vector

Murine IFN-β (mIFN-β) gene was artificially synthesized by Integrated DNA Technologies (Iowa, USA), according to the reference sequence from GenBank (Accession No. NM_010510). The synthesized mIFN-β gene was cloned into ClaI and NotI sites of deficient retrovirus vector, pDON-5-Neo (Takara, Tokyo, Japan) and transfected to packaging cells, PT67 producing essential proteins for retrovirus components protein such as Gag, Pol and Env (Clontech by Takara, Tokyo, Japan) using Effectene Transfection Reagent (QIAGEN, Tokyo, Japan), followed by G418 (Roche, Basel, Swizerland) selection. Produced temporary infectious recombinant retrovirus containing mIFN-β gene was infected to C57 mouse embryonic fibroblast cells (C57), followed by G418 selection and designated as therapeutic cells, C57-mIFN-β used for the cell therapy. The pDON-5-Neo was also retrovirally introduced to C57 using same procedure then designated as C57-EV used as control in the study.

Enhanced green fluorescent protein (EGFP) gene was cloned into BamHI and NotI sites of deficient retrovirus vector, pLPCX (Clontech) containing puromycin resistance gene as a selection marker, then transfected PT67 cells, followed by puromycin (Sigma-Aldrich) selection. Produced temporary recombinant retrovirus was infected to C57-mIFN-β, followed by puromycin selection, then designated as C57-mIFN-β-EGFP. Producing EGFP was verified by fluorescence microscope BZ-9000 (Keyence, Osaka, Japan) and used to evaluate the production of mIFN-β in survival therapeutic cells during the therapy.

2.3.2. Expression and production of mIFN-β in C57-mIFN-β

To validate the mIFN-β gene expression, RT-PCR was carried out by following method. Total RNA was extracted from C57-mIFN-β using RNeasy Plus Mini Kit (QIAGEN), and 5 μg of total RNA were used for cDNA synthesis in a total volume of 50 μl using Omniscript RT Kit (QIAGEN). Five out of the 50 μl RT product was submitted for PCR in a total volume of 50 μl, followed by in one cycle of initial activation at 94°C for 15 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems by Life Technologies). The oligonucleotide primers used for the PCR are as follows: mIFN-β (forward primer 5'-TCACCTACAGGGCGGACTTCA-3', reverse primer 5'-AGATCTCTGCTCGGACCACCAT-3'), mGAPDH (forward primer 5'-GACGGCCGCATCTTCTTGTG-3', reverse primer 5'-GCCCCGGCCTTCTCCAT-3'). The PCR products were electrophoresed with 3% agarose (TaKaRa agarose L03) in TBE buffer. Then the products were stained with GelRed (Wako, Osaka, Japan), and detected with UV trans illuminator. The concentrations of mIFN-β protein in over night culture supernatants of C57-mIFN-β cells, tumor tissues, and murine serum were measured by mouse IFN-β ELISA kit (PBL Assay Science), according to the instruction manual.

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2.4. Anti-tumor effect of mIFN-β secreting therapeutic cell supernatant

To evaluate the anti-tumor effects of C57-mIFN-β cells on B16 melanoma cells *in vitro*, 50µl of the over night cultured supernatant of C57-mIFN-β was added to cultured B16 melanoma cells (3000 cells/ well in 50µl DMEM) then cell growth was evaluated by MTT assay (Roche, Basel, Switzerland) after 48 hours. The supernatant of C57-EV was used as control.

2.5. Anti-tumor effects by cell therapy in murine melanoma model

2.5.1. Murine melanoma model and cell therapy

B16 melanoma cells (0.5×10^6 cells/50 µl PBS) were inoculated into footpad of 6-week-old male C57BL/6NJcl mice then C57-mIFN-β cells (0.5×10^6 cells/50 µl PBS) were inoculated as therapeutic cells around the B16-derived tumor after a week of the melanoma cell inoculation. As control, C57-EV were injected instead of C57-mIFN-β. The size of tumor was daily measured by the thickness of inoculated footpad in consideration of uninoculated. For histological assessment of cell therapy, tumor tissues were resected at 7 days after the cell therapy, then embedded by paraffin, followed by hematoxylin-eosin (HE) staining.

2.5.2. Flow cytometry analysis (FCM) of immune cells induced by the cell therapy

Infiltrated immune cells induced by C57-mIFN-β cell therapy were analyzed by FCM. The method of isolation of cells from the tissues was performed basically in accordance with the previous study.[16] The resected tumor tissues were cut into 3 mm small pieces and fractionated in 2 ml of PBS by scissors. The fractionated tissues were treated with collagenase D (final concentration 2 mg/ml: Roche) for 1 hour at 37°C, then filtrated through the 70 µm pore size cell strainer (BD, NJ, USA). Single cell suspension from the tissues was washed with PBS, and then incubated with 2.4G2 mAb (anti-FcγRIII/II) to block FcγRIII/II receptor mediated non-specific binding of primary mAb. After washing, the cell suspension was split into two tubes, and each samples were stained with mixed antibodies set A and B. The components are follows; set A: FITC conjugated anti-mouse CD3e , violet fluor 450 conjugated anti-mouse CD4 , PE conjugated anti-mouse CD8a , and APC conjugated anti-mouse NK1.1; Set B: FITC conjugated anti-mouse CD11b , APC conjugated anti-mouse F4/80, and PE conjugated anti-mouse CD45R. Every conjugated antibody was purchased from Bay bioscience (Hyogo, Japan). Stained cells were washed with FACS buffer, then stained with 7-AAD (BD Pharmingen) to distinguish viable and inviable cells. The cells were applied to FACS Caliber flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (FlowJo, LLC).

2.6. Analysis of therapeutic cell function and side effects using EGFP-expressing cells

2.6.1. Viability and IFN-β production of therapeutic cells during the therapy

To assess how the inoculated therapeutic cells work in the tissues, the viability of the therapeutic cells and the concentration of mIFN-β in the tissues were evaluated. EGFP-expressing therapeutic cells, C57-mIFN-β-EGFP cells, were inoculated into mouse foot pad (0.5×10^6 cells/mouse). The inoculated feet were resected at 14 days after the inoculation, then resected tissues were embedded in parafin and sectioned to 3 µm thickness. To evaluate the viability of the inoculated cells, immunostaining was performed using anti-EGFP antibody (abcam, UK) and VECTASTAIN ABC Rabbit IgG kit (VECTER LABORATORIES). IFN-β production in inoculated and tumor cells, was also evaluated by ELISA method.

2.6.2. Evaluation of systemic side effects of cell therapy

The systemic side effects of cell therapy were assessed, by using blood counts and liver enzyme tests. The blood samples were harvested from mice at 7 or 14 days after the inoculation of C57-mIFN-β-EGFP. Blood counts of each blood samples were performed by automated blood cell counter (MEK-6450, Nihon Kouden, Japan). Liver enzymes, ALT and AST, were evaluated with IDTox ALT Colour Endpoint Assay Kit (ID Labs, UK) and IDTox AST Colour Endpoint Assay Kit (ID Labs) according to the instruction manuals, respectively.

2.7. Statistical analysis

Data are expressed as the means ± SD or SEM. Statistical analysis was performed with the KaleidaGraph software (HULINKS, Tokyo, Japan). The comparison of more than two groups was analyzed with one-way ANOVA followed by Turkey's multiple comparison test. For comparison of two groups, Student's t-test was performed. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of C57-mIFN-β used as therapeutic cells in the therapy

The expression and secreted production of retrovirally introduced mIFN-β in C57- mIFN-β and C57-EV were evaluated by RT-PCR and ELISA, respectively (Figure 1A and 1B). Resulted 348 bp specific RT-PCR band and approximate 6600pg/ml of mIFN-B protein were detected in C57-mIFN-B but undetected in C57-EV.

3.2. Anti-tumor effect of mIFN-β secreting therapeutic cell supernatant

The culture supernatant of C57-mIFN-β significantly suppressed the proliferation of B16 melanoma cells *in vitro* ,compared to the supernatant of C57-EV or control (mock) (Figure 2). Approximate 50% suppression of the B16 cell growth was observed with supernatant of C57-mIFN-β.

Figure 1

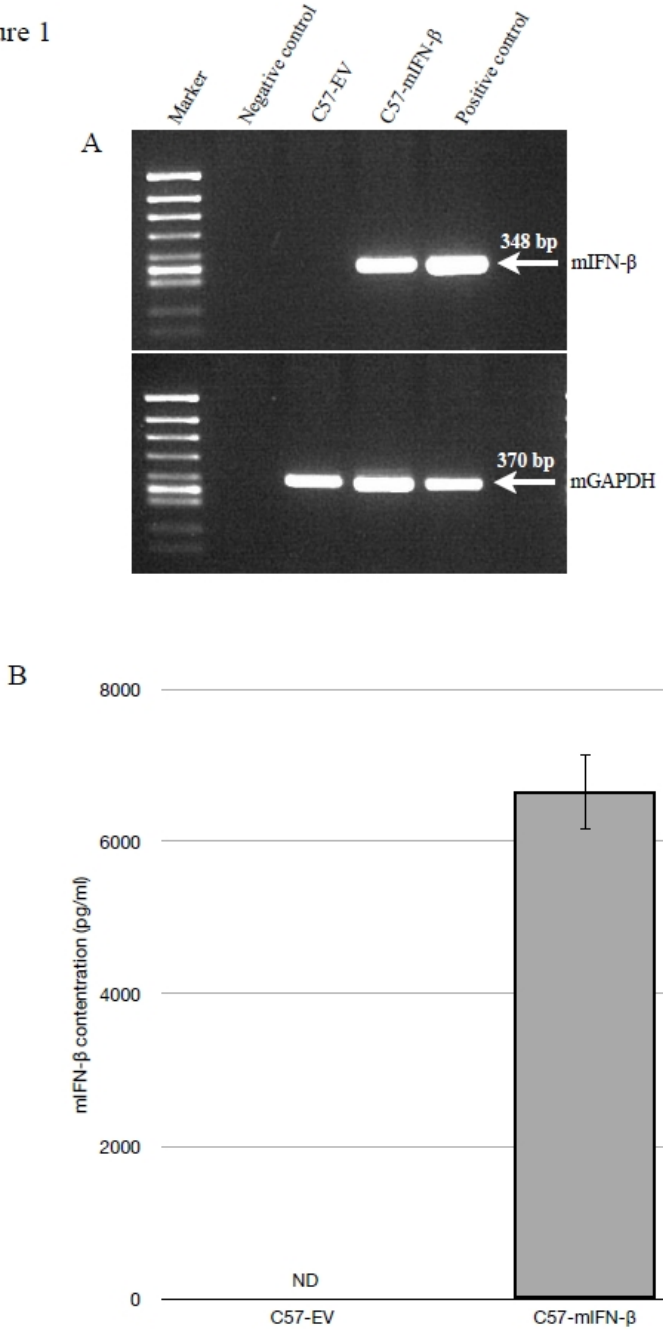

Figure 1: Characterization of C57-mIFN-β cells.

RT-PCR showed the expression of mIFN-β gene as the specific band of 348bp in C57-mIFN-β cells, but not in C57-EV cells (A). Positive control was mIFN-β DNA cloned in pDON-5-Neo vector, and negative control was PCR mixture without DNA template. The concentration of mIFN-β protein in the supernatant of C57-mIFN-β cells was approximately 6600 pg/ml, whereas not detected (ND) in the supernatant of C57-EV cells (B). Data were shown as mean ± s.d. [n = 3].

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Figure 2

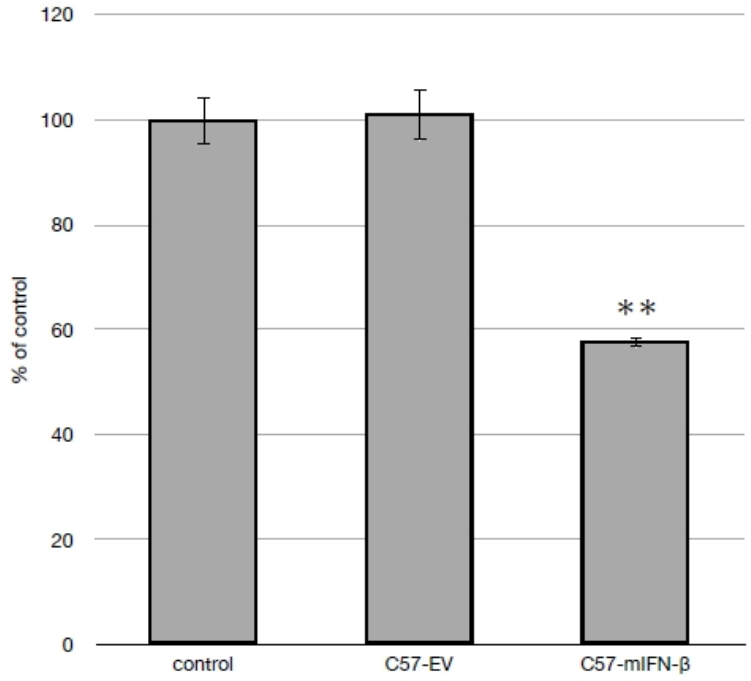

Figure 2: Suppressive effect of secreted mIFN-β from C57-mIFN-β on the proliferation of B16 melanoma cells.

The viability of B16 melanoma cells was assessed by MTT assay after the treatment with culture supernatant of C57-mIFN-β cells for 48 hours. The supernatant of C57-mIFN-β cells significantly suppressed the proliferation of B16 melanoma cells compared with control (without adding supernatant), whereas the supernatant of C57-EV cells did not suppress. Data were shown as mean ± s.d. [n = 3]. **P < 0.01.

Figure 3

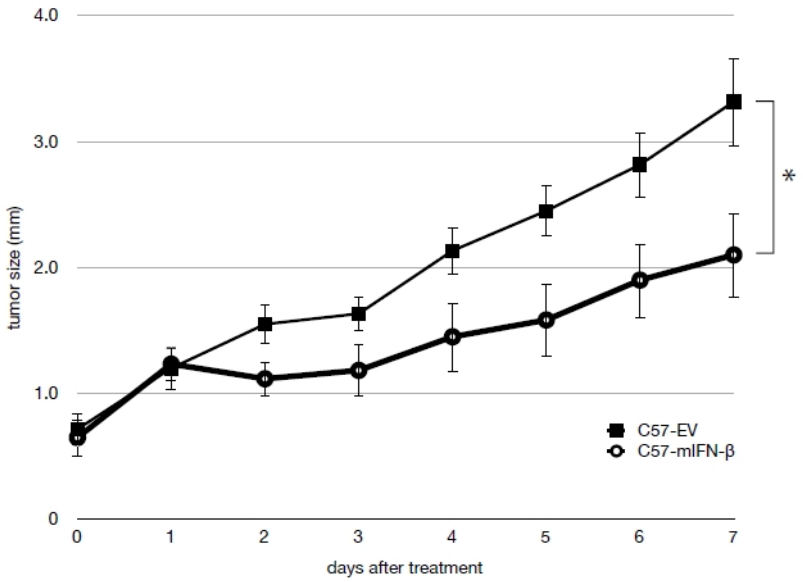

Figure 3: Anti-tumor effects of C57-mIFN-β cells on murine B16-derived melanoma model *in vivo.*

The size of B16-derived melanoma was measured after the inoculation of C57-mIFN-β cells. C57-mIFN-β cell therapy significantly suppressed the tumor growth compared with the treatment by C57-EV. Tumor size was estimated by subtraction of the thickness of the normal footpad from the affected footpad. Data were shown as mean ± s.e. [n = 6]. *P < 0.05.

Figure 4

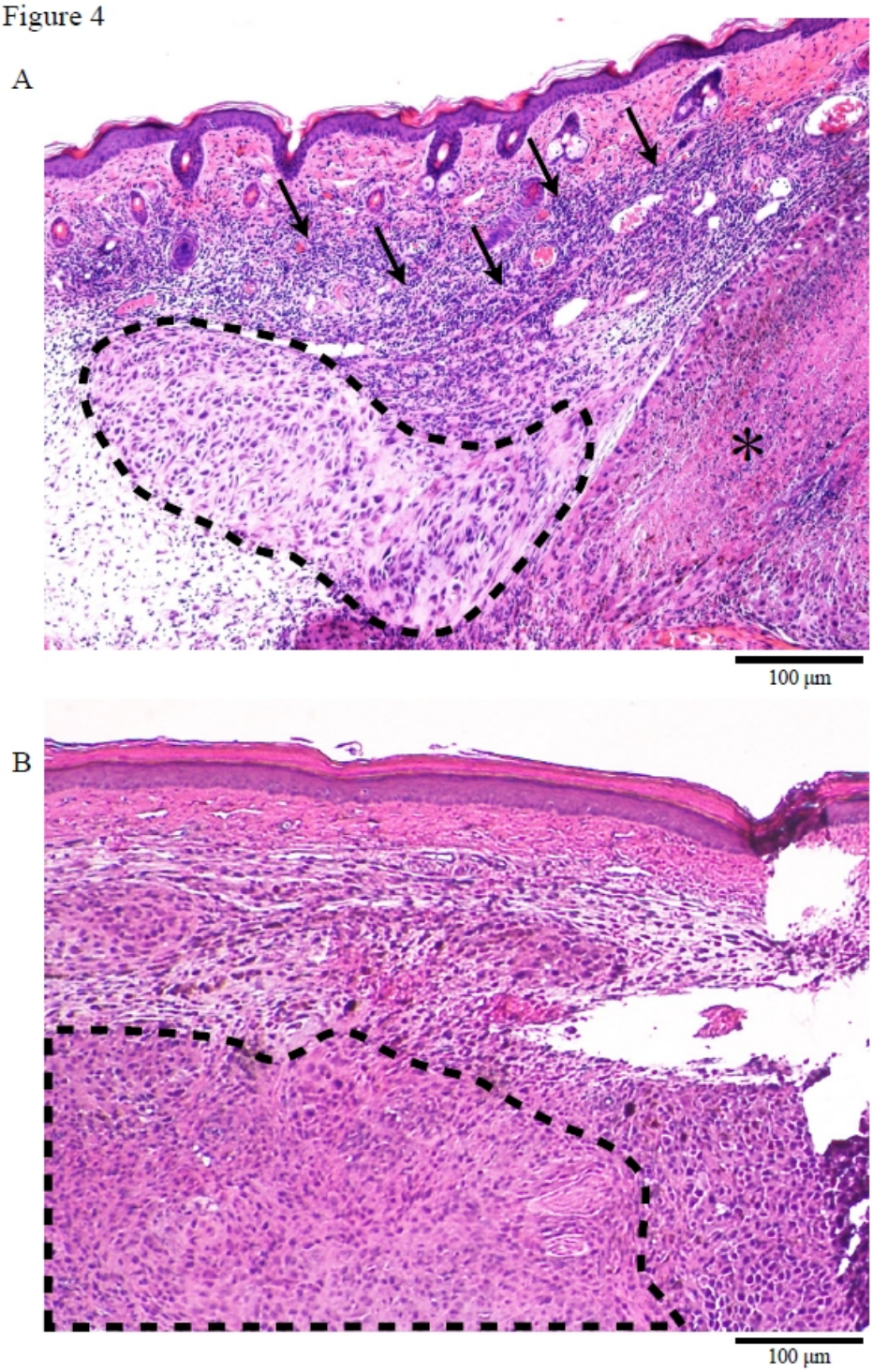

Figure 4: Histological analysis of the cell therapy by C57-mIFN-β cells.

The edge area of the resected tumor tissues was stained by HE staining. The right area is covered by B16-derived tumors, and the circled area is constituted by inoculated C57-mIFN-β cells (A) or C57-EV (B). Between the tumor tissues and C57-mIFN-β cells, there was intense lymphocytic infiltration (arrows). In addition, degeneration of tumor was observed in the tissue treated by C57-mIFN-β cells (*).

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Figure 5

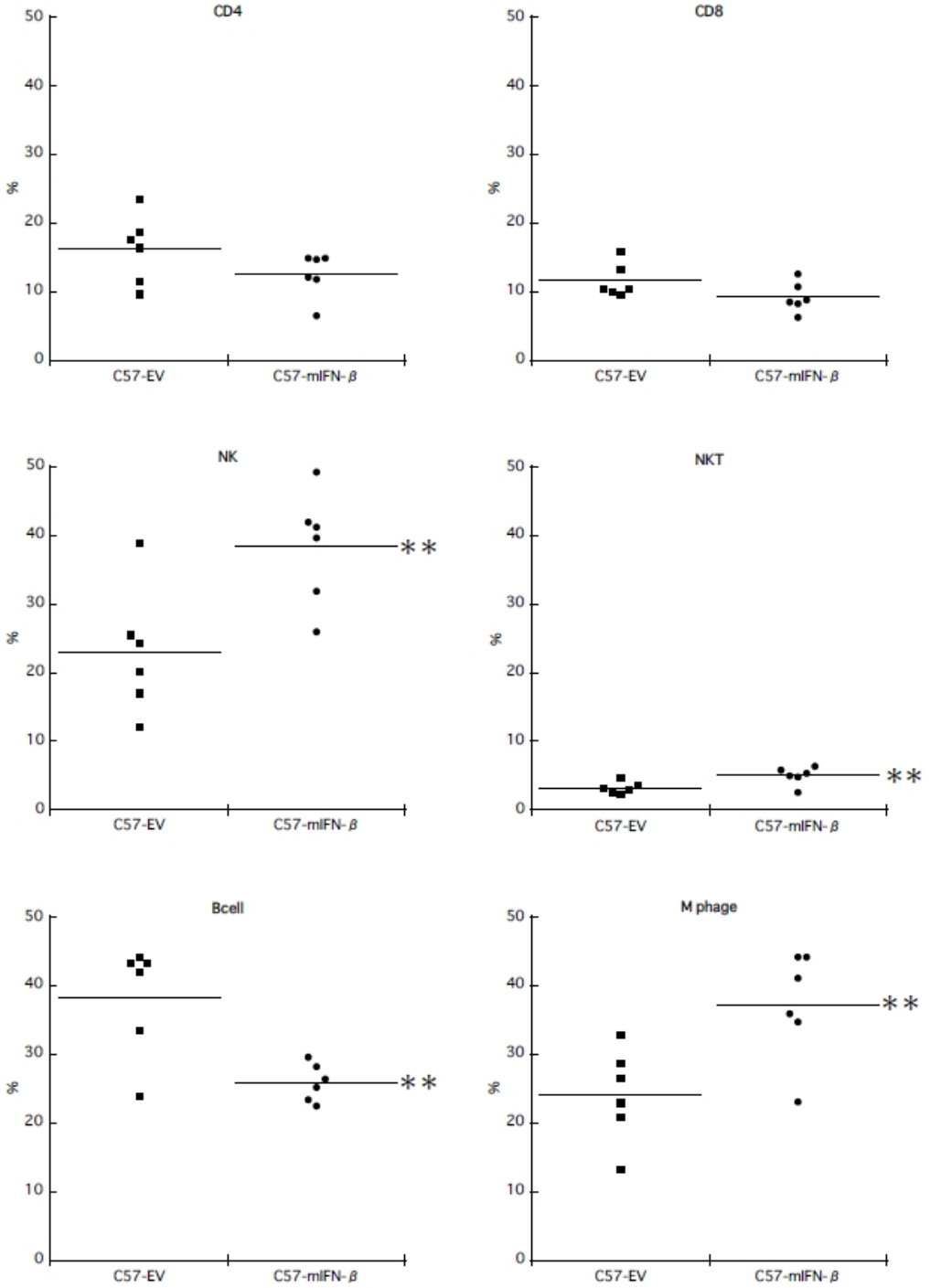

Figure 5: Flow cytometry analysis of infiltrating immune cell by the cell therapy.

The percentage of each lymphocytes content in the infiltrating lymphocyte was investigated by FCM. NK cells, NKT cells and macrophage contents were significantly increased in the tissues treated with C57-mIFN-β cells. For CD4 T cells and CD8 T cells, there was no difference C57-EV and C57-mIFN-β. B cells were significantly decreased in the tissues treated with C57-mIFN-β cells. **P < 0.01.

Figure 6

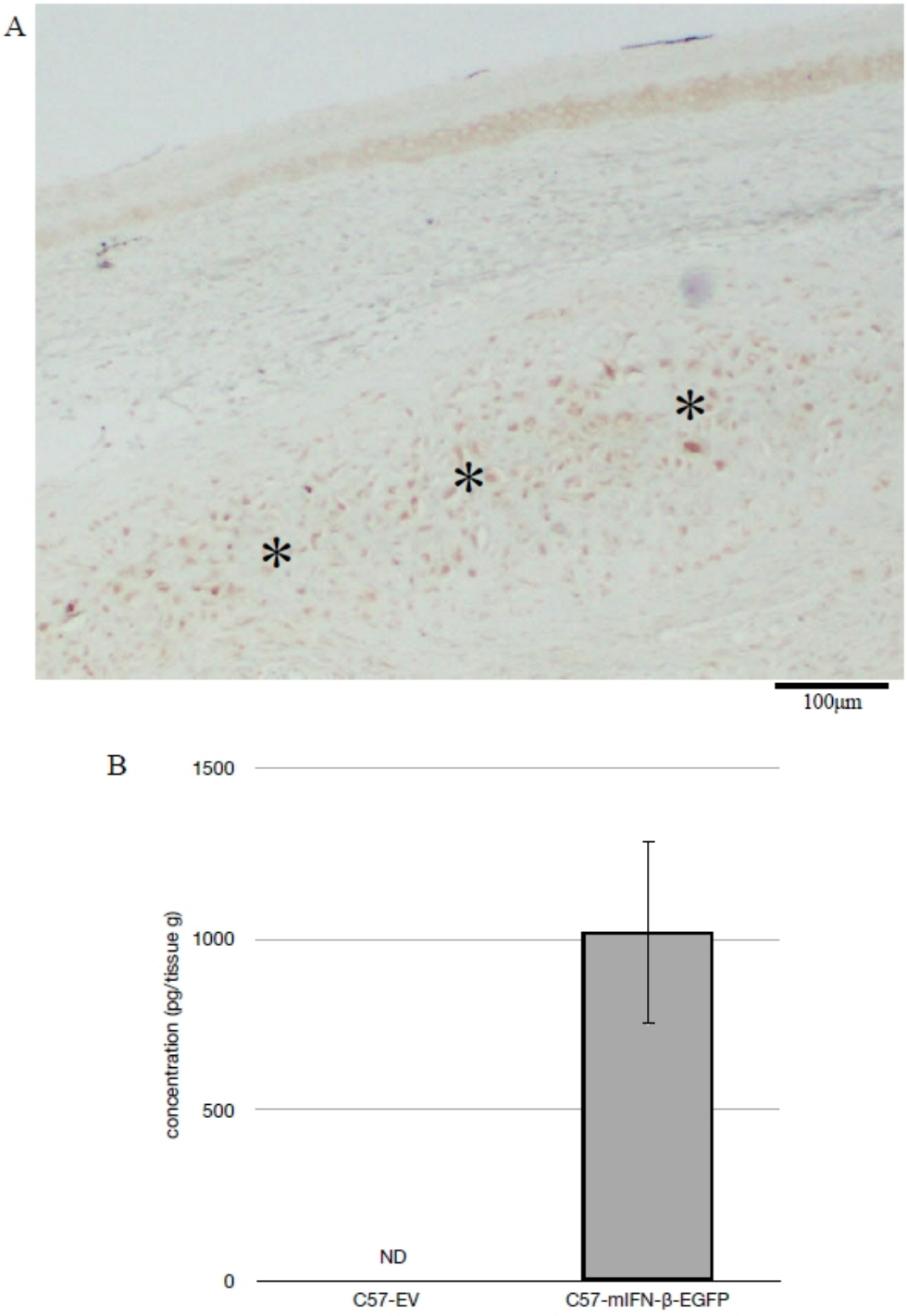

Figure 6: The viability of C57-mIFN-β-EGFP cells in the tissues.

The survival of C57-mIFN-β introduced with EGFP was determined by immunohistochemistry with anti-EGFP antibody (A). At 14 days after the inoculation of C57-mIFN-β-EGFP cells, EGFP-expressing cells were detected as living cells (*). The concentration of mIFN-β in the tissues after the injection of cells was assayed by ELISA (B). Approximately 1000 pg mIFN-β was detected per 1 g of tissue treated with C57-mIFN-β cells , whereas mIFN-β was undetectable (ND) in the tissue inoculated with C57-EV cells. Data were shown as mean ± s.e. [n = 3].

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Figure 7

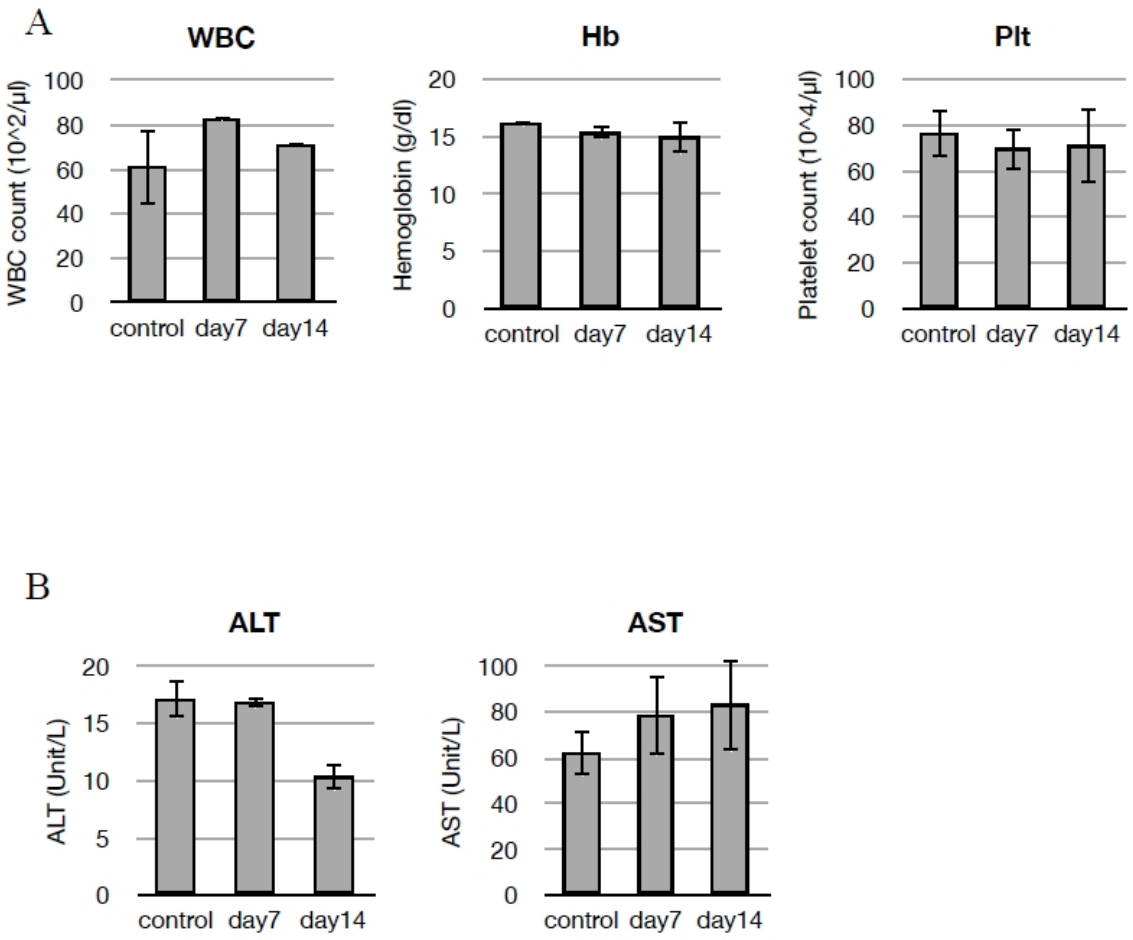

Figure 7: The analysis of side effects by the inoculation of C57-mIFN-EGFP cells.

Blood counts including white blood cells (WBC), hemoglobin (Hb) and platelet (Plt) were determined at 7 and 14 days after the local inoculation of C57-mIFN-β-EGFP. Every blood counts was not suppressed by the inoculation of C57-mIFN-β cells (A). Liver enzymes including ALT and AST were measured in the serum of mice inoculated with C57-mIFN-β cells. Neither ALT nor AST were elevated by the inoculation of C57-mIFN-β cells (B). Data were shown as mean ± s.e. [n = 3].

3.3. Anti-tumor effects by cell therapy in murine melanoma model

3.3.1. Suppression of tumor growth by C57-mIFN-β cell therapy

The tumor size on the mice foot derived from B16 cells, was significantly suppressed by the C57-mIFN-β cell therapy after 7 days of the therapy in comparison with the C57-EV inoculation (Figure 3). The histological analysis was also carried out using the section from the tumor after 7 days of inoculation with C57-mIFN-β or C57-EV. As shown in Figure 4, there was significant infiltration of lymphocytes around the B16-derived tumor tissues after C57-mIFN-β cell therapy, compared to C57-EV inoculation. The degeneration of tumor cells was also observed in the marginal area of the tumor tissues.

3.3.2. FCM analysis of infiltrated lymphocytes

The component of infiltrated lymphocytes was assessed by FCM. Significant increase of NK, NKT and macrophages were observed by C57-mIFN-β cell therapy in comparison with C57-EV inoculation and no difference was observed of CD4+ and CD8+ T cells (Figure 5). Interestingly, B cells were significantly decreased by the therapy.

3.4. Evaluation of viability, continuous IFN-β production and side effects using C57-mIFN-β-EGFP

Viability, IFN-β production and side effects were evaluated after 14 days of C57-mIFN-β-EGFP inoculation by immunostaining with anti-EGFP antibody, ELISA and blood test, respectively. Anti-EGFP immunostaining showed the viability of C57-mIFN-β-EGFP cells at 14

days after the inoculation (Figure 6A), accompanied with continuous hight concentration of mIFN-β (approximate 1000pg/1g tissue, Figure 6B), whereas undetectable amount of mIFN-β protein were observed in C57-EV. The blood sample was simultaneously taken for mIFN-β ELISA and side effects evaluation. Resulted undetectable amounts of mIFN-β and no significant change of blood test and liver enzyme value were observed (Figure 7).

4. Discussion

Our result indicated mIFN-β cell therapy successfully showed anti-tumor effects against melanoma both *in vitro* and *in vivo*. The suppressive effect on melanoma cell growth *in vitro* and melanoma-derived tumor development *in vivo* might be considered as direct anti-tumor effect *via* IFN-β receptor, followed by activation of complicated signal transduction including apoptosis and growth arrest.[1, 17] In addition, induction of lymphocytes especially for NK, NKT and macrophages might be considered as indirect anti-tumor effect. The strong anti-tumor effect of mIFN-β cell therapy might be composed from those direct and indirect manners, owing to local and continuous high concentration of mIFN-β production. Interestingly, liposome-mediated mIFN-β gene therapy also induced NK cell activation but not for CD4 and CD8.[18]

The mIFN-β cell therapy also showed continuous mIFN-β production after 14 days of the inoculation, without any side effects. The risk for oncogenicity of therapeutic cells themself still remained for the cell therapy. We have previously tried to use amniotic stem cell MHC double negative. It is, however, introduction of target gene still remained problem because of its characterization of primary cells. Therefore, recently, ganciclovir induced suicide C57 introduced herpes simplex virus thymidine kinase gene (C57-TK), was developed as therapeutic cells in our laboratory, and using C57-TK may avoid the oncogenicity and increase the safety. We have also observed the inhibition for proliferation of various cancer derive cells such as murine lung cancer LLC and prostate cancer RM9 (data not shown). These data suggested the mIFN-β cell therapy may apply for the various cancer treatment.

IFN-β is strongly implicated in the regulation of angiogenesis in various kinds of tumors.[19, 20] The tumor angiogenesis is one of the important processes in tumor progression and metastasis. The cell therapy using our IFN-β-producing cells could have an ability to inhibit tumor angiogenesis, followed by the prevention of tumor metastasis. It has recently reported IFN-β exacerbated CXCL10 production from melanoma and lymphatic endothelial cells.[21] CXCL10 is known to play the suppression of invasiveness and proliferation of melanoma,[22] therefore the mIFN-β cell therapy is expected to suppress the metastasis of melanoma *via* CXCL10. Dendritic cell-base vaccination has been studied in cancer immunotherapy especially in late stage melanoma.[23] Recent study reported that type I IFN also plays an important role in dendritic cell vaccines against murine melanoma metastasis model.[24] Therefore, the mIFN-β cell therapy in combination with the dendritic cell vaccination may contribute to improve the treatment of late stage melanoma.

The present study provides important information regarding the therapeutic potential of genetically engineered IFN-β-producing cells in the local tissues of tumors, with minimized systemic side-effects. Taken together with previous studies, IFN-β is likely to have multiple anti-tumor effects related to the activation of immune cells, regulation of cell cycle and anti-angiogenesis in tumors. Cell-based therapy targeting IFN-β could be a candidate as a novel anti-tumor therapy for the treatment of melanoma as well as many types of cancers.

Conflict of interest

The authors declare that they have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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