

Immunologically augmented skin flap as a novel dendritic cell vaccine against head and neck cancer in a rat model

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Advanced stage head and neck squamous cell carcinomas (HNSCC) are difficult to treat and have a high mortality rate.⁽¹⁾ Because the radical resection of HNSCC is impossible in most cases due to the complicated anatomical structures that are involved, multi-disciplinary approaches including surgery and adjuvant chemo-radiotherapy are routinely employed.⁽²⁾ In addition, wide local excision of tumors as well as functional and cosmetic restoration have become possible through immediate reconstructive surgery using an autologous flap transfer.⁽³⁾ Nevertheless, the relatively high recurrence rate, either local or metastatic, must be addressed,^(4–7) and this recurrence rate underscores the importance of newly emerging therapeutic modalities. Cancer immunotherapy is one of several new options available for patients seeking less invasive and more expedient alternatives.^(2,8,9)

A variety of types of cancer immunotherapy, such as epidermal growth factor receptor (EGFR) signaling inhibitors, cetuximab, dendritic cell (DC) vaccines, adoptive CTL transfer and allogeneic hematopoietic stem cell transplantation with donor lymphocyte infusion, have been performed in pilot studies for

Local recurrence is a major clinical issue following surgical resection in head and neck cancer, and the dissemination and lymph node metastasis of minimal residual disease is relatively difficult to treat due to the lack of suitable therapeutic approaches. In the present study, we developed and evaluated a novel immunotherapy using a skin flap transfer treated with sensitized dendritic cells (DC), termed the “immuno-flap,” in a rat tumor model. After the local round area of skin was resected, SCC-158 cells (a rat head and neck cancer cell line) were inoculated into the muscle surface; lastly, the groin skin flap injected with mature DC was overlaid. Two weeks after the second DC injection, systemic immunological reactions and tumor size were measured. The DC-treated group showed a significant reduction in tumor size compared with the control. Although the induction of CTL activity in spleen cells was marginal, Th1 cytokines such as interleukin-2 and interferon- γ were elevated in the DC-treated group. These results suggest that a novel immunotherapy based on the immuno-flap method has the potential for clinical application to prevent the local recurrence of head and neck cancer patients.

head and neck patients.^(4,10,11) Currently, the major focus of cancer-specific DC-based immunotherapy is shifting toward the improvement of overall survival and quality of life, including performance status.^(8,9)

As long as the systemic remission of recurrent cancer is a major concern, DC can be theoretically administered either subcutaneously or intravenously and effectively present tumor antigens.⁽¹²⁾ Conversely, regional DC vaccination (i.e. the intra/para-tumoral injection of DC) has shown better regression of local residual or recurrent tumors compared with systemic DC administration, as is reported in animal experiments and also clinical trials.⁽¹³⁾ Although the putative mechanisms underlying this phenomenon have not been fully elucidated, regional DC vaccination seems to be more efficient in simulating both antigen presentation and the local mobilization of cytotoxic immune cells.⁽¹⁴⁾

Based on the above, we hypothesized that regional DC encapsulated in autologous flap tissue adjacent to HNSCC can effectively induce a local immune response against cancer and strongly prevent its growth at the primary site. To test this

hypothesis, we developed a novel rat model of a skin flap with augmented anti-tumor immunity, termed the “immuno-flap,” where a DC vaccine was locally administered in conjunction with a pedicled groin skin flap overlaying the tumor site. In this immuno-flap model, DC-treated rats showed significant suppression of tumor growth beneath the flap. The specific immunological mechanisms involved in the anti-tumor effect *in vivo* were investigated.

Materials and Methods

Animal experiments and cell lines. For the tumor-bearing rat model and for the collection of rat DC, pathogen-free 5–6-week-old female Fisher rats were purchased from Clea (Shizuoka, Japan). The rats were housed humanely, according to guidelines for the welfare and use of animals in cancer research (*Br J Cancer* in 2010), and the procedures were approved by the Animal Care and Use Committee of the Shizuoka Cancer Center Research Institute. SCC-158 cells were purchased from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan), and cultured in DMEM (SIGMA, St. Louis, MO, USA) supplemented with 10% FBS (Life Technology, Carlsbad, CA, USA), 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin. The C6 cell line was obtained from JCRB and was cultured in Ham’s F10 medium containing 15% horse serum (Life Technologies) and 2.5% FBS.

Cytokines and reagents. For the generation of mature rat DC, recombinant rat granulocyte macrophage colony-stimulating factor (rGM-CSF) and rat interleukin-4 (rIL-4) were purchased from Peprotech (Rocky Hill, NJ, USA). Recombinant rat IL-2 was obtained from Miltenyi Biotech GmbH (Bergisch Gladbach, Germany). FITC-labeled mouse anti-CD11c antibody and phycoerythrin (PE)-labeled anti-CD103 (OX62) monoclonal antibody (MoAb) were purchased from AbD Serotec (Oxford, UK) and eBioscience (San Diego, CA, USA), respectively. Biotin-labeled mAb against rat MHC-class II antigen RT1B and PE-labeled mAbs against rat CD54, CD80 and CD86 were purchased from BD Biosciences (Franklin Lakes, NJ, USA) for FACS analysis. Anti-rat CD8 mAb (LSBio, Seattle, WA, USA), anti-rat Foxp3 (eBiosciences, San Diego, CA, USA), anti-rat granzyme B mAb (Abcam, Cambridge, MA, USA), and anti-rat CD11c mAb (BD Biosciences) were purchased for immunohistochemistry (IHC) analysis.

Culture of bone marrow-derived dendritic cells and tumor antigen pulsing. Unfractionated rat bone marrow (BM) cells were resuspended in RPMI 1640 medium containing 2% FBS, and incubated with 10 ng/mL rGM-CSF and 10 ng/mL rIL-4 at a concentration of 2.5×10^6 /mL in a 6-well culture plate for 7 days. Non-adherent DC-enriched cells were collected by gentle pipetting. SCC158 tumor-derived lysate preparation was performed using a freeze–thaw method that has been described previously.⁽¹⁵⁾ Briefly, SCC-158 cell suspensions in PBS (–) at the concentration of 1×10^7 /mL were frozen in a -150°C freezer and thawed at 56°C ; this was repeated five times. The lysates from disrupted cells were centrifuged at 13,000 g for 10 min. Endotoxin levels in tumor lysates were found to be negative (<0.8 pg/mL). On day 7, 1×10^7 DC-enriched cells were resuspended in RPMI1640 medium containing 2% FBS. Then, 400 µg of SCC-158 tumor lysates were mixed with the same volume of Opti-MEM medium (Life Technology, Carlsbad, CA, USA), and incubated at room temperature (RT) for 15 min. The mixture was then added to a total of 2 mL DC suspensions in a 6-

well culture plate, and incubated for 24 h at 37°C in a CO_2 incubator. On day 8, lipopolysaccharide (LPS, E. coli K12-derived, InvivoGen, San Diego, CA, USA) was added to the culture at a final dose of 1 µg/mL and incubated for 24 h at 37°C . On day 9, DC were collected and washed two times with serum-free RPMI 1640 medium, and were then resuspended at 2×10^7 /mL for injection into rats.

Mixed leukocyte reaction assay. To quantify the ability of DC to stimulate leukocyte proliferation, rat BM-derived immature (non-LPS-treated) or mature (LPS-treated) DC with rat splenic $\text{CD}3^+$ T cells were co-cultured. Spleen $\text{CD}3^+$ T cells were obtained by cell sorting using a FACSaria (BD Biosciences). Then, 1×10^4 DC and 1×10^5 T cells were mixed in each well of a 96-well micro-culture plate (Corning, NY, USA) and incubated in RPMI 1640 medium containing 10% FBS for 3 days. The growth-stimulating effect of DC was measured using a WST-1 proliferation assay (Dojindo, Kumamoto, Japan).

Animal experiments. Two types of DC-based immunotherapy *in vivo* models were used. In a subcutaneous tumor model (A), SCC-158 tumor cells were subcutaneously transplanted at the flank, and DC were injected into the para-tumor area. In an immuno-flap model (B), the pedicled island groin skin flap was elevated as previously reported.⁽¹⁶⁾ Briefly, a rat was anesthetized with vaporized isoflurane (DS Pharma Animal Health, Osaka, Japan) and shaved. A skin paddle 1.5 cm in diameter was made using scissors in the left groin region, including its irrigating blood vessels, circumflex iliac artery and veins. The subcutaneous inguinal lymph nodes were also included in the flap. The flap was transferred to the tumor inoculation site in the flank through a subcutaneous tunnel and was then locally injected with sensitized mature DC. The skin incision was sutured onto the flank skin using a needle and nylon thread. In both the A and B experiments, the 2×10^5 tumor cells were transplanted in each rat and 2×10^6 DC were injected two times weekly. The tumor inoculation was temporarily set as day 0. DC injections were administered on day 1 and 8 or day 7 and 14 in experiment A, and on day 0 and 7 in experiment B. Tumor growth was monitored for 3 weeks after tumor inoculation in B, and the flap was resected on day 21 for analyses. On the same day, rat spleens were harvested for use in CTL assays and cytokine RT-PCR analysis. For the DC tracking experiment, the graft was resected 24 h after DC were injected into the skin flap via the feeding artery, and DC status was evaluated using IHC analysis.

Immunohistochemistry. The formalin-fixed paraffin-embedded flap tissue block was cut along the line where the maximal tumor area was obtained. To quantitate tumor suppressive effects, the total area of tumors in each section stained with H&E was calculated using the image-analyzing software Winroof (Mitani Corporation, Tokyo, Japan). Tumor-infiltrating lymphocytes were analyzed using anti-CD8, Foxp3 and granzyme B mAbs on appropriate sections from control and DC-treated groups. In the tracking experiment, rat DC localization was evaluated by IHC using anti-rat CD11c subset MoAb.

Rat CTL assay. To estimate the induction of cytotoxic T-lymphocytes, spleens were harvested from three rats per group 14 days after the last DC injection. Spleens were dissociated, erythrocytes were lysed with ACK lysis buffer, and splenocytes were filtered through cell strainers and resuspended in RPMI 1640 medium containing 2% FBS. Spleen cells were co-cultured at 2.5×10^6 /mL with irradiated (180 Gy) SCC-158 cells at 2.5×10^5 /mL in RPMI 1640 with 10% FBS in a 6-well culture plate (Corning, NY, USA) for

7 days in the presence of 3 ng/mL rat IL-2. Restimulated splenocytes were used as effector cells, and SCC-158 and C6 tumor cells were used as target cells. The effector/target (E/T) ratio ranged from 100:1 to 11:1. CTL killing was assessed with the DELFIA non-radioactive cytotoxicity assay (Perkin-Elmer, Waltham, MA, USA) as previously reported.⁽¹⁷⁾ The percentage of specific lysis was determined by the following formula: percentage of specific lysis = (experimental release – spontaneous release)/(maximal release – spontaneous release) × 100.

Rat interferon- γ production assay. Antitumor activity induced by the immuno-flap was further evaluated by quantifying IFN- γ production. Restimulated spleen cells (1×10^5) and SCC-158 or C6 target cells (1×10^5) were co-incubated in RPMI1640 medium containing 10% FBS in a round bottomed-96-well microculture plate for 24 h. Finally, supernatants were collected and rat interferon- γ (IFN- γ) levels were measured using an ELISA kit specific for rat IFN- γ (Thermo Scientific, Waltham, MA, USA).

Rat cytokine RT-PCR. For profiling of the immune response induced by immuno-flap transfer, the expression of cytokines, such as IL-2, IL-4, IL-10, IL-17A, IFN- γ , Foxp3 and NKR-P1A, was analyzed using RT-PCR. Total RNA was isolated from rat splenocytes stimulated with SCC-158 cells using the NucleoSpin RNA kit (MACHEREY NAGEL, Düren, Germany). Complementary DNA was synthesized using SuperScript III RTase and Oligo (dT)20 primer (LifeTechnologies, CA, USA). RT-PCR was performed as previously described.⁽¹⁸⁾ The primer sequences used are shown in Table 1. All primers for rat cytokine expression, including IL-2, IL-4, IL-10, IL-17A, IFN- γ , Foxp3 and NKR-P1A, were designed in our laboratory and synthesized.

Statistical analysis. Statistically significant differences were analyzed using Student's paired two-tailed *t*-test. Values of $P < 0.05$ were considered significant.

Results

Dendritic cell characterization *in vitro*. The rat DC were characterized by flow cytometric analyses, and their ability to stimulate T cells *in vitro* was analyzed by mixed leukocyte reaction (MLR) assay. The rat DC gate is shown as a large and widely distributed population in flow cytometric analysis (Fig. 1a). The mean percentage of DC that were determined to be CD11c⁺ was 84.4%, as shown in Figure 1(a). Morphologically, the cells were large round cells with eccentric nuclei (Fig. 1b). The frequencies of cells expressing specific DC markers were as follows: MHC class II 49.1%, CD54 58.5%, CD80 33.5%, CD86 9.9% and CD103 99.8%. Most DC expressed a high level of CD103,

a rat-specific DC marker, but the expression of co-stimulatory molecules CD80 and CD86 was not high (Fig. 1c). In the MLR assay, mature DC significantly stimulated the T cell proliferation more than immature DC (Fig. 1d). Collectively, the profile of cell surface markers and the ability to stimulate T cell proliferation indicated that the cells obtained were DC.

Dendritic cell functions in a subcutaneous tumor model. Next, the ability of rat DC to induce CTL activity *in vivo* was evaluated in a subcutaneous tumor model without a skin flap. For each group (control and DC-treated), five rats were subcutaneously transplanted with SCC-158 tumor cells on day 0. Then, 2×10^6 rat DC was injected on days 1 and 8 as in the previous tumor experiment. In another experiment using an established tumor, DC were injected on days 7 and 14. In both experiments, DC treatment significantly suppressed the growth of SCC-158 tumors (Fig. 2a,b). A CTL assay was performed using splenocytes, which showed moderate SCC-158-specific killing activity at an E/T ratio of more than 5 (Fig. 2c). In accordance with that, IFN- γ production from DC-treated splenocytes was significantly stimulated to a greater than in the control group (Fig. 2d). Those data indicated that rat DC from the current study were able to induce tumor-specific CTL activity *in vivo*.

Tumor suppression in the immuno-flap. Next, the anti-tumor properties of the immuno-flap model were explored. Four rats per group were inoculated with SCC-158 tumor cells (2×10^5) in the area of the skin incision, and the immuno-flap was overlaid on the inoculation site at the same time. Then, 2×10^6 rat DC were injected into the immuno-flap on days 0 and 7 in the DC-treated group. The flap and surrounding tumor-containing tissue was resected 14 days after the last DC injection. In the DC-treated group, the tumor growth under the skin flap was dramatically inhibited in all rats compared to the control (Fig. 3a). In particular, two of the grafts showed a remarkable reduction in tumor size (Fig. 3b). CTL activity against SCC-158 tumor was activated in DC-treated rats in accordance with *in vivo* tumor inhibition (Fig. 4a).

CTL-related gene upregulation in spleen cells by immuno-flap. To test whether the immuno-flap can stimulate systemic immunity, spleen cells were collected from the rat model and the expression profiles of CTL-related genes were analyzed. RT-PCR analyses were performed for rat IL-2, IL-4, IL-10, IL-17A, IFN- γ , Foxp3 and NKR-P1A using total RNA from spleen cells stimulated with SCC-158 cells (Fig. 4b). In the majority of DC-treated spleens (three of four), CTL-related genes were upregulated: Th1 cytokines, such as IL-2 and IFN- γ , as well as Th2 cytokines, such as IL-4 and IL-10, were activated, and a natural killer (NK) cell-activating marker, NKR-P1A, was also upregulated. In contrast, among three of

Table 1. Rat PCR primer sequence

| Gene | Forward | Reverse |
|---------------|--------------------------|--------------------------|
| IL-2 | TGTTGCTGGACTTACAGGTGCTC | CAAATCCAACACACGCTGCAGAG |
| IL-4 | TATGTACCGGGAACGGTATCCAC | CGTGGGAAGTAAAATTTGCGAAG |
| IL-10 | ATAACTGCACCCACTTCCCAGTC | TGGCAACCCAAGTAACCCCTTAAA |
| IL-17A | GAAGGCCCTCAGACTACCTCAA | AAAGTGAAGGGGCACTTCTCAG |
| IFN- γ | TTACTGCCAAGGCACACTCATT | AGGTGCGATTGCATGACACTTA |
| NKR-P1A | CCCTGGTTGGGATGAGTATTTTAG | ATGAAAGCATTATCTCGTGTGA |
| Foxp3 | CCACACCTCCTCTTCTCTTCTGAA | ATGTGCGTCTACAGTGGAGAGCTG |

IFN- γ , interferon- γ ; IL, interleukin.

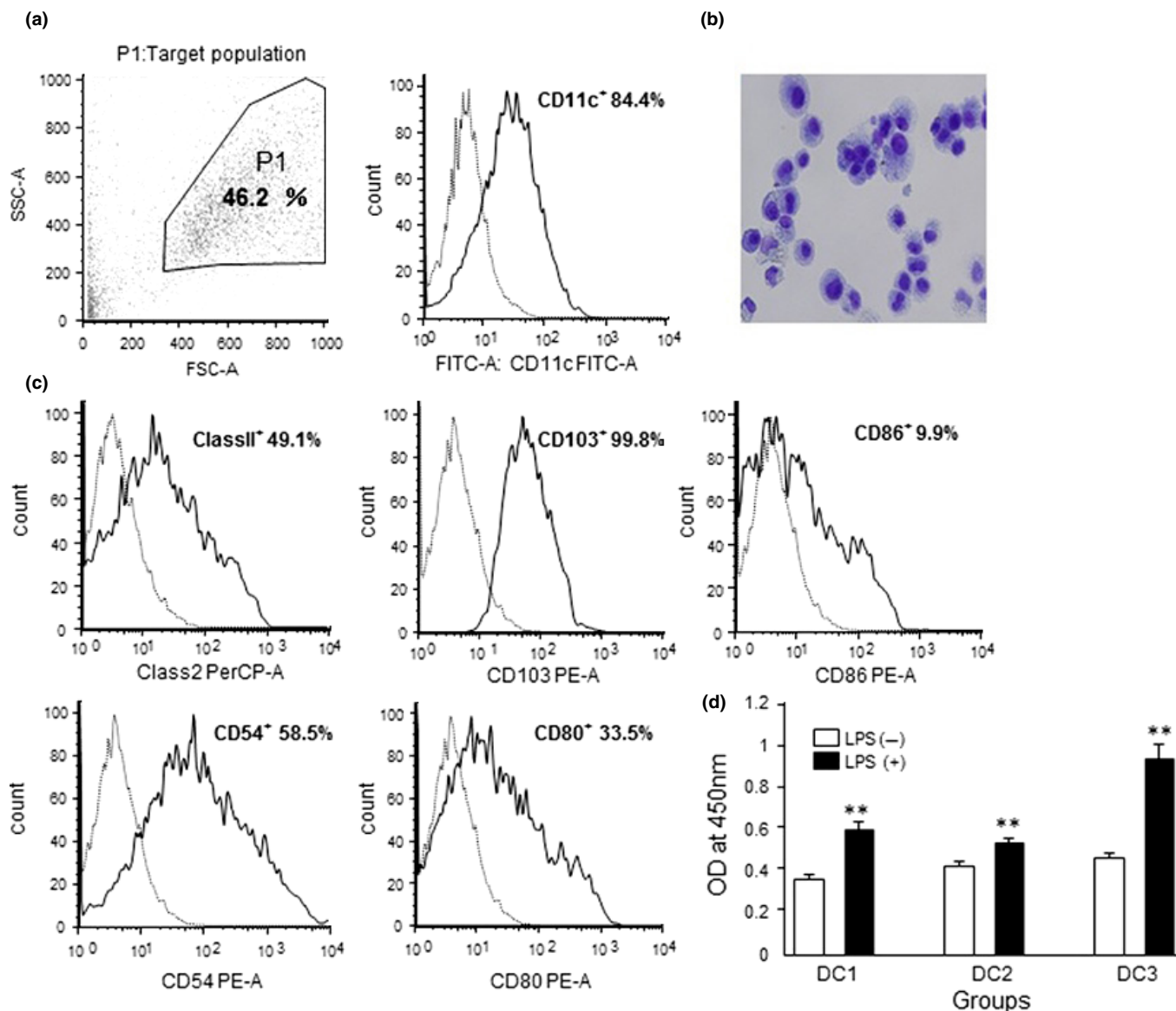


Fig. 1. Characterization of cultured rat dendritic cells (DC) by flow cytometry. The mean percentage of DC rated as CD11c⁺ cells is shown in (a). (a) Dot gram of whole cultured cells. The DC gate is shown as a large and widely distributed population. (b) The morphological feature of DC. (c) Expression of DC-specific markers (CD54, CD80, CD103 and MHC-class II). (d) Mixed leukocyte reaction assay. Three groups of DC cultures were tested for the ability to stimulate leucocyte proliferation. Each column shows the mean value of five samples. **P < 0.01, statistically significant compared with immature DC.

the untreated spleens, two of them did not show the upregulation of these genes.

Characterization of tumor-infiltrating lymphocytes in the immuno-flap. Immunohistochemistry analysis of infiltrating lymphocytes in the tumor revealed an obvious infiltration of a large number of CD8⁺ T cells and a small number of granzyme⁺ T cells were shown (Fig. 5), consistent with tumor regression. However, no significant T cell infiltration was seen in the tumors from the control group. Foxp3⁺ T cells were not detected in either the control or the DC-treated tumor group (data not shown), indicating no signs of local accumulation of regulatory T cells and subsequent immune tolerance.

DC-tracking experiment. To determine whether the DC vaccine was administered diffusively over the flap for optimal immune response, the fate of DC injected in the flap was

tracked *in vivo*. DC were injected via an artery rather than subcutaneously, for that purpose. After injection via the flap-feeding artery, the distribution of DC was explored using IHC. Then, 24 h after the DC injection, many CD11c⁺ DC were found to be resident in the subcutaneous tissue, which was not observed in the flap without DC injection (Fig. 6).

Discussion

The classical flap transfer has been used as a source of donor skin and adipose tissue for the recipient site without any particular function. The few exceptions are those flaps designed to restore physical function, such as innervated muscle transfer for the reanimation of the face.⁽¹⁹⁾ Despite evidence demonstrating that the flap may contain physiological immune cells,⁽²⁰⁾ a flap with anti-tumor immunity was not achieved

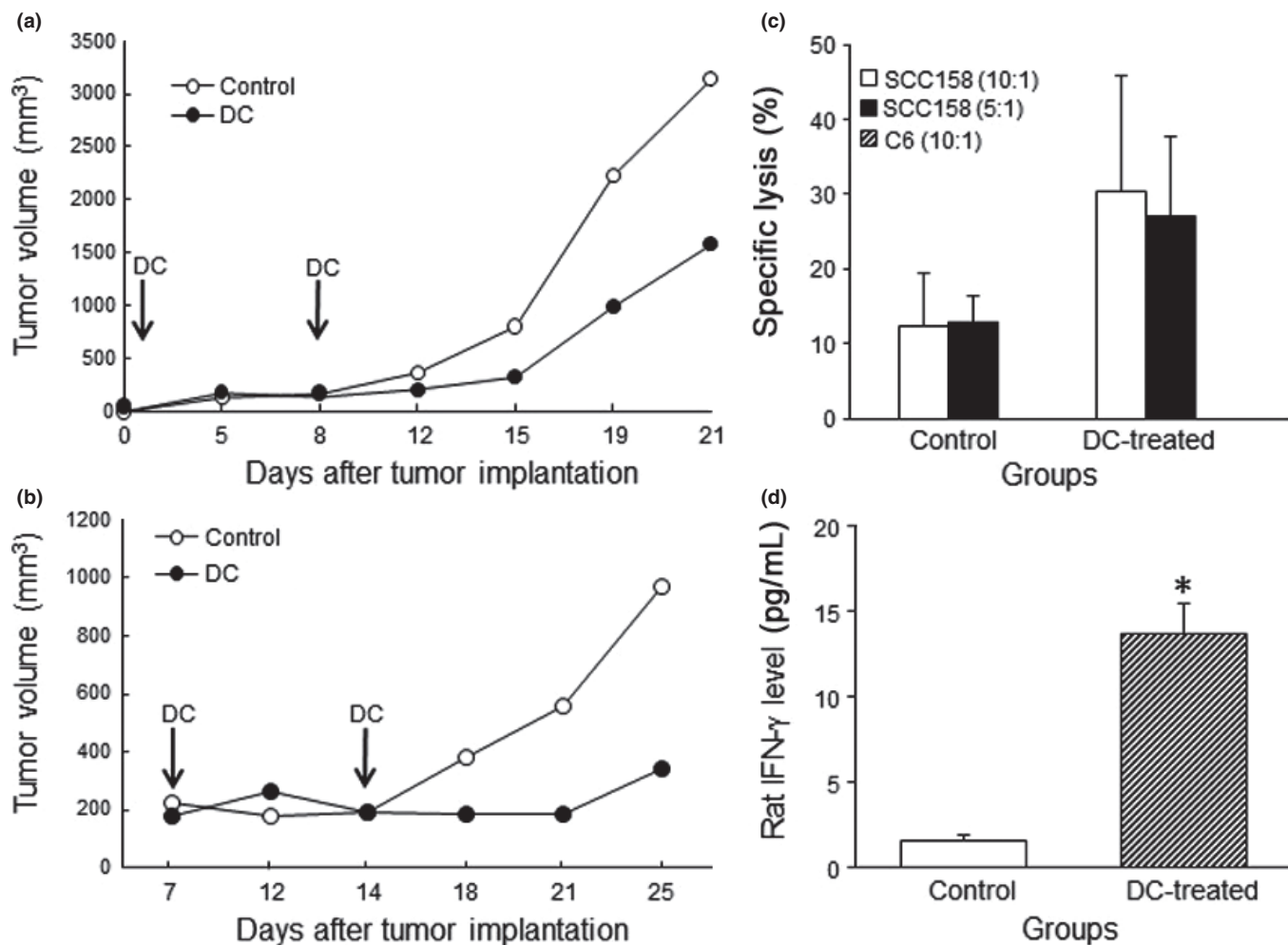


Fig. 2. Rat dendritic cell (DC)-based immunotherapy of the subcutaneous tumor model. Each of five rats was subcutaneously transplanted with SCC-158 tumor cells in the control and the DC-treated group each on day 0. On days 1 and 8, 2×10^6 rat DC were injected in an earlier tumor experiment (a). Rat DC were injected on days 7 and 14 in an established tumor experiment (b). (c) The induction of CTL activity against tumor cells using non-radioactive cytotoxicity assay and (d) interferon- γ (IFN- γ) production in co-cultures of boosted spleen cells with SCC-158 tumor cells. Rat spleen cells were derived from the earlier tumor experiment. Each point shows the mean value of the triplicate samples. * $P < 0.05$, statistically significant compared with control.

until Dempsey *et al.*⁽²¹⁾ designed one by means of *in vivo* transfection to express IL-12 within. Tumor suppression by the transfected flap was successfully demonstrated in a rat model, but genetic modification will likely hamper its clinical application because of concerns regarding biological safety. Consequently, we injected tumor antigen-sensitized mature DC instead of gene constructs into the flap, which could be regarded as less hazardous. As a result, sufficient numbers of DC to exert an anti-tumor effect were delivered to the tumor margin, as shown in the DC tracking experiment. Tumor reduction and the migration and accumulation of cancer-specific CTL were also observed around the flap, similar to in the genetic flap model.

In the immuno-flap, the DC vaccine is administered locally in conjunction with the flap being overlaid onto the site of the resected tumor. Local immunotherapy is being investigated in several clinical trials^(22,23) for the following reasons: (i) the minimal side effects as a result of local immunotherapy; and (ii) the intratumor immunosuppression induced by Tregs and MDSC, which is a promising target for anti-immune checkpoint MoAb therapy, such as anti-CTLA-4 and anti-PD-1

mAbs.^(24,25) Taking this into account, local immunotherapy has an obvious advantage in that it can easily access and manipulate the tumor microenvironment through the administration of immune effector cells or reagents in a paratumoral or intratumoral manner.

Bourdeaux *et al.*⁽²⁶⁾ established male skin graft transplantation experiments in CBA female mice and demonstrated that local immunostimulation led to the rejection of foreign tissue by re-stimulation with potent anti-male mouse antigen HY T cells. They eventually concluded that the restored local immunostimulation could be applied to reactivate anti-cancer immunity similar to skin graft rejection. In their model, as well as the genetic flap model, T cell migration using immunostimulation by a cytokine combination and the subsequent induction of Th1 cytokines, such as IL-12 and IFN- γ , were important for the anti-tumor response.⁽²¹⁾ The local DC administration accompanying the immuno-flap technique is also meant to evoke a local immune response at the graft site by augmented antigen presentation instead of cytokine injection. The gene expression profiles revealed in our study also suggested that a shared mechanism between

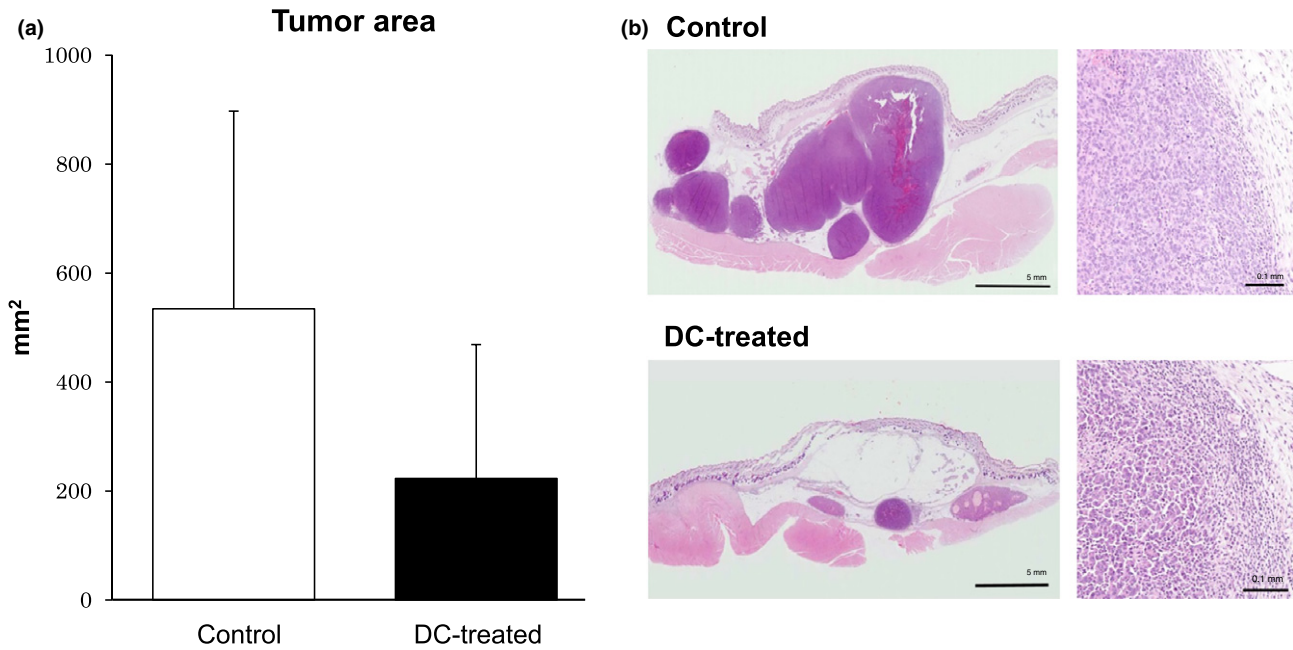


Fig. 3. Tumor inhibitory effect in the skin flap tumor models. Four rats per group were used. (a) Quantitated tumor area in the section where maximal tumor area was obtained in the control and dendritic cell (DC)-treated group. Each column shows the mean value for the four rats. (b) Pictures of representative sections from the control and DC-treated group.

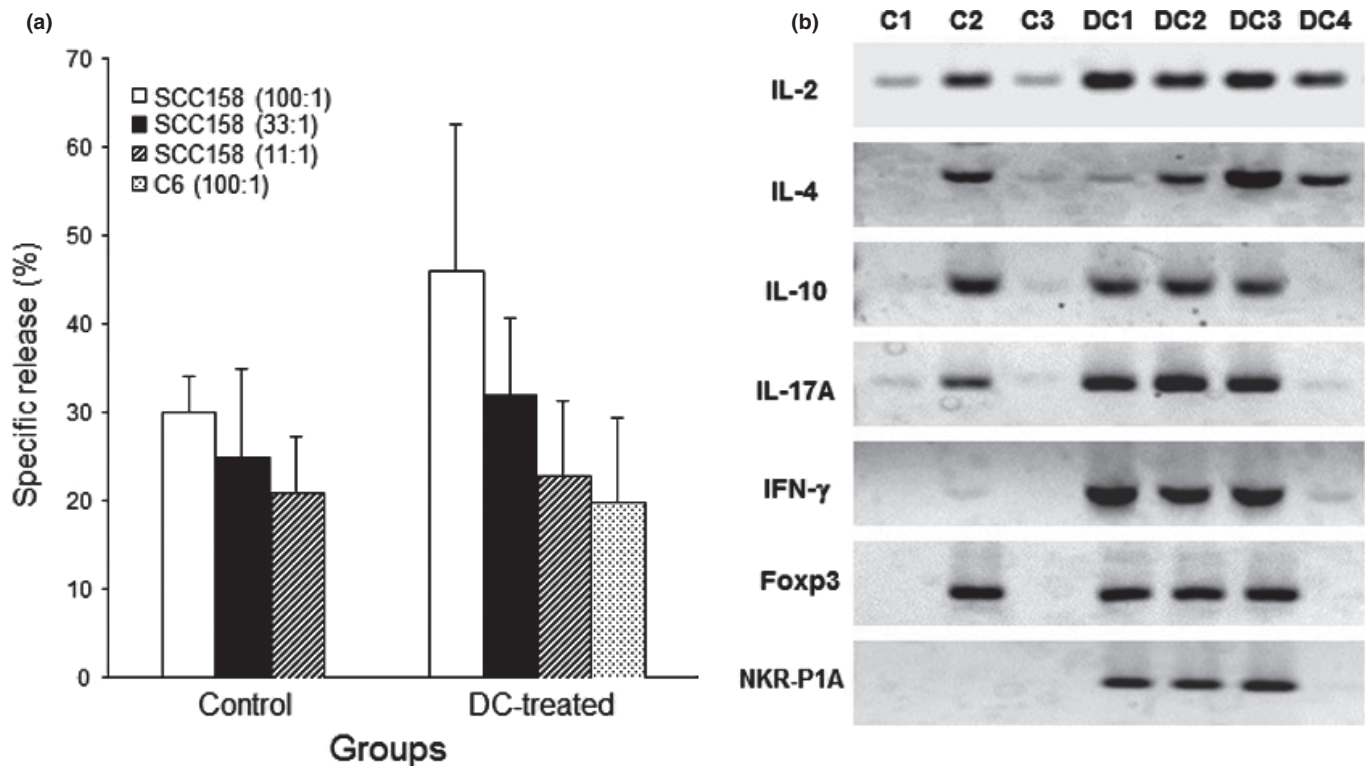


Fig. 4. Immunological monitoring using spleen cells from the skin flap experiments. (a) The induction of CTL activity against SCC-158 tumor cells using a non-radioactive cytotoxicity assay. The E/T ratio ranged from 100:1 to 11:1. Each column shows the mean value derived from four rats. (b) Rat cytokine expression analysis as measured by RT-PCR.

Bourdeaux's model and an immuno-flap should be working in our model.

Many studies of systemic DC vaccines against advanced head and neck cancers have been attempted, but have not been

successful, and no evidence in relation to survival benefit for cancer patients has been obtained.^(27,28) However, some clinical studies of DC-based local immunotherapy have been found to be effective.^(29,30) In those cases, mature DC are thought to

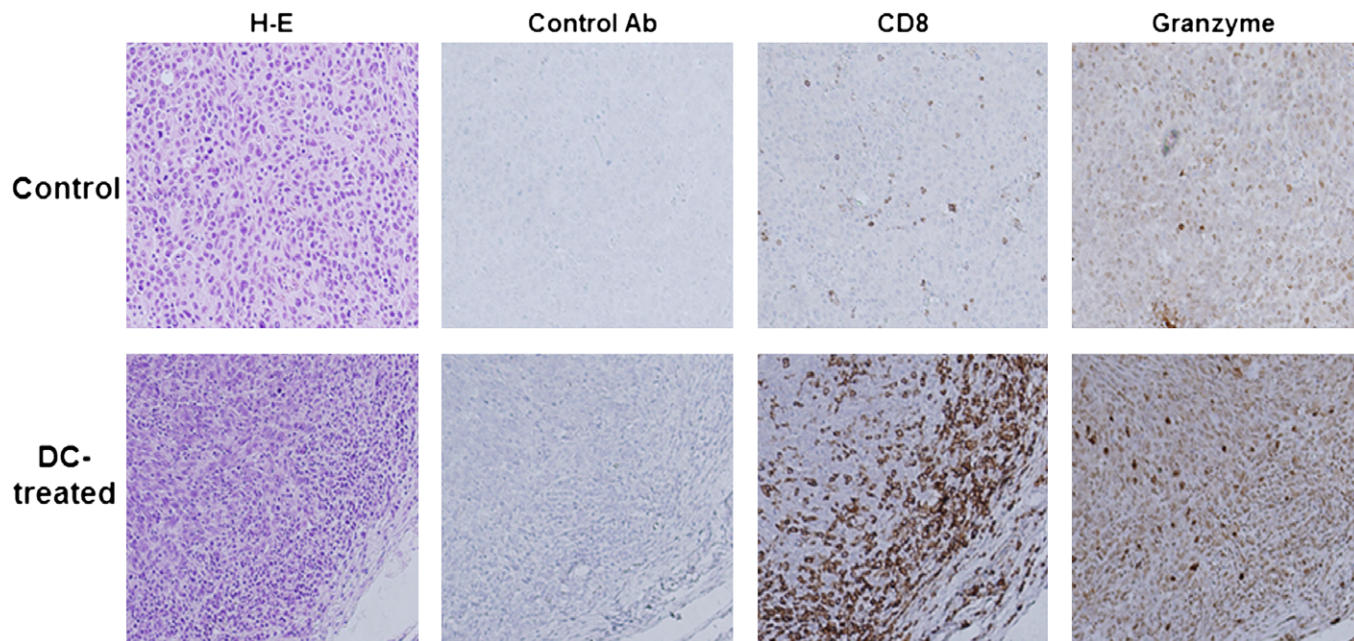


Fig. 5. Immunohistochemistry analysis of lymphocytes infiltrating the tumor site. A large number of CD8⁺ T cells were observed in the dendritic cell (DC)-treated group; however, no significant T cell infiltration was observed in the tumors of the control group. Indirect staining using anti-CD8, anti-granzyme B and anti-Foxp3 MoAbs as primary Ab and goat anti-mouse Ab as secondary Ab was performed. Magnification $\times 100$.

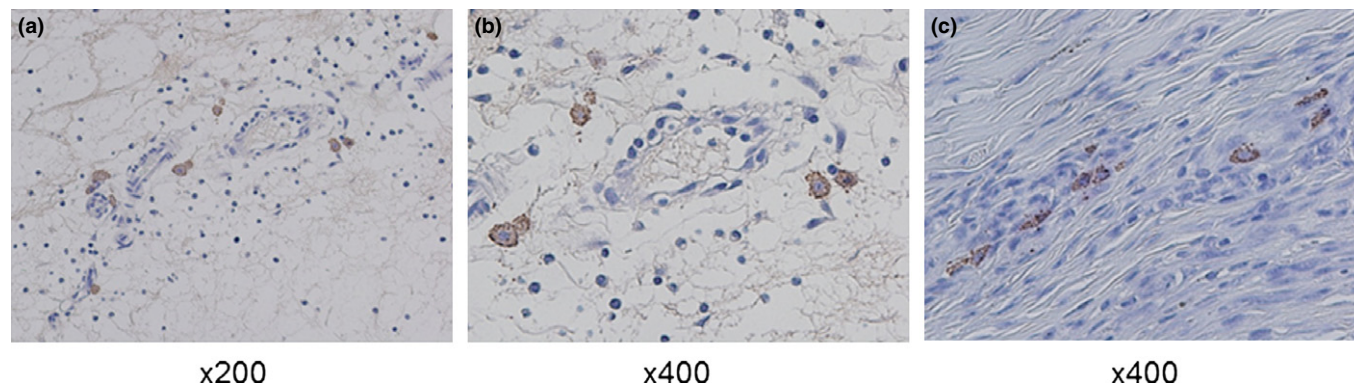


Fig. 6. Distribution of injected rat dendritic cells (DC) in subcutaneous tissue of the immuno-flap. For the DC tracking experiment, 24 h after DC were injected into the skin flap via artery, the graft was resected, and DC status was evaluated using immunohistochemistry analysis. Magnification $\times 200$ and $\times 400$.

migrate to regional lymph nodes and induce CTL activity, as preclinical studies demonstrate using efficient cell-labeling method such as indium or fluorescence.^(31,32) However, it has also been reported that the local injection of DC around the head and neck region can induce diverse and discrepant immunologic responses, either effective or anergic, depending on the injection site, such as the nasal submucosa or the oral floor.⁽³³⁾ The immuno-flap contains regional lymph nodes, and the flap tissue can stay attached to the resection margin after the transfer. Although further DC and CTL tracking experiments will be needed, the immuno-flap can induce a local immunological response via accumulated DC and CTL, particularly in the local region where tumor recurrence is most likely.

In the current study, we developed an *in vivo* model of new DC-based immunotherapy using a skin flap transfer. Based on the progress of the present study, it will be necessary to refine

the current therapeutic protocol of the model so that it can be applied clinically.

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Disclosure Statement

The authors have no conflict of interest to declare.

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