

Original Articles

Generation of Functional Monocyte-Derived Dendritic Cells from HTLV-I Infected Rabbits : Therapeutic Approach Using a Dendritic Cell Vaccine

Ziyad M Al-Dahoodi¹⁾, Shigeki Takemoto¹⁾, Hisanori Machida¹⁾, Masato Furuya²⁾,
Isao Miyoshi¹⁾ and Hirokuni Taguchi¹⁾

Dendritic cells (DC) are essential antigen-presenting cells (APC) with a unique capacity to initiate an immune response. We described the *in vitro* generation of rabbit monocyte-derived DC (Mo-DC) in the presence of granulocyte macrophage-colony stimulating factor and interleukin-4. Autologous DC, which had phagocytosed apoptotic Ra-1 cells, acted as an APC for cellular response following their injection into human T lymphotropic virus type I (HTLV-I)-infected rabbits. PKH-26 labeled DC preferentially homed to the T cell area of the draining lymph node following s.c. injection, whereas i.v.-injected DC accumulated in the spleen. We demonstrated that rabbit Mo-DC functioned as potent APC *in vivo* and that the route of DC administration was inconsequential for cytotoxic T lymphocytes response. Although Mo-DC generated from patients with adult T-cell leukemia/lymphoma (ATLL) show a functional impairment, this study provides evidence that a new, supporting immunotherapy using the donor's DC generates a more efficient anti-HTLV-I infected cell/ATLL cell immune response following allogeneic stem cell transplantation.

Key words dendritic cells, HTLV-I, vaccine, allo-SCT

INTRODUCTION

Dendritic cells (DC) are currently a focus of attention because of their central role in immune therapy. DC with antigen-presenting cell (APC) function need to be isolated, purified and propagated from peripheral blood using recombinant cytokines for study of them. This has been demonstrated in mice and humans *in vitro* by culturing peripheral blood mononuclear cells (PBMC) with granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4)¹⁻³. Pro-inflammatory cytokines, such as

IL-1 β , IL-6, and tumor necrosis factor α (TNF- α) are able to stimulate DC into more mature stages with a strong T cell stimulatory potential^{4,5}. After being stimulated by bacterial products, inflammatory cytokines or CD40 ligation, DC undergo a maturation process that results in enhanced antigen-presenting capacity. Major histocompatibility complex (MHC) and costimulatory molecules expressed in DC migrated to secondary lymphoid organs and primed naive T cells^{6,7}. One of the crucial functions of DC is to migrate specifically into T cell areas of secondary lymphoid organs. They have been shown to efficiently stimulate both primary and secondary CD4⁺ and CD8⁺ T cell immune responses⁸.

Apoptotic tumor cells are known to be a source of tumor antigen for DC⁹⁻¹¹. After DC phagocytose necrotic and apoptotic tumor cells, as well as virus infected cells, they became able to stimulate T cells and to induce the cytotoxic T lymphocyte (CTL) response¹². These properties

Received : Oct 15, 2003

Revised : Jan 6, 2004

Accepted : Jan 7, 2004

¹⁾ Department of Hematology and Respiratory Medicine

²⁾ Institute of Laboratory Animals, Kochi Medical School, Kochi, Japan

Address correspondence and reprint request to Shigeki Takemoto
Department of Hematology and Respiratory Medicine, Kochi Medical School, Kohasu, Oko-cho, Nankoku, Kochi 783-8505, Japan

of mature DC are thought to be ideal to generate a primary immune response against cancer, viral infection and other diseases.

Human T lymphotropic virus type I (HTLV-I) is the etiologic agent of adult T-cell leukemia/lymphoma (ATLL) and is able to infect and immortalize not only human, but also animal lymphocytes¹³⁻¹⁶. However, no animal model of ATLL has been successfully established because HTLV-I and Tax are insufficient for ATLL development, as we learned natural course of this disease in human¹⁷. HTLV-I infected rabbits were established and HTLV-I transmission and prevention were investigated previously in our department^{15,18}. We demonstrated that HTLV-I infected both Japanese monkeys and rabbits more effectively than rats or mice. Thereafter, rabbits were chosen for this study for economy, safety and laboratory space.

ATLL occurs in a very small percentage of HTLV-I carriers after a long latency¹⁹. The number of infected T cells is probably restricted by viral antibodies and CTL during asymptomatic stage of HTLV-I infection²⁰. Combination chemotherapy for ATLL could also achieve complete or partial remission for the most part of patients. However, it remains difficult to eliminate HTLV-I infected cells and/or ATLL cells from HTLV-I infected individuals. Recently, allogeneic stem cell transplantation (allo-SCT) improved the survival of ATLL patients and the graft versus leukemia/lymphoma cell effect played an important role in this therapy²¹.

In this study, we generated monocyte-derived DC (Mo-DC) from HTLV-I infected rabbits to investigate whether autologous DC from HTLV-I carriers activated immunity to anti-HTLV-I infected /ATLL cell after vaccination. We found that DC migrated to the T cell area of draining lymph node (LN) and contacted CD4⁺ T cells after injection. We also found that lymphocytes from immunized HTLV-I infected rabbits showed cytolytic activity against Ra-1 cells. Because not only T cells, but also DC from ATLL, are functionally impaired, as we recently have reported²², our findings suggest that Mo-DC from a donor might eliminate a recipient's residual ATLL cells after allo-SCT.

MATERIALS AND METHODS

HTLV-I infected rabbits

Ten non-inbred white Japanese rabbits weighing approximately 2.5 kg were studied. They were purchased from Kitayama Rabesu (Nagano, Japan) and maintained in specific, pathogen-free conditions in the Institute of Laboratory Animals of Kochi Medical School. The first rabbit (#1) was i.v. inoculated with mitomycin C-treated Ra-1 cells, HTLV-I-producing rabbit lymphoid cells²³. Subsequently, 7-10 ml of blood and PBMC from rabbit #1 was transfused into four other rabbits (#2-5), as described previously¹⁵. HTLV-I infection was confirmed by PCR (pX region)¹⁸ and detection of HTLV-I antibodies by enzyme-linked immunosorbent assay (ELISA) for IgG Abs against disrupted MT-2 cells (Eisai, Tokyo, Japan).

Generation of apoptotic Ra-1 cells

Apoptotic Ra-1 cells were prepared by treatment with 50 μ g/ml mitomycin C for 30 min at 37°C, then the cells were twice frozen at -80°C and thawed quickly at 37°C. Cell death was assessed using a trypan blue exclusion assay. For further confirmation, apoptotic cell death was assayed with a Vybrant™ Apoptosis assay kit #4 (Molecular Probes, Eugene, OR) according to the manufacturer's protocol.

Generation of DC and culture with apoptotic Ra-1 cells

Heparinized blood was collected and PBMC were isolated by density centrifugation using Ficoll-Hypaque. Cells (4×10^6 /ml) were suspended in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 μ g/ml streptomycin and 100 U/ml penicillin G, and incubated in 12-well culture plates (Costar, NY). Plastic adherent cells were obtained by culturing PBMC for 2 h. After washing out non-adherent cells with pre-warmed medium, the remaining adhered monocytes were cultured in the presence of 50 μ g/ml recombinant human (rh) GM-CSF and 20 μ g/ml rhIL-4 (Sigma). On day 3, non-adhered B and T cells were gently removed and fresh medium, containing rhGM-CSF and rhIL-4, was added and changed every 2 days. On day 7,

immature DC were co-cultured with apoptotic Ra-1 cells at a ratio of 1 : 3. After 16 h of incubation at 37°C in 5% CO₂ they were harvested, purified with Ficoll-Hypaque and washed twice with phosphate-buffered saline (PBS). DC were matured by adding TNF- α (20 ng/ml, Sigma) for 18 h and then evaluated by May-Giemsa (M-G) and Hematoxylin-Eosin (H-E) staining. Cell viability was examined by trypan blue exclusion assay.

Assay of phagocytosis

The phagocytic activity of DC was assessed according to the method previously described, with some modification²⁴. Briefly, Ra-1 cells were labeled with fluorescent dye PKH-26 (Sigma) according to the manufacturer's instructions. Cells were washed twice with PBS to remove FBS and re-suspended in PKH-26 staining solution for 5 min. RPMI 1640 medium containing 0.1% bovine serum albumin was added to stop the staining reaction, followed by extensive washing with the same medium, 4 times, to remove unbound dye. Loosely adhered immature DC were co-cultured with apoptotic labeled Ra-1 cells and harvested, as mentioned above. Flow cytometric analysis was performed to evaluate the phagocytosis of apoptotic labeled Ra-1 cells using FACSCalibur (Becton Dickinson, San Jose, CA).

Allogeneic and autologous mixed lymphocyte reaction (MLR)

Allogeneic and autologous MLR was conducted. Immature DC were pulsed with apoptotic Ra-1 cells and TNF- α . Then DC were harvested, treated by irradiation at 30 Gy (Gamma cell, Ontario, Canada) and used as stimulator cells. After 2 h culture in PBMC, lymphocytes were separated from adhered monocytes and used as responder cells. Stimulator cells were incubated in graded doses with 1×10^5 responder cells in 96-well, round-bottom tissue culture plates (Costar). Cultures were maintained in a humidified atmosphere at 37°C and 5% CO₂. Thymidine incorporation was measured after 4-day incubation by pulsing for 18 h with 1 μ Ci/well of ³H-thymidine. The results were expressed as the main difference in counts per minute obtained from triplicate cultures using a liquid scintilla-

tion counter.

Immunization and cytolytic assay

After DC were co-cultured with apoptotic Ra-1 cells and supported by addition of TNF- α , 1.5×10^6 DC/0.5 ml PBS were injected into HTLV-I infected rabbits twice with a one-week interval by both s.c. (two rabbits) and i.v. (two rabbits). One day before the first injection and one week after the second injection harvested, non-adhered lymphocytes were used as effector cells. Target Ra-1 cells were labeled with 50 μ l of ⁵¹Cr (ICN Biomedicals, Irvine, CA) for 30 min at 37°C and washed three times with RPMI 1640 medium. 1×10^4 target cells were cultured with effector cells at the various ratios in triplicate and incubated for 16 h. 100 μ l of supernatant was harvested per well and the amount of released ⁵¹Cr was counted using a Betaplate Scintillation Counter (Wallec). The percent specific cytolysis was calculated as $100 \times [(\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximal cpm} - \text{spontaneous cpm})]$. Spontaneous release was determined from wells containing target cells and medium alone. Maximal release was defined as controls from target cells incubated with 1% NP40.

Injection of PKH-26-labeled DC and fluorescent tissue labeling

DC were labeled with PKH-26 dye according to the manufacturer's protocol, as described above. Labeled DC were incubated with apoptotic Ra-1 cells at a 1 : 3 ratio. After 16 h incubation at 37°C, DC were harvested, purified with Ficoll-Hyperque and washed twice with PBS. Two normal rabbits and 4 HTLV-I infected rabbits were separated to two groups and autologous labeled DC (1.5×10^6) suspended in 0.5 ml PBS were injected s.c. at a site lateral to the proximal axillary LN or by i.v. in an ear vein, respectively. Two days after injection, all rabbits were sacrificed and the regional axillary LN and the spleen were examined. Frozen tissues were cryosectioned into 6 mm-thick sections, fixed in 100% acetone and stained with 1 : 20 PBS-diluted mouse anti-rabbit FITC-CD4 (Serotec Ltd., Oxford, UK) for 30 min at 37°C. Specimens were washed with PBS for 30 min and the solution was changed every 10 min. Slides were examined using a fluorescence microscope.

Non-lymphoid tissues were used as a negative control and lymphocytes were used as a positive control.

Fluorescence microscopy

Slides were examined directly using a Zeiss Axiophot microscope fitted with appropriate filters. Fluorescence was visualized using a cooled CCD camera (RS photometrics, Cool SNAP, Germany). Fluorescence signals were collected independently and stored as 8 bit RGB images. Data were analyzed and colors were assigned to each signal using Lumina Vision software (Fukui, Japan). Images were edited using Adobe Photoshop (Adobe Systems, Mountain View, CA).

RESULTS

HTLV-I infection of rabbits

Blood and PBMC from the first HTLV-I infected rabbit were transfused into 4 rabbits and HTLV-I infection was confirmed in 3 of them by ELISA and PCR (Table 1). Seroconversion occurred 4-10 weeks after blood transfusion and we finally established 4 HTLV-I infected rabbits (#1-4).

Generation of mature rabbit DC

The generation and maturation stages of DC were examined morphologically by light micros-

copy using M-G and H-E staining (Fig. 1). We found that immature DC were larger than monocyte progenitors following 6 day-stimulation of adhered monocytes with rhGM-CSF and rhIL-4, as shown by M-G staining (Fig. 1A). Immature DC co-cultured with apoptotic Ra-1 cells exhibited functional phagocytosis, as illustrated in Fig. 1B. Following treatment of cultures with TNF- α , the typical morphology of mature DC, with numerous cytoplasmic extensions (dendrites) and a large rounded nuclei, was found, as in Fig. 1C. Uptake efficiency of FITC-dextran by immature DC was compared with monocytes and a decrease by mature DC was observed (data not shown). The efficiency of DC priming shows that they are potent stimulators of primary MLR and induce proliferation of allogeneic lymphocytes *in vitro*. For confirmation, allogeneic MLR was performed and compared with the results of autologous MLR (Fig. 2). The stimulatory capacity of DC was shown in representative experiments, suggesting that functional mature DC were generated.

Lymphocytes activated by Ra-1-specific DC from HTLV-I infected rabbits

To examine the immune mechanism involved in the stimulation of immune response, a cytotoxicity assay was performed before and after DC immunization. One day before the first immunization and one week after the second immunization with Ra-1-specific autologous DC, PBMC

Table 1 Animal characteristics

Animal No.	Sex	Weight (kg)	HTLV-I inoculation	Seroconversion (Days)	PCR
1	Female	3.0	Ra-1(2.0x10 ⁷)	34	(+)
2	Female	2.5	Blood(7 ml)+ PBMC from 3ml of blood	38 (24)*	(+)
3	Female	2.5	Blood(10 ml)	38	(+)
4	Male	2.5	Blood(7 ml)+ Blood(10 ml)	69 (25)*	(+)
5	Male	2.5	Blood(7 ml) Blood(10 ml)	(-) (-)*	(-)
6	Male	3.0	(-)	(-)	(-)
7	Male	2.5	(-)	(-)	(-)
8	Female	2.5	(-)	(-)	(-)
9	Male	2.3	(-)	(-)	(-)
10	Female	2.3	(-)	(-)	(-)

(*) indicates seroconversion from 2nd inoculation.

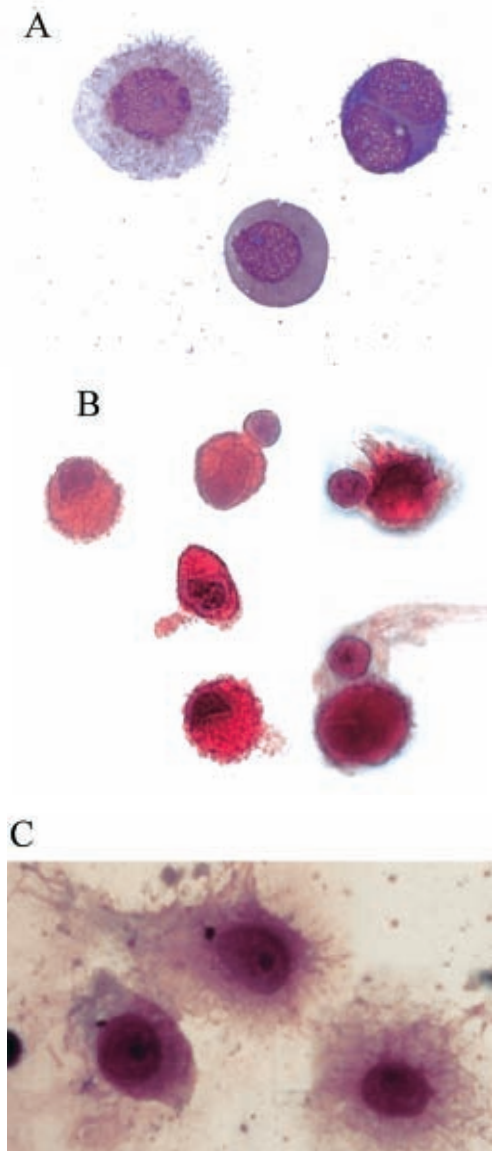


Fig. 1. Light microscopic examination of isolated Mo-DC in different maturational stages. (A) Immature DC after 6 d-culture of adhered monocytes treated with rhGM-CSF and rhIL-4, M-G stain; (B) DC phagocytosed apoptotic Ra-1 cells during co-culture, H-E stain; (C) DC co-culture with apoptotic Ra-1 cells followed by TNF- α showing a typical morphology with abundant veiled, dendritic protrusions, M-G stain (Magnification: A, $\times 750$; B, $\times 500$; C, $\times 1100$).

were recovered from HTLV-I infected rabbits. Non-adhered lymphocytes were used as effector cells and showed cytolytic activity against Ra-1 cells in all rabbits after vaccination s.c., as well as i.v. The efficiency of cytotoxicity was increased compared with lymphocytes recovered before

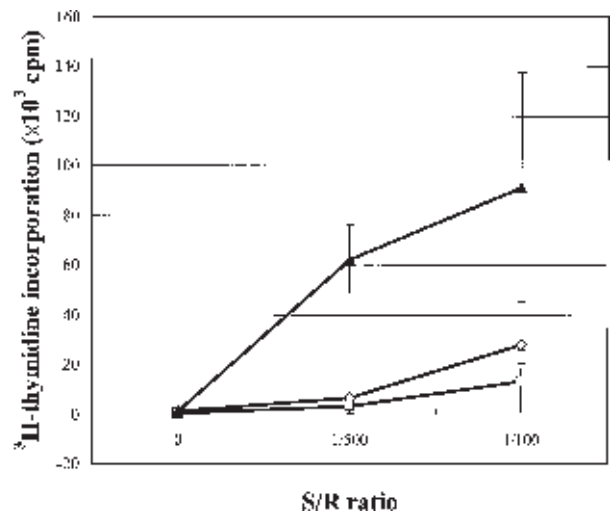


Fig. 2. Lymphocyte-stimulatory capacity of Mo-DC. DC and lymphocytes (Ly) from 4 normal rabbits were used as stimulator (S) and responder (R), respectively, and co-cultured in the indicated ratio (▲, DC of rabbit #7 and Ly of rabbit #8; ◇, autologous MLR of #7; □, autologous MLR of #8). Data shown represent mean values \pm SD of triplicate experiments.

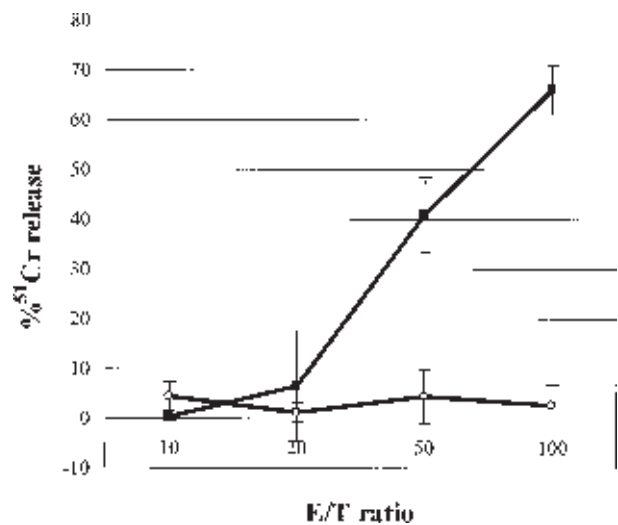


Fig. 3. Cytolytic analysis of Ra-1 cells. HTLV-I-infected rabbits were immunized with autologous Mo-DC-phagocytosed apoptotic Ra-1 cells. Two rabbits were injected s.c. or i.v., respectively. Non-adhered lymphocytes harvested one day before the first injection (○) and one week after the second injection (■) were used as effector cells (E) and Ra-1 cells were used as target cells (T) in a chromium release assay. Data represent mean values \pm SD of four rabbits. Triplicate experiments were performed.

immunization (Fig. 3). Our data indicated that injection of DC that had phagocytosed apoptotic Ra-1 cells induced efficient CTL activity.

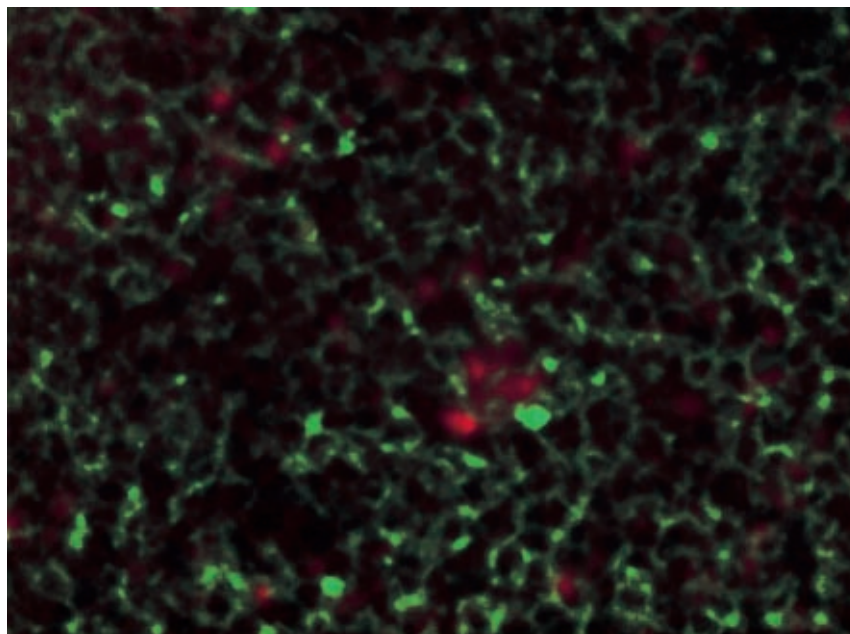
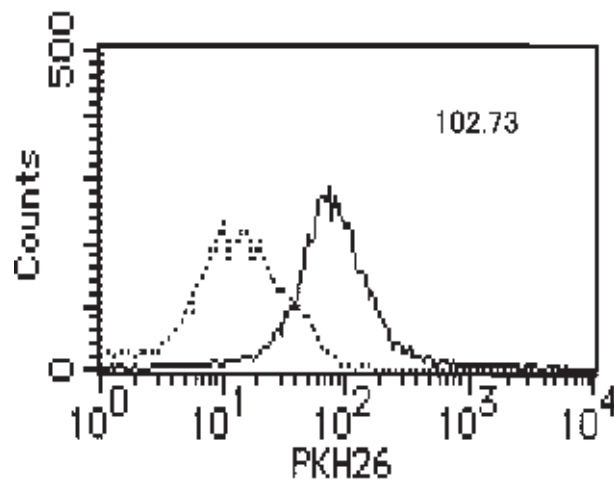


Fig. 4. Phagocytosis of apoptotic PKH-26-labeled Ra-1 cells and migration to draining LN by autologous Mo-DC. (A) Flow cytometric analysis of DC co-cultured with PKH-26-labeled Ra-1 cells (—) and unlabeled Ra-1 cells (---). The numbers show mean fluorescent intensity. (B) Cryosection of axillary LN after s.c. injection of DC. PKH-26-labeled DC (red) contacted CD4⁺ T cells (green) in the paracortical region. Slides were examined by fluorescence microscopy with appropriate filters.

Distribution of autologous PKH-26-labeled DC following s.c. and i.v. injection

To assess the interaction between DC and resident T cells in the secondary lymphoid tissues the location of DC was examined after 48 h of vaccination in HTLV-I infected rabbits s.c. or i.v. It was shown that the process of labeling DC did not affect their trafficking ability. We confirmed, by flow cytometry, that approximately

25% of apoptotic Ra-1 cells were phagocytosed after mixing DC with apoptotic PKH-26 labeled Ra-1 cells (Fig. 4A). PKH-26 labeled DC were identified in the T cell area of the paracortical region of axillary LN after s.c. injection (Fig. 4B). Furthermore, migrated DC were identified in the white pulp area of the spleen, but not in axillary LN, after i.v. injection in both normal and HTLV-I infected rabbits (data not shown). It was also confirmed that injected DC made contact

with CD4⁺ T cells in the paracortical region of axillary LN (Fig. 4B).

DISCUSSION

This is the first morphological and functional characterization of DC derived from PBMC of normal and HTLV-I infected rabbits. Because there are no cytokines and antibodies available for rabbit studies, we used human cytokines and so could not examine any DC-specific or DC-related antigens on the surface. However, allogeneic MLR demonstrated that mature DC as potential lymphocyte-stimulators were generated from rabbits' Mo. We also found that autologous DC that had phagocytosed apoptotic Ra-1 cells at a 1 : 3 ratio and matured with TNF- α were able to activate lymphocytes against Ra-1 *in vivo*. Up-regulation of co-stimulatory molecules and subsequent presentation with high expression of both MHC class I and II complexes may be responsible for this effect, as reported²⁵.

After 10 d from a second DC injection in HTLV-I infected rabbits, no changes were observed in the titer of HTLV-I antibody compared to that before the first injection (data not shown). This implies that no stimulation for humoral immunity occurred. DC loaded with soluble antigenic protein or ingested apoptotic tumor cells triggered effective anti-tumor cellular immune responses but not humoral immunity^{26,27}. DC loaded with killed allogeneic melanoma cells primed naive T cells to differentiate into CTL specific for a broad spectrum of shared tumor antigens²⁸. The capacity of DC to migrate to the T cell area of LN was a key event in initiating immunity⁸. Injected DC migrated to the T cell area *in vivo* (Fig. 4B) and induced a CTL response (Fig. 3). This suggests that both CD4⁺ and CD8⁺ T cells are needed to interact with the same APC to generate the CTL response, as previously reported²⁹. We observed that DC injected s.c. preferentially sought the T cell area of draining LN, whereas DC injected i.v. were detected in the spleen, as in some prior reports^{30,31}. The stability and the capacity of rabbit DC to migrate to secondary lymphoid tissues may determine the efficacy of DC-based vaccines.

We used an apoptotic, HTLV-I-infected cell line, but not tumor peptides, to activate DC because apoptotic bodies phagocytosed by DC

from a poorly immunogenic tumor cell line led to tumor regression and induced specific long-term protection¹⁰. Although tumor antigens specific for ATLL cells remain to be studied, reconstruction of the immune system is most important to achieve complete remission for the improved survival of ATLL patients. Because we recently found that Mo-DC from HTLV-I carriers were functionally impaired²², Mo-DC should be established from the PBMC of healthy donors, if possible, and might be suitable to eliminate minimal residual disease from recipients following allo-SCT²¹. When DC vaccine and allo-SCT are combined, they may induce strong immune responses against ATLL progression and provide a new approach to improve clinical outcomes.

ACKNOWLEDGEMENTS

We wish to thank Sayo Kataoka, Ken-ichi Yagyu and Hiroyoshi Takatsuji for their technical assistance. This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan (#12218104).

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