Fas-Deficient Fully Allogeneic Dendritic Cells Administered Via an Intratumoral Injection Route Show Efficient Antitumor Effects in Murine models

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https://doi.org/10.15017/26076

出版情報：福岡醫學雜誌. 104 (1), pp.15-26, 2013-01-25. 福岡医学会
バージョン：
権利関係：

Fas-Deficient Fully Allogeneic Dendritic Cells Administered Via an Intratumoral Injection Route Show Efficient Antitumor Effects in Murine models

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Abstract

Dendritic cell (DC)-based immunotherapy is a potent, active and specific cancer immunotherapy, as DCs are preferable professional APCs (pAPCs) that prime the tumor-associated antigen (TAA)-specific $CD8^+$ T-cell response. In DC-based immunotherapy, allogeneic DCs may be an alternative source of DCs for patients in whom it is difficult to obtain a sufficient number of quality-guaranteed, autologous DCs. However, the usefulness of fully allogeneic DCs in DC-based immunotherapy is controversial, and many investigators have failed to demonstrate that fully allogeneic DCs can induce an efficient antitumor effect in various experimental settings. In this study, we found that the injection of Fas-deficient fully allogeneic DCs via an intratumoral injection route exerted efficient antitumor effects, as did syngeneic DCs, but wild-type fully allogeneic DCs did not. Intratumoral injection therapy using Fas-deficient syngeneic DCs does not show superior tumor growth suppression compared to that using wild-type syngeneic DCs, suggesting that the inhibition of functional Fas may be critical for overcoming the unfavorable factor related to allogeneic DCs, especially overcoming the rejection response to alloantigens, in therapy using fully allogeneic DCs. In addition, the intratumoral injection therapy using Fas-deficient fully allogeneic DCs induced the generation of a significant tumor-specific $CD8^+$ T-cell response, which is restricted by a host-derived major histocompatibility antigen. Therefore, intratumoral injection therapy using fully allogeneic DCs of which functional Fas is inhibited may be an alternative in clinical DC-based immunotherapy, under circumstances that do not allow the use of autologous DCs.

Key words : Dendritic cells, Fully allogeneic, Cancer vaccines, Cytotoxic T cells, Immunotherapy

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Grant support
This work was supported by a Grant-in-Aid from the Japan Society for the Promotion of Science (to S.O., # 19590395).
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Nonstandard abbreviations used in this paper :
DC, dendritic cell ; pAPC, professional antigen presenting cell ; i.t., intratumoral(ly) ; TAA, tumor-associated antigen ; C57BL/6, BL6 ; Balb/c, B/c; ITADT, intratumoral activated dendritic cell therapy ; PBL, peripheral blood leukocyte ; TRP, tyrosinase-related protein ; LCMV, lymphocytic choriomeningitis virus ; GP, glycoprotein ; TADC, tumor-associated dendritic cell.

Introduction

Studies that seek to identify tumor-associated antigens (TAA), including cancer-testis antigens, mutations of normal genes, and viral antigens in tumors have focused on inducing tumor-specific T-cell responses for the treatment of malignant tumors as an active and specific immunotherapy for cancer[1)2)]. In early experimental studies and subsequent clinical trials, to elicit tumor-specific T-cell responses, TAAs were directly administered *in vivo* in the context of adjuvants[3)] which provide 'danger signals'[4)] and a 'depot' effect allowing the slow release of antigen[5)] targeting the most efficient professional antigen-presentation cells (pAPCs) *in situ*, i.e., dendritic cells (DCs), which play a central role in the presentation of antigens to naive T cells and in the induction of primary immune response[6)]. Developments in *ex vivo* generation systems enabled the use of large numbers of DCs for immunotherapy[7)8)]. In DC-mediated cancer immunotherapy, the effective priming of TAA-specific $CD8^+$ T cells is the most important concern, since the frequency of functional TAA-specific effector $CD8^+$ T cells is positively correlated with the clinical response or survival[9)10)]. Although, in general, the *ex vivo*-generated DCs are administered via a subcutaneous route, we and others have reported that the intratumoral route (i.t.) is an alternative route for DC-based immunotherapy for the induction of efficient antitumor responses (we refer to this DC-based cancer immunotherapy as intra-tumoral activated DC therapy, or ITADT)[11)~16)]. The i.t. route has the advantage of not requiring *ex vivo* pulsation with tumor lysates or tumor antigens, since the i.t.-injected DCs can engulf tumor antigens *in situ*[12)]. Therefore, neither the identification of TAAs nor tumor isolation are required, and thus antigen-loss variants or tumors in which the TAAs have not been identified can be treated.

In DC-based immunotherapy, it is occasionally difficult to obtain a sufficient number of quality-guaranteed DCs for some patient groups such as pediatric cancer patients[17)], cancer patients with pancytopenia, and patients with a hematological malignancy. In such patients, allogeneic DCs may be an alternative source. When allogeneic DCs are used for cancer immunotherapy, three important factors need to be considered : (1) major histocompatibility complex (MHC) -compatibility of the DCs used in the context of antigen presentation[18)19)], (2) the survival of the injected allogeneic DCs, which can be shortened by T cell-and/or NK cell-mediated rejection, and (3) the function of host-derived *in situ* pAPCs. Some preclinical studies using murine subcutaneous tumor models have shown that subcutaneous immunization using fully allogeneic DCs failed to induce antitumor effects[11)20)], and thus the use of allogeneic DCs in DC-based immunotherapy may be limited. We reported that host-derived pAPCs function well for the priming of TAA-specific CTL (cytotoxic T lymphocyte) responses in ITADT, but the availability of fully allogeneic DCs is also limited (even in ITADT), as is DC-based immunotherapy via other routes[21)]. However, we also found that if the rejection of the injected DCs by an alloresponse of host T cells can be controlled, ITADT using fully allogeneic DCs exhibits antitumor responses indirectly through host-derived in situ DCs. Therefore, in the case of ITADT, the most crucial aspect is the control of the alloresponse to injected DCs, resulting in the prolongation of the survival of the injected DCs[21)].

Fas is a member of the tumor necrosis factor (TNF) receptor family, and the Fas ligand-Fas signal triggers apoptotic cell death and plays a crucial role in the control of the immune system[22)]. Autoimmune lymph-proliferative syndrome (ALPS) patients have a heterozygous inherited mutation in the *FAS* gene[23)]. Mice lacking Fas or Fas ligand and ALPS patients develope progressive lymphadenopathy with an accumulation of abnormal T cells and autoimmune phenomena[22)23)]. Experiments using the deletion

of Fas in select cell types revealed that loss of Fas on B cells elicited lymhadenopathy, hypergammagloblinemia and autoantibody production[24]. Interestingly, loss of Fas on DCs also elicited lymphoid hyperplasia and the production of antinuclear autoantibodies[23)25)]. Chen et al. reported that DCs with Fas mutation, or the expression of an anti-apoptotic baculoviral caspase inhibitor, p35, enhanced the survival of DCs *in vivo*, and exhibited resistance to apoptosis of DCs by a CTL response of alloreactive T cells, resulting in enhancement of the expansion of antigen-specific T cells[26)]. Therefore, it is expected that Fas-deficient DCs may be useful for the induction of TAA-specific T cells, especially in DC-based immunotherapy using fully allogeneic DCs.

Here, we aimed at evaluating the availability of Fas-deficient fully allogeneic DCs for DC-based immunotherapy. We found that the injection of Fas-deficient fully allogeneic DCs via the i.t. injection route in mice induced an efficient antitumor response and a significant tumor-specific $CD8^+$ T-cell response which had a host MHC restriction.

Materials and Methods

Mice

Female C57BL/6 (BL6, H-2^b) and female Balb/c (B/c, H-2^d) of Charles River grade were obtained from KBT Oriental (Tosu, Japan). Female MRL/MpJ-lpr/lpr (MRL lpr, H-2^k), female MRL/MpJ-+/+ (MRL, H-2^k), and female C57BL/6J-lpr/lpr (BL6 lpr, H-2^b) were obtained from Japan SLC (Shizuoka, Japan). All mice were maintained in semi-clean facilities and were fed standard rodent chow and tap water. All mice were used at 6-12 weeks old. The animal experiments were reviewed by the Ethics Committees for Animal Experiments and Recombinant DNA Experiments, Kyushu University, and were carried out under the 'Guidelines for Animal Experiments' of Kyushu University.

Tumor cell lines

A murine malignant melanoma cell line, B16.F10, and a T cell lymphoma cell line, EL-4, both of which originate from C57BL/6 mice ; and a colon cancer cell line, CT26, which originates from Balb/c mice, were purchased from American Type Culture Collections (ATCC, Manassas, VA). These cell lines were maintained in complete medium (RPMI 1640 ; Sigma-Aldrich, St. Louis, MO) supplemented with 10% FCS (Gibco Life Technologies, Osaka, Japan), 100 IU/ml penicillin (Meiji Seika, Tokyo, Japan), and 100 μg/ml streptomycin (Meiji Seika, Tokyo, Japan) under a humidified atmosphere containing 5% CO_2 at 37°C.

Preparation of bone marrow-derived DCs

Murine bone marrow-derived DCs were generated as described with minor modifications.[12)] Briefly, bone marrow cells (BMCs) were flushed from the femoral and tibial bones of the mice, using a 10-ml syringe fitted with a 22-gauge needle (Terumo, Tokyo), and the cell suspensions were washed twice with culture medium. RBCs and lineage-positive cells (B220, CD5, CD11b, Gr-1, TER119, 7/4) were depleted using the SpinSep mouse hematopoietic progenitor enrichment kit (StemCell Technologies, Vancouver, BC, Canada) or the BD™ IMag Hematopoietic Progenitor Cell Enrichment Set-DM (BD Biosciences, San Diego, CA). These lineage-negative cells ($5\text{-}10 \times 10^4$/ 5 ml / well) were cultured in 50 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF ; PeproTech, Hamburg, Germany) and 25 ng/ml interleukin (IL)-4 (PeproTech) in endotoxin-free complete medium in 6-well plates. On Day 3 of culture, half of the culture medium was replaced by fresh medium supplemented with GM-CSF and IL-4 at the same concentrations. DCs were harvested on Day 6. All DCs were incubated with 100 ng/ml of lipopolysaccharide (LPS, Sigma-Aldrich) for 8 h, followed by incubation with 50 μg/ml of polymyxin B (50 μg/ml) for 30 min at 37°C. Finally, the

DCs were washed three times in endotoxin-free phosphate-buffered saline (PBS, Sigma-Aldrich) for use in subsequent experiments. The maturation state of the DCs was confirmed by flow cytometric analysis as described[12].

DC-based immunotherapy for established subcutaneous tumors

Intratumoral activated DC therapy (ITADT) : C57BL/6 mice were subcutaneously injected with 1×10^5 B16.F10 cells into the right flank on Day 0, and the established tumors were injected with 1×10^6 DC in 100 μl of PBS via an i.t. injection route on Days 3, 10, and 17. The right flanks of Balb/c mice were subcutaneously injected with 1×10^5 CT26 colon carcinoma cells on Day 0, and the tumors were subsequently treated with 1×10^6 DC in 100 μl of PBS via an i.t. injection route on Days 5, 12, and 19.

In all experiments, control groups received 100 μl of PBS alone, instead of DCs. The size of the tumors was assessed three times a week using microcalipers, and tumor volume was calculated using the following formula : (tumor volume ; mm^3) = 0.5236 × (long axis) × (short axis) × (height)[27].

^{51}Cr release assay for cytolytic activity of cytotoxic T lymphocytes (CTLs)

Assays for CTL activity were performed as described with minor modifications[12,28]. Six days after the third immunization with DCs, splenocytes were obtained and erythrocytes were depleted using 0.83% ammonium chloride. Splenocytes (4×10^6) were cultured with 1 μM tyrosinase-related protein (TRP)-$2_{180-188}$ synthetic peptide (H-2K^b-restricted antigenic epitope : SVYDFFVWL ; produced by Genenet, Fukuoka, Japan) for the B16 melanoma model in 1 ml of complete medium in a 24-well culture plate. Two days later, 30 IU/ml human recombinant interleukin 2 (rIL-2) was added to the medium. After 5 days, the cultured cells were collected and used as CTL effector cells.

To detect B16 melanoma-specific CTL activity, we used TRP-2-peptide-pulsed EL-4 target cells, or EL-4 cells pulsed with lymphocytic choriomeningitis virus (LCMV) glycoprotein $(GP)_{34-41}$ peptide (H-2K^b-restricted peptide: AVYNFATC GI ; produced by Genenet), as a third-party control. The target cells were labeled with 100 μCi $Na_2{}^{51}CrO_4$ for 1.5 h, and the ^{51}Cr release assay was performed as described[12]. The percentage of specific ^{51}Cr release was calculated as follows : % cytotoxicity = [(Cr release of experimental medium − culture medium background) / (maximum Cr release − culture medium background)] × 100. Each data point was obtained from triplicate wells.

Analysis of the survival of injected DCs within the tumors of treated mice

Tumor tissues were resected and minced into small pieces. The fragmented tissues were digested with 0.4 mg/ml of Liberase CI (Roche, Mannheim, Germany) and 1% (wt/vol) DNase I (Roche) for 30 minutes at 37°C before the digestion was terminated by the addition of ice-cold PBS supplemented with 10% FCS (Gibco Life Technologies) and 2 mM EDTA (Sigma-Aldrich). Collected cells were centrifuged and incubated with 100 μl of the supernatant from a cultured hybridoma line producing anti-mouse CD16/32 mAb (2.4G2 ; ATCC) or with a commercial anti-mouse CD16/32 mAb (BioLegend Japan, Tokyo) for 30 minutes at 4°C (Fc-blocking).

After the Fc-blocking, the cells were stained with PE-conjugated anti-CD11c mAb (HL3 ; BD Biosciences), fluorescein isothiocyanate (FITC) -conjugated anti-CD45.2 mAb (104 ; BD Biosciences), and Apc-conjugated anti-CD45.1 mAb (A20 ; eBioscience Inc., San Diego, CA) for an analysis of the survival of the injected DCs. Finally, 125 ng of propidium iodide was added to 250 μl of the cell suspension immediately prior to its application onto the cytometer to detect and exclude dead cells from the analysis. The labeled cells were analyzed using a FACSCalibur cyto-

meter with CellQuest software (Becton Dickinson, San Jose, CA). Data were assessed using the FLOWJO program (TREE STAR, San Carlos, CA).

Statistical analysis

Tumor growth was evaluated using a two-way analysis of variance (ANOVA), and the significance was calculated using Bonferroni's post hoc test. A probability value of $p < 0.05$ was considered significant. All data were analyzed using GraphPad PRISM®4 software (version 4, GraphPad Software, San Diego, CA).

Results

ITADT using either syngeneic or Fas-deficient fully allogeneic DCs showed significant antitumor effects, but ITADT using wild-type fully allogeneic DCs in a subcutaneous B16 melanoma model did not

Apoptosis in DCs helps regulate self-tolerance, and Fas-deficient DCs are resistant to apoptosis induced by the CTL response of alloreactiveT cells[26]. These findings prompted us to investigate whether Fas-deficient fully allogeneic DCs could be used for cancer immunotherapy in the setting of ITADT. In the present study, we used two models : a B16 melanoma model and a CT26 colon adenocarcinoma model. C57BL/6 mice were subcutaneously injected with B16.F10, and an i.t. injection of DCs was given 3 days later, followed by two further injections at 1-week intervals. Consistent with previous reports[11)12)21)], ITADT using syngeneic C57BL/6 DCs (BL6 DC ; H-2^b) induced an efficient antitumor effect, resulting in significant suppression of tumor growth compared to the PBS control (Fig. 1, $p < 0.001$), but ITADT using fully allogeneic DCs (MRL : MRL DC ; H-2^k) did not show any significant antitumor effect (Fig. 1). As expected, ITADT using Fas-deficient MRL DCs (MRL lpr DC ; H-2^k) exhibited a significant antitumor effect compared to the PBS control, as did ITADT using syngeneic DCs (Fig. 1, $p < 0.001$). This advantage of using Fas-deficient DCs is exclusive to ITADT using allogeneic DCs, because ITADT using Fas-deficient syngeneic DCs (BL6 lpr DC ; H-2^b) did not enhance the tumor growth suppression compared to ITADT using BL6 DCs (Fig. 1).

We also evaluated the antitumor effects of ITADT using Fas-deficient fully allogeneic DCs in a subcutaneous CT26 tumor model. Balb/c mice were subcutaneously injected with CT26,

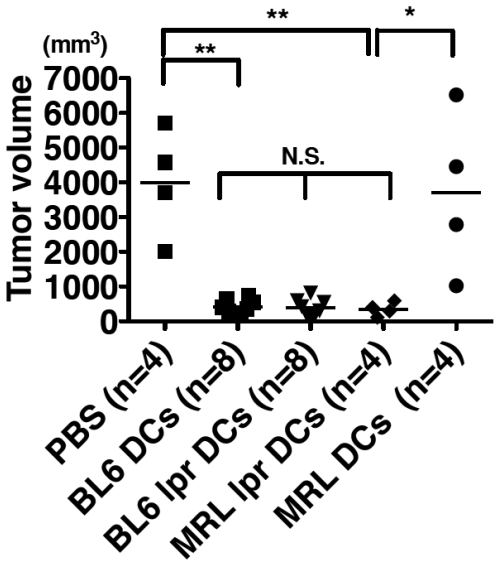

Fig. 1 ITADT using either syngeneic or Fas-deficient fully allogeneic DCs showed significant antitumor effects, but ITADT using wild-type fully allogeneic DCs in a subcutaneous B16 melanoma model did not. On Day 0, BL6 female mice (H-2^b) were subcutaneously inoculated with B16.F10 cells and the subsequently established melanomas were treated with ITADT via intra-tumoral injection on Days 3, 10, and 17, using DCs from syngeneic BL6 mice (BL6 DCs, H-2^b), syngeneic BL6 lpr mice (BL6 lpr DCs, H-2^b), fully allogeneic MRL mice (MRL DCs, H-2^k) .or fully allogeneic MRL lpr mice (MRL lpr DCs, H-2^k). Control groups received PBS alone. Tumor volumes were calculated as described in Materials and Methods on Day 24 after tumor inoculation. The data were obtained from three separate experiments and the pooled data are indicated. The 'n' numbers are indicated on the X-axis. The horizontal lines represent the mean tumor volume in the indicated experimental groups. The statistical significances (p value) between the indicated groups are shown in the graph (*$P < 0.01$, **$P < 0.001$). Similar significant differences in tumor volume were obtained after Day 21 (data not shown). N. S. = no significant difference between the indicated groups.

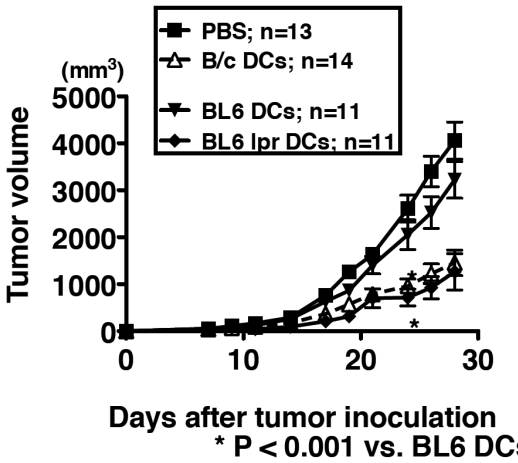

Fig. 2 ITADT using either syngeneic or Fas-deficient fully allogeneic DCs showed significant antitumor effects, but ITADT using wild-type fully allogeneic DCs in a subcutaneous CT26 colon adenocarcinoma model did not. On Day 0, B/c mice (H-2^d) were subcutaneously inoculated with CT26 cells, and the established tumors were treated with ITADT on Days 5, 12, and 19 using DCs from syngeneic B/c mice (B/c DCs, H-2^d), fully-allogeneic BL6 mice (BL6 DCs, H-2^b), and fully allogeneic BL6 lpr mice (BL6 lpr DCs, H-2^b). The control group received PBS alone. Tumor volumes were calculated as described in Materials and Methods. The dot plot data show mean tumor volume ± SEM at the indicated time points. The data were obtained from three separate experiments, and the pooled data are given. The n-numbers are indicated in the mark legends within the graph. Significant differences in tumor volume were observed on Day 24 in the indicated groups compared with those that underwent ITADT using fully allogeneic BL6 DCs (*$p < 0.01$).

and an i.t. injection of DCs was given 5 days later, followed by two further injections at 1-week intervals. ITADT using syngeneic Balb/c DCs (B/c DC ; H-2^d) induced an efficient antitumor effect, resulting in significant suppression of tumor growth compared to the PBS controls (Fig. 2 ; $p < 0.001$), but ITADT using fully allogeneic DCs (BL6 DC ; H-2^b) failed to induce tumor growth suppression compared to the PBS controls (Fig. 2). Also in this case, ITADT using Fas-deficient fully allogeneic BL6 lpr DCs exhibited significant tumor growth suppression at a level similar to that of ITADT using syngeneic DCs (Fig. 2, $p < 0.001$). These findings suggest that ITADT using Fas-deficient fully allogeneic DCs exhibits sufficient antitumor effects, as does ITADT using syngeneic DCs.

ITADT using Fas-deficient fully allogeneic DCs induce host MHC-restricted TAA-specific CTL

T cells are essential for an antitumor effect by ITADT, because ITADT using syngeneic DCs induced no effective antitumor response against CT26 tumors in nude mice, or against B16 melanomas in T-cell receptor β chain-deficient mice (data not shown). We also reported that $CD8^+$ T cells are required for antitumor responses in a B16 melanoma model[12]. Since the tumor cells express host MHC class I, it is important for efficient antitumor effects to prime the host MHC class I-restricted TAA-specific CTL response in syngeneic tumor models. Although fully allogeneic DCs do not express host MHC, not only the injected syngeneic DCs but also host-derived in situ APCs function well as professional APCs in the setting of ITADT, resulting in an efficient antitumor effect as we reported[21].

It is thus expected that TAA-specific CTLs recognizing the complex of the peptide of a TAA and host class I can be induced in ITADT using Fas-deficient fully allogeneic DCs. Therefore, we assessed the CTL response to TRP-$2_{180-188}$ in the B16 melanoma model. H-$2K^b$ -restricted CTL responses recognizing a dominant epitope of TRP-$2_{180-188}$ were detected in the spleens of B16 melanoma-bearing mice treated with ITADT using BL6 DC and BL6 lpr DCs, but not in mice treated with fully allogeneic MRL DCs (Fig. 3). It was noteworthy that the TRP-$2_{180-188}$ peptide-specific CTL response was sufficiently induced in the ITADT using fully allogeneic MRL lpr DCs (Fig. 3).

Longer survival time of Fas-deficient fully allogeneic DCs compared to fully allogeneic wild-type DCs after ITADT

It has been proposed that the survival time of

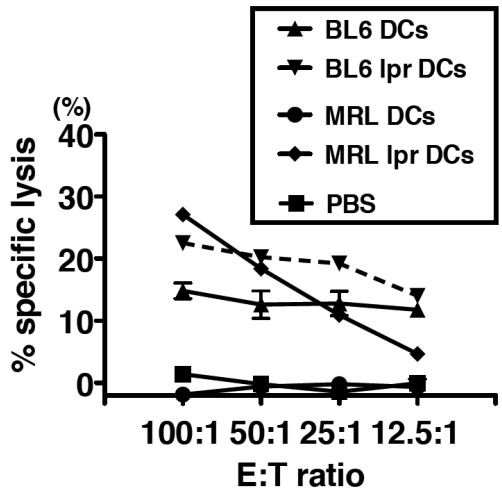

Fig. 3 ITADT using Fas-deficient fully allogeneic DCs induced the generation of a significant H-2K^b restricted TRP-2$_{180-188}$ -specific CD8$^+$ T-cell response. Established B16.F10 tumors on BL6 mice were treated with ITADT using syngeneic BL6 mice (BL6 DCs, H-2^b), syngeneic BL6 lpr mice (BL6 lpr DCs, H-2^b), fully allogeneic MRL mice (MRL DCs, H-2^k), or fully allogeneic MRL lpr mice (MRL lpr DCs, H-2^k) on Days 3, 10, and 17. Control groups received PBS alone. Six days after the third round of ITADT, splenocytes were isolated and restimulated with TRP-2 peptide *in vitro* for 5 days, and a standard ^{51}Cr-release assay was performed as described in Materials and Methods. Specific cytotoxic activity against TRP-2 is indicated. The data were obtained by subtracting the background activity to LCMV peptide-pulsed EL-4 from the cytotoxicity activities to TRP2 peptide-pulsed EL-4. The dot plot data indicate mean % specific lysis ± SEM from triplicate wells at the indicated ET ratio in each group. The data are representative of two independent experiments.

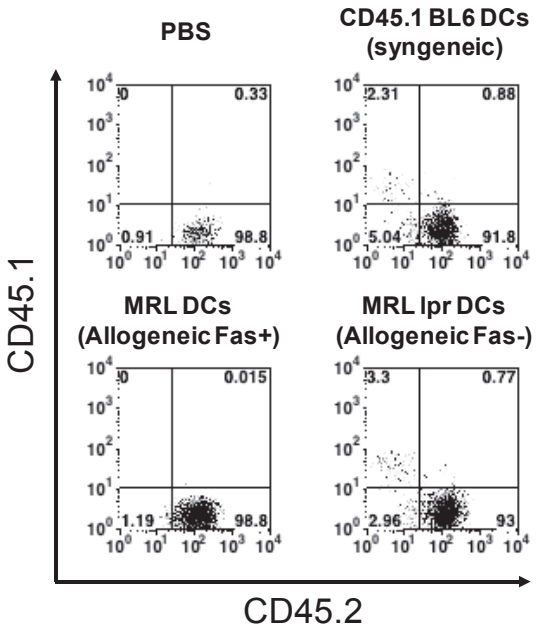

Fig. 4 Prolongation of the survival of i.t.-injected DCs using Fas-deficient fully allogeneic DCs. Established B16.F10 tumors in CD45.2 BL6 mice (H-2^b) were treated with ITADT on Days 3 and 10 after tumor inoculation using DCs from CD45.1 congenic BL6 (H-2^b, the upper right dot plot), fully allogeneic MRL mice (MRL DCs, H-2^k, the lower left dot plot), or fully allogeneic MRL lpr mice (MRL lpr DCs, H-2^k, the lower right dot plot) mice, all of which express CD45.1. One and a half days after the second round of ITADT, the tumors were resected and digested as described in Materials and Methods. Cells were then stained with PE-conjugated anti-CD11c mAb, FITC-conjugated anti-CD45.2 mAb, and Apc-conjugated CD45.1 mAb. The tumors in the control group were injected with PBS alone (the upper left dot plot). Dot plots show the percentage of CD45.1 or CD45.2 in the CD11c$^+$ -gated population. The percentages are indicated within each quadrant. The data are representative of three independent experiments.

injected DCs may be an important factor for efficient anti-tumor effects in DC-based cancer immunotherapy[29]. We have also reported that the injected DCs tend to remain at the tumor site, and we found that the survival rates of i.t. -injected fully allogeneic DCs within the tumor site is a critical factor for priming a TAA-specific CD8 T-cell response via host-derived DCs and the antitumor effect, in ITADT for tumor models on bone marrow transplant recipient mice, which are tolerant to the fully allogeneic antigens[21]. We therefore evaluated the survival of injected Fas-deficient fully allogeneic DCs in the established tumors in the present study. Subcutaneously established B16.F10 tumors in CD45.2 C57BL/6 mice were treated with ITADT on Days 3 and 10 after tumor inoculation using DCs from CD45.1 congenic BL6 (H-2^b), fully allogeneic MRL (MRL DCs, H-2^k), or fully allogeneic MRL lpr mice (MRL lpr DCs, H-2^k) mice, all of which express CD45.1. The tumors were resected, and the survival of the injected DCs was examined 1.5 days after a second round of i.t. injection of DCs. A significant percentage of CD45.1$^+$-injected DCs was detected within the total CD11c$^+$ DC population in the tumors on Day 1.5 ; however, no CD45.1$^+$ DCs were observed

within the total $CD11c^+$ DC population in the tumors from the mice treated with ITADT using fully allogeneic MRL DCs (Fig. 4).

As expected, a significant percentage of $CD45.1^+$-injected DCs was detected in the tumors of the mice treated with ITADT using Fas-deficient fully allogeneic MRL lpr DCs (Fig. 4), suggesting that Fas-deficient fully allogeneic DCs are resistant to alloresponse, and the efficient antitumor effects with the TRP-2 specific CTL response via host-derived APCs are due to the prolongation of the survival of the injected DCs within the tumor.

Discussion

In DC-based immunotherapy, allogeneic DCs are considered an important source of DCs for patients for whom it is difficult to obtain a sufficient number of quality-guaranteed DCs. However, the preclinical data obtained regarding the use of allogeneic DCs for immunotherapy have been negative, where peptide or tumor-lysate pulsed fully allogeneic or semi-allogeneic DCs were subcutaneously injected[11)20)30)31)]. We also reported that the effectiveness of ITADT using fully allogeneic DCs is limited for inducing sufficient antitumor effects[21)]. The present study is the first report that Fas-deficient fully allogeneic DCs are available for DC-based immunotherapy in the setting of ITADT. This finding may provide an alternative potential source of DCs for clinical DC-based immunotherapy. That is to say, fully allogeneic DCs that are genetically modified to suppress Fas expression or apoptosis by transduction of siRNA to Fas or anti-apoptotic molecules such as the baculoviral caspase inhibitor p35[26)] may be available for DC-based immunotherapy. Since gene-modified DCs can be stored for long periods with the appropriate storage methods[32)], DC-based immunotherapy can be performed promptly for patients for whom the pre-vaccination period is limited.

We demonstrated that ITADT is an alternative route for DC-based immunotherapy and can exhibit sufficient antitumor effects for various cancers in preclinical settings[12)21)33)]. It has also been suggested that ITADT is a safe method and can induce clinical antitumor effects in clinical immunotherapy[34)35)]. ITADT has the advantage of not requiring *ex vivo* pulsation with tumor lysates or tumor antigens, since the i.t.-injected DCs can engulf tumor antigens *in situ*[12)]. We have also suggested that injected DCs can enhance the antitumor effect through the efficient mobilization of host-derived APCs and the subsequently enhanced TAA-specific $CD8^+$ T-cell responses in ITADT[21)]. However, fully allogeneic DCs rapidly disappeared *in vivo* due to a T cell-mediated alloresponse, resulting in insufficient antitumor effects[21)].

Using a bone marrow transplantation (BMT) model, we also demonstrated that the survival time of the injected DC is more important than MHC compatibility in ITADT, since the antitumor effects were induced by host-derived APCs expressing MHC molecules restricting T cells, even when fully allogeneic DCs were injected intratumorally into BMT recipients tolerant to alloantigens as a result of mixed chimerism[21)]. In the present study, we found that ITADT using Fas-deficient fully allogeneic DCs induces a host MHC-restricted TAA-specific CTL response, notwithstanding that the DCs used did not express host MHC (Fig. 3). This finding strongly supports our hypothesis that the prolongation of the survival of fully allogeneic DCs can induce efficient antitumor effects, probably through indirect activation of *in situ* host-derived DCs.

An alloreactive T-cell response to the alloantigens expressed by the injected DCs themselves was expected to provide the injected DCs with additional danger signals via costimulatory-related molecules (such as CD40-CD40L signaling[36)~38)]) or a bystander production of T-cell-growth factors, resulting in enhanced priming of T-cell responses[39)]. However, there

are also conflicting reports suggesting that antitumor effects are impaired by concurrent alloreactivity[30)31)]. Whether the concomitant T-cell mediated alloresponse to allogeneic DCs has a positive or negative effect on the induction of TAA-specific T cells may depend on the experimental setting. At the least, the alloresponse has a negative effect on the antitumor effects of ITADT, and fully allogeneic DCs are available for ITADT when the Fas of injected DCs can be controlled.

In conclusion, ITADT using Fas-deficient fully allogeneic DCs can induce an efficient antitumor response with a concomitant host MHC-restricted TAA-specific CTL response. In DC-based immunotherapy, this alternative method may be very helpful for treating patients from whom it is difficult to obtain large numbers of DCs or for whom the pre-vaccination period is limited.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgement

We thank Katsuo Sueishi for cooperation and advice on the research direction and Chie Arimatsu for the animal care.

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(Received for publication December 10, 2012)

（和文抄録）

Fas 欠損アロ樹状細胞を用いた腫瘍内樹状細胞投与療法は有効な抗腫瘍効果を惹起する

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樹状細胞はプロフェッショナル抗原提示細胞で強力な腫瘍関連抗原特異的 CD8T 細胞応答を惹起するため，それを利用した樹状細胞免疫療法は強力で特異的ながん免疫療法となることが期待できる．樹状細胞免疫療法において，アロの樹状細胞は，質的量的に樹状細胞が採取困難な患者における代価細胞として期待できるが，その有効性は議論の余地があり，実際，種々のプロトコールで良好な結果が得られていない．本研究において，我々は，Fas が正常なアロの樹状細胞の腫瘍内投与は全く抗腫瘍効果を発揮しないが，Fas 欠損アロ樹状細胞を腫瘍内投与すると，同系マウスの樹状細胞に匹敵する抗腫瘍効果を発揮することを初めて報告する．この時，Fas が欠損している同系のマウスから得られた樹状細胞は同系の樹状細胞より強い効果を発揮することはなかったことから，Fas の欠損はアロ樹状細胞にのみにおこる不利な応答を克服するのに必要な分子であることが示唆された．更に，この Fas 欠損アロ樹状細胞を用いた腫瘍内樹状細胞療法は，宿主の主要組織適合抗原に拘束される腫瘍関連抗原特異的 CD8T 細胞応答を惹起した．よって，Fas 分子の機能抑制を施したアロ樹状細胞は，自己樹状細胞が採取困難な患者の代価樹状細胞として有用である可能性が示唆された．