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Immortalized Dendritic Cell Line with Efficient Cross-Priming Ability Established from Transgenic Mice Harboring the Temperature-Sensitive SV40 Large T-Antigen Gene

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The uptake of an antigen and its presentation to specific T cells by dendritic cells (DCs) is a primary event in initiation of humoral and cellular immune responses as well as the induction of cytotoxic T cells (CTLs). DCs are induced by culturing bone marrow cells in the presence of GM-CSF. However, the resulting DCs are short-lived and the culture usually contains CD11c-negative non-DC cells, which adversely affects reproducibility and makes interpretation of the experimental results difficult. Therefore, it would be useful if DCs could be readily immortalized with their functions being retained. In this study we established a novel, immortalized murine DC line with antigen-presenting capacity *in vitro* as well as an augmenting effect on humoral and cellular immune responses *in vivo*, utilizing bone marrow cells from transgenic mice harboring the temperature-sensitive SV40 large T-antigen gene. In the presence of GM-CSF, the resulting DC line, termed SVDC, could be continuously subcultured for more than 12 months. When pulsed with OVA alone or OVA-IgG immune complexes *via* Fcγ receptors, SVDC augmented OVA-specific T cell proliferation efficiently *in vitro*, and elicited OVA-specific IgG production *in vivo* on the adoptive transfer of pulsed SVDC into naive mice. Interestingly, SVDC exhibited significantly high cross-priming ability compared to DCs in a short-term culture, thus leading to their extremely high effectiveness in inducing anti-tumor immunity *in vivo*. Thus, SVDC is useful for the detailed characterization of antigen presentation, and for research on the various therapeutic benefits of DC vaccination to elicit specific immune responses in immunodeficiencies, infectious diseases and cancer.

Key words: antigen presentation, dendritic cells, Fc receptor, immortalized cell, vaccination.

Abbreviations: APC, antigen-presenting cell; B6, C57BL/6; BMDC, short-term cultured dendritic cells derived from bone marrow cells of C57BL/6 mice; DC, dendritic cell; IC, IgG-immune complex; SVDC, temperature-sensitive SV40 large T antigen-transduced dendritic cell line; tsSV40LT, temperature-sensitive mutant of SV40 large T transgenic.

The uptake and processing of exogenous antigens by antigen-presenting cells such as dendritic cells (DCs) is the first critical step in the chain of events leading to the activation of T and B cells, which evoke humoral and cellular immune responses (*1*, *2*). The antigens are concentrated by DCs through both high-rate fluid phase pinocytosis and receptor-mediated uptake *via* receptors for heat shock proteins (*3*, *4*), mannose receptors (*5*), and Fc receptors (*6–8*). Exogenous antigens are presented on MHC class II molecules on DCs, while intracellular components are presented on MHC class I molecules. Exogenous antigens are sometimes presented on MHC class I and induce $CD8^+$ T cells, a process called cross presentation, which is believed to be important for eliminating viruses that do not infect DCs. To elucidate the molecular mechanisms underlying these antigen presentation and cross presentation pathways in DCs, it is necessary to establish stable DC lines with normal *in vitro* and *in vivo* functions because primary culture DCs as well as those derived from bone marrow are short-lived, and their phenotypes are not stable.

In this study we established and characterized an immortalized DC line with normal functions, namely, antigen processing and antigen presentation *in vitro*, and induction of humoral and cellular immune responses *in vivo*. We utilized a temperature-sensitive mutant of SV40 large T-transgenic (tsSV40LT) mice as the source of bone marrow–derived DCs. tsSV40LT mice have several advantages for the establishment of immortalized cell lines. These transgenic mice show the stable and detectable expression of the SV40 large T gene in all tissues, and cultured cells can be readily immortalized by activating the SV40 large T gene at 33°C, a permissive temperature (*9*). The immortalized cells proliferate at 33°C because

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large T-antigen binds to the tumor suppressor gene product, p53 protein, and/or the retinoblastoma gene product, Rb, which normally regulate cell proliferation (*9*). At temperatures over 37°C, which are non-permissive, large T antigen becomes unstable and is degraded, which stops the growth of the cells. Several cell lines, such as hepatocytes (*10*), smooth muscle cells (*11*), and gastric surface mucosal cells (*12*), have been established from tsSV40LT mice, and these cell lines exhibit specific differentiated phenotypes. However, lymphoid or myeloid cell lines with specialized functions have not yet been established from tsSV40LT mice.

We successfully established an immortalized myeloid DC line from this transgenic strain. The established DC line continuously proliferated in response to GM-CSF and actively incorporated a soluble antigen, ovalbumin (OVA), at 33°C. The antigen could be efficiently presented to OVA-specific T cells *in vitro* at 37°C. Upon adoptive transfer into naive mice, these cells gave rise to induced anti-OVA IgG responses as well as antigen-specific cytotoxic T cells. Interestingly, the DC line exhibited significantly high cross-priming ability as to exogenous antigens and was found to be effective in inducing anti-tumor immunity *in vivo*. In conclusion, the large T antigen-transduced DC line, now termed SVDC, had essential functions characteristic of DCs that lead to the activation of $CD4^+$ T cells as well as to significant cross-priming of $CD8^+$ T cells.

MATERIALS AND METHODS

Animals—C57BL/6 (B6) mice were purchased from Charles River Japan Inc. (Kanagawa, Japan). The founder mice for the tsSV40LT mice (*9*) were a kind gift from Dr. K. Ishibashi (Daiichi Parmaceutical Co., Tokyo, Japan). Mice were housed and bred in the Animal Facility of The Institute of Development, Aging and Cancer (IDAC, Tohoku University, Sendai, Japan), an environmentally controlled and specific pathogen-free facility, according to guidelines for experimental animals defined by the facility, and animal protocols were reviewed and approved by the IDAC Animal Studies Committee. All the experiments were performed on 6- to 8-wk-old female mice.

Antibodies and Flow Cytometry—Rabbit anti-OVA IgG was obtained from BioDesign International (Saco, ME). mAb to $OVA_{257–264}$ peptide SIINFEKL in conjunction with $H\text{-}2K^b$ (25-D1.16) (*13*) was a kind gift from Dr. Ronald N. Germain (NIH). Rat anti-mouse FcγRIIB/III (2.4G2) (*14*) mAb was purified from the ascites of hybridomas by ion exchange chromatography on DEAE-cellulose (Merck, Darmstadt, Germany) (*15*), and by affinity isolation on a protein G column (*16*). For immunostaining, we used the following mAbs: FITC-, PE-, or biotin-conjugated anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD16/32 (2.4G2), anti-CD45 (30-F11), anti-CD3 (145-2C11), anti-B220 (RA3-6B2), anti-NK1.1 (PK136), anti-$I\text{-}A^b$ (M5/114.15.2), anti-B7.2 (GL1), anti-CD40 (3/23), anti-GL7 (Ly-77), and anti-CD4 (RM4-5) (PharMingen, San Diego, CA). TR-conjugated streptavidin (Caltag, Burlingame, CA) was used for staining for biotin-Ab. Cell surface staining was carried out according to the standard technique, and flow cytometric analysis was performed with a FACSCalibur using CellQuest software (BD, San Jose, CA).

Generation of an OVA-Specific Helper T Cell Line—An OVA-specific helper T cell line was established by the procedure described previously (*17*) with modifications (*18*). Briefly, female B6 mice were immunized with 100 μg of OVA (Sigma Chemical Co., St. Louis, MO, USA) in complete Freund's adjuvant (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Ten days later, the lymph nodes were removed, and single cell suspensions were enriched as to T cells using nylon wool columns. The cells were then cultured with 1×10^6 syngeneic spleen cells, mitotically inactivated with 25 μg/ml of mitomycin C (Kyowa Hakko Kogyo Co., Tokyo, Japan), and 50 μg/ml OVA in 24-well plates (Greiner Labotechnic, Co., Ltd., Tokyo, Japan) at 4×10^6 cells/well in 1 ml of RPMI-1640 supplemented with 10% heat-inactivated FBS, 10 μM 2-ME, 0.1 U/ml penicillin, and 0.1 μg/ml streptomycin. On day 4, 0.8 ml of the medium was removed and replaced with fresh medium. After 7 days, the cells were harvested and recultured with 5×10^6 mitotically inactivated splenocytes and 50 μg/ml OVA in 24-well plates at 2×10^5 cells/well in 1.5 ml of medium. OVA-reactive T cells were maintained by means of this 4-d antigen re-stimulation followed by a 7-d resting culture, and they have now been cultured for a long period of time (> 12 mo; > 98% $CD4^+$ cells by flow cytometric analysis).

Preparation of DCs from Bone Marrow Cells of B6 or tsSV40LT Mice—Bone marrow–derived DCs (BMDC) were prepared as previously described (*19*) with some modifications (*20*). Bone marrow cells from B6 or tsSV40LT mice were depleted of red blood cells using 0.144 M ammonium chloride, and also depleted of lymphocytes and Ia-positive cells using rabbit complement (Cedarlane Inc., Ontario, Canada) and anti-CD4, anti-CD8, anti-$I\text{-}A^b$, and mouse anti-rat Ig mAbs (TIB207, 211, 154, and 216, respectively; American Type Culture Collection, Rockville, MD). The cells were plated at 1×10^6 cells/well in 24-well plates in 1 ml of RPMI supplemented with 5% FBS, 10 μM 2-ME, 0.1 U/ml penicillin, 0.1 μg/ml streptomycin, 20 ng/ml murine rGM-CSF (Peprotech, Inc., Rocky Hill, NJ, USA), and 5 mM HEPES, and then incubated at 37°C (for cells from B6 mice) or 33°C (for cells from tsSV40LT mice) in a humidified incubator containing 5% CO_2. On day 4, most of the medium was removed and replaced with fresh medium. On day 6, in some experiments for maturation analysis, aggregated cells from B6 mice were gently collected and applied to 6-ml columns of 50% FBS-RPMI and then left on ice for 30 min to enrich immature DCs; about 80% of the cells at the bottom of the tube were $CD11c^+$ DCs. Otherwise, BMDC from B6 mice were used at this day 6 stage. For the long-term culture of tsSV40LT DCs, the medium was replaced every 4–5 d, the cells were subcultured every 3 weeks at 5×10^5 cells/ml/well, and this medium replacement and subculture were continued for over 12 mo. The resultant SVDC was used for experiments. The proliferation of SVDC was measured by MTT assay at 7.5×10^3 cells/100 μl/well in 96-well culture plates using methylthiazoletetrazolium (Sigma Chemical Co.) according to the manufacturer's instructions.

Antigen Uptake and DC Maturation—To measure the maturation of DCs, most of the medium was removed

from the Day 6 culture of BMDC or the long-term culture of SVDC, and replaced with fresh medium containing 10 μg/ml OVA. After 2 d of antigen pulsing for both BMDC (at 37°C) and SVDC (at 33°C), or after 1 d of 2 μg/ml LPS stimulation, non-adherent mature DCs were collected and stained with CD11c, CD86, CD40 and I-A^b, and then analyzed by flow cytometry. IL-12p70 production by SVDC or BMDC was measured by ELISA 1 d after LPS stimulation. For antigen uptake analysis of DCs, the cells were incubated with 10 μg/ml FITC-conjugated OVA (Molecular Probes, Eugene, OR) for 2 d, washed, and then stained with anti-CD11c and assessed by flow cytometry.

Antigen Presentation Assay In Vitro—OVA-specific $CD4^+$ T cells were cultured at 1×10^5 cells/well in 96-well flat-bottomed culture plates (Greiner) with 5×10^3 BMDC or SVDC, which had been exposed to 15 Gy X-ray irradiation, in the presence of graded doses of OVA or OVA-IgG imune complexes (ICs). OVA-containing ICs were prepared by incubation at 37°C for 1 h of OVA and rabbit anti-OVA IgG (BioDesign International) under antibody excess conditions so as to prevent the formation of a precipitate. After 24-h culture, supernatants were harvested and subjected to cytokine measurement. The IL-4 secretion by OVA-specific T cells was measured by ELISA according to the manufacturer's instructions (BD PharMingen). For T cell proliferation, the culture was performed for 66 h, [methyl-^{3}H]thymidine (Amersham Pharmacia Biotech, Co., Tokyo, Japan) being added to the cells at 20 kBq/well for the final 18 h of the culture. The cells were then harvested on glass fiber filters (Hewlett-Packard, Co., Tokyo, Japan), using a cell harvester (Hewlett-Packard), and the incorporation of [^{3}H]thymidine was measured using a gas scintillation counter (Hewlett-Packard).

Adoptive Transfer with Antigen-Pulsed DCs—DCs were cultured with 10 μg/ml OVA-ICs or OVA. The antigen-pulsed DCs were washed three times in PBS and then administered to naive B6 mice at the dose of 1×10^6 cells/mouse in 200 μl PBS intravenously through a tail vein.

Assay for Detection of Serum Anti-OVA Antibodies—Serum Ab titers were measured by the modified ELISA described previously (*18*). Briefly, a 96-well microplate (Falcon) was coated with 50 μl/well of a 50 μg/ml solution of OVA in PBS at 4°C overnight, washed three times with PBS containing 0.02% Tween 20 and 0.05% BSA, and then blocked with 200 μl/well of PBS containing 0.5% BSA at 4°C overnight. The diluted serum (1:100 to 1:500) was added at 50 μl/well and allowed to react at 4°C overnight. The wells were washed three times with PBS containing 0.02% Tween 20 and 0.05% BSA, incubated with 50 μl of a 1/1,000 dilution of goat anti-mouse IgG1, IgG2a, or IgG2b coupled to horseradish peroxidase (Medical and Biological Laboratories, Co., Ltd., Nagoya, Japan) at room temperature for 2 h, washed three times with PBS containing 0.02% Tween 20 and 0.05% BSA, and then developed at room temperature for 30 min with 50 μl of TrueBlue Peroxidase Substrate (Kirkegaard & Perry Lab., Gaithersburg, MD). The $OD_{450\ nm}$ was read with a Biolumin 960 Microplate Reader (Molecular Dynamics Japan Inc., Tokyo, Japan).

Immunohistochemistry—Frozen spleen sections (5 μm) from immunized mice were prepared, fixed in 10% (vol/vol) neutral buffered formalin at 4°C for 30–60 min, washed six times with PBS containing 0.05% Tween 20, and then blocked with PBS containing 3% skim milk at room temperature for 30 min. The sections were then incubated at 4°C with FITC-conjugated GL7 overnight. After washing six times in PBS and once in distilled water, the sections were mounted on slides and then examined under a fluorescent microscope (Olympus Optical Co., Ltd., Tokyo, Japan) for GL7-positive germinal centers.

Cytotoxicity Assays—Groups of three mice were injected intravenously through a tail vein with DCs (1×10^6 in 200 μl PBS/mouse) loaded with OVA or OVA-ICs. After 1 wk, the mice were sacrificed and T-enriched cells were prepared from their spleens. Splenocytes were incubated *in vitro* for 5 d with irradiated (100 Gy) E.G7 cells at an effector-stimulator ratio of 10:1. After restimulation, the target E.G7 cells were labeled with 100 μCi/ml $Na_2{}^{51}CrO_4$ for 1 h and then incubated with the stimulated splenocytes for 4 h at 37°C. Cytotoxic T cell (CTL) activity was measured using an auto well γ system (Aloka, Tokyo, Japan).

In Vivo Anti-Tumor Protection Effect—Groups of seven–eight mice were injected intravenously into a tail vein with OVA-pulsed DCs (5×10^5/200 μl PBS/mouse) or PBS alone, and the same immunization was repeated once more 1 wk later. Seven days after the second vaccination, the mice were injected subcutaneously with 1×10^5 E.G7 cells in 100 μl PBS into the left rear leg. A tumor of a diameter smaller than 5 mm was regarded as tumor-free, with monitoring for 75 d.

RESULTS

Proliferation and Morphology of SVDC—An immortalized DC line, termed SVDC, was obtained from bone marrow cells of tsSV40LT mice with a B6 background. We were able to maintain SVDC even after multiple passages for 12 mo in the presence of GM-CSF at 33°C. On the other hand, short-term–cultured DCs derived from bone marrow cells of B6 mice (BMDC) allowed subculture for a few times, but the cell number progressively decreased (data not shown), indicating that BMDC show limited growth potential after a few passages. Flow cytometric analysis indicated that SVDC are positive for a hematopoietic marker, CD45, and also positive for DC markers, CD11c, CD11b, and CD16/32, while they were negative for lymphocyte markers, CD3, B220, and NK1.1, showing that they are DC lineage cells (Fig. 1A). Morphological examination by means of Wright-Giemsa staining of SVDC revealed they were slightly larger than BMDC (Fig. 1B). We examined the proliferation ability of SVDC at 33°C (a permissive temperature), 37°C (a semi-permissive temperature), and 39°C (a non-permissive temperature) (Fig. 1C). SVDC proliferated at 33°C, as judged on MTT assay (Fig. 1C, closed circles). As expected, at 37°C they stopped proliferation and became prone to death (Fig. 1C, open circles) because they were vulnerable to further passages (data not shown). SVDC were found to start dying at 39°C because MTT absorbance was significantly decreased (Fig. 1C, closed triangles). As shown in Fig. 1D, SVDC required GM-CSF for their growth. The optimum concentration for maintaining SVDC was 20 ng/ml, a concentration used to produce

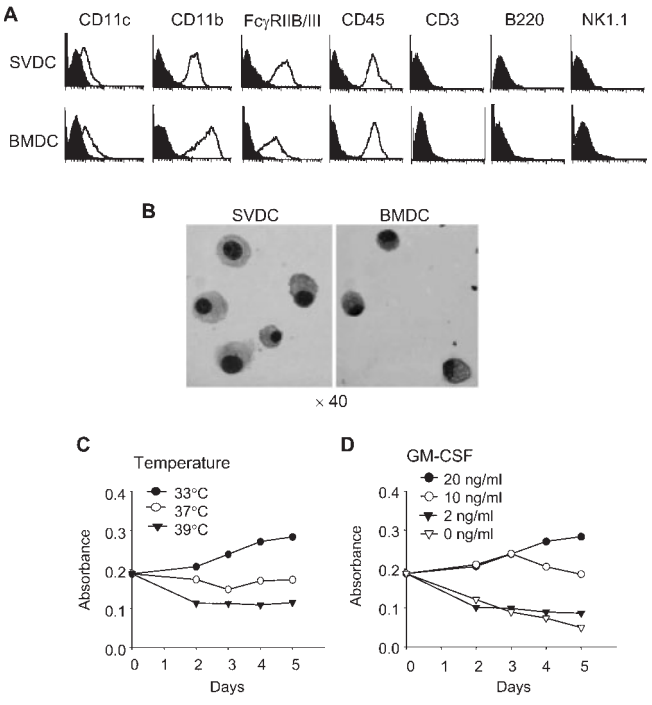

Fig. 1. **Surface markers, morphology, and growth characteristics of SVDC.** (A) Flow cytometric analysis of CD11c, CD11b, CD16/32 (2.4G2; FcγRIIB/III), CD45, CD3, B220, and NK1.1 expression on SVDC or BMDC. The open histograms show the binding of specific antibodies, whereas that of isotype-matched control antibodies or secondary reagents is represented by the closed histograms. The results are representative of at least three independent experiments. (B) Light microscopic morphology of SVDC or BMDC after Wright-Giemsa staining. Original magnification, ×40. (C) Temperature-dependent growth of SVDC. (D) GM-CSF-dependent growth of SVDC. Growth was monitored by means of MTT assay.

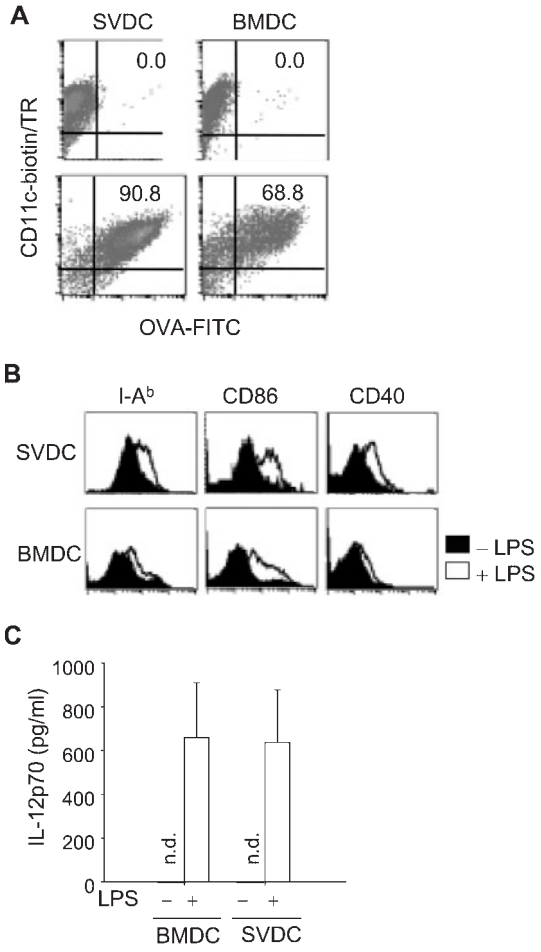

Fig. 2. **Incorporation of soluble antigens into or LPS stimulation of SVDC, and their subsequent maturation.** (A) SVDC efficiently engulfed OVA. SVDC or BMDC were co-cultured with 10 μg/ml FITC-conjugated OVA for 2 d at 33°C (for SVDC) or 37°C (for BMDC), and then washed with EDTA for 10 min to eliminate non-specific binding of OVA-FITC to the cell surface. SVDC and BMDC incubated with OVA-FITC were labeled with anti-CD11c and then analyzed by flow cytometry. DCs internalizing antigen were defined as CD11c and OVA double-positive cells. The upper panels are the negative controls for which no OVA-FITC was added. (B) Maturation of SVDC and BMDC after LPS stimulation. Cells were incubated at 33°C (SVDC) or 37°C (BMDC) in the presence of LPS (2 μg/ml) for 1 d. The cells were then stained with anti-I-A^b, anti-CD86, or anti-CD40 after gating anti-CD11c positive cells, and analyzed by flow cytometry. The results are representative of at least three independent experiments. (C) IL-12 p70 production by SVDC or BMDC after LPS stimulation. IL-12 p70 was measured by ELISA as described under "MATERIALS AND METHODS". n.d., not detected.

DCs from bone marrow cells. In the absence of GM-CSF, they did not show any growth (Fig. 1D).

Antigen and LPS Stimulation of SVDC, and Their Maturation—We next examined whether SVDC can take up OVA *via* fluid-phase pinocytosis. We added FITC-labeled OVA to an SVDC culture at 10 μg/ml, followed by culture at 33°C for 2 d and then flow cytometry. Interestingly, SVDC engulfed OVA-FITC more efficiently than BMDC cultured at 37°C for 2 d in the presence of 10 μg/ml OVA-FITC (Fig. 2A). We compared the antigen uptake capacities of SVDC and BMDC at the optimum temperatures for their growth, namely 33°C and 37°C for SVDC and BMDC, respectively, because the antigen uptake should require active metabolism in the cells. We confirmed by a confocal microscopy that the vast majority of the observed FITC signals were co-localized with the lysosomal marker LAMP1, indicating that OVA-FITC are incorporated into lysosomes, and not adsorbed on the cell surface (*21*). In addition, we verified that this enhanced OVA-FITC incorporation by SVDC was not due to the lower incubation temperature than that for BMDC because incorporation was not enhanced when BMDC were incubated with OVA-FITC at 33°C (data not shown).

We then investigated whether antigen uptake or LPS stimulation could induce SVDC maturation. In the absence of OVA or LPS stimulation, the expression levels of a MHC class II molecule (I-A^b) and co-stimulatory molecule CD86 on SVDC were relatively higher than those on BMDC (Fig. 2B, closed histograms). After OVA loading of BMDC or SVDC for 2 d as described above, the expression of I-Ab, CD86, and CD40 was slightly up-regulated in both BMDC and SVDC, although SVDC showed lower up-regulation of these maturation markers (1.5–1.8 fold, estimated by comparing peak values) than BMDC (2.0–2.6 fold) (not depicted in the figure). On the other hand, LPS stimulation (2 μg/ml) of SVDC at 33°C for one day successfully induced significant up-regulation of the mat-

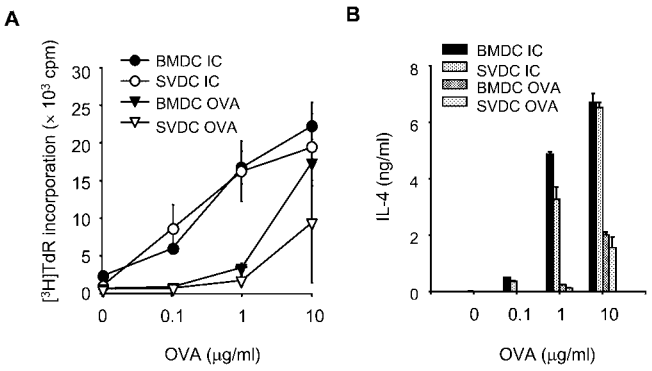

Fig. 3. **The antigen presentation of OVA and OVA-ICs by BMDC and SVDC.** (A) T cell proliferation. SVDC or BMDC (5×10^3) were cultured at 37°C with the OVA-specific T cell line (1×10^5) and various concentrations of OVA or OVA-ICs (triangles and circles, respectively), which were obtained by incubating OVA and OVA-specific IgG at a weight ratio of 1:10. [^{3}H]thymidine incorporation was measured after 66 h. Data are shown as the means for triplicate samples ± SD. The results are representative of at least three independent experiments. (B) IL-4 production by the OVA-specific CD4$^+$ T cell line co-cultured with SVDC or BMDC pulsed with various amounts of OVA. The IL-4 content in the culture supernatant of the above antigen-presentation culture was determined by ELISA. SVDC and BMDC pulsed with OVA alone induced little production of IL-4, while those pulsed with OVA-ICs efficiently induced IL-4 production by antigen-specific T cells. Data are shown as the means of triplicate determinations ± SD.

uration markers (3.0–3.6 fold, Fig. 2B, open histograms in upper panels), which is higher than that of BMDC (1.7–3.7 fold, Fig. 2B, open histograms in lower panels), and the production of IL-12 (Fig. 2C), which is comparable to that of BMDC.

Antigen Presentation of OVA Epitopes on SVDC to CD4$^+$ T Cells In Vitro—We then examined the efficiencies of antigen presentation of OVA or OVA-ICs on SVDC to OVA-specific CD4$^+$ T cells (Fig. 3A). When pulsed with OVA alone, presentation of OVA epitopes by SVDC to specific T helper cells was observed at a relatively high (10 μg/ml) OVA concentration, and the efficiency was slightly lower than in the case of BMDC, indicating that antigen presentation after uptake of OVA alone *via* the fluid phase is inefficient even in SVDC, which can engulf OVA efficiently, as shown in Fig. 2A. In contrast, the antigen presentation efficiency of SVDC as well as that of BMDC was significantly enhanced when OVA was complexed with IgG (Fig. 3A), demonstrating the very efficient antigen presentation after OVA-IC uptake *via* Fcγ receptors, as we previously demonstrated on DCs (*20*). The enhancement of the efficiency was roughly estimated to be 100-fold for SVDC (Fig. 3A).

Antigen presentation efficiencies *in vitro* have often been estimated as the cytokine production of activated T cells (*22*). To confirm our results, we measured IL-4 production by CD4$^+$ T cells stimulated by BMDC or SVDC that had taken up OVA or OVA-ICs, instead of measuring T cell proliferation, and compared it with that in the case of BMDC. The results were essentially the same as those of the T cell proliferation assay (Fig. 3B). SVDC were able to activate and induce the IL-4 release by OVA-specific CD4$^+$ T cells very efficiently when incorporated as ICs.

Adoptive Transfer of SVDC Pulsed with ICs Efficiently Induces Humoral Responses in Naive Mice—The adoptive transfer of antigen-pulsed DCs into naive mice can induce the humoral immune responses, and the efficiency is greatly enhanced by loading DCs with ICs (*20*). Although SVDC cannot proliferate well at the semi-permissive temperature of 37°C, we tried to see the effect of adoptive transfer of antigen- or IC-loaded SVDC. As shown in Fig. 4A, the adoptive transfer of OVA-pulsed SVDC induced a weak IgG2a immune response, but no significant IgG1 or IgG2b response. The BMDC loaded with OVA alone induced little responses. However, upon OVA-IC loading, SVDC successfully elicited OVA-specific IgG1 and IgG2a responses comparable to those in the case of BMDC (Fig. 4A).

B cell activation and antibody production occur in the germinal center. We detected splenic germinal centers positive for GL-7, an activation marker for germinal center B cells (*23*), in naive mice transferred with OVA-IC–loaded SVDC as well as those transferred with BMDC (Fig. 4B), suggesting that Fcγ receptor-mediated

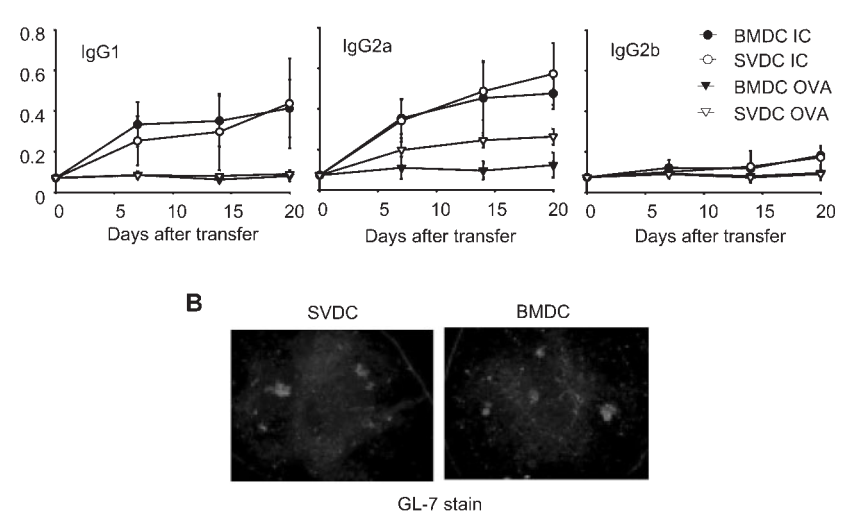

Fig. 4. ***In vivo*** **anti-OVA humoral response and germinal center formation upon transfer with antigen-pulsed SVDC.** (A) Anti-OVA antibody response in naive B6 mice transferred with OVA- (triangles) or OVA-IC-pulsed (circles) BMDC (closed symbols) or SVDC (open symbols). BMDC and SVDC were cultured for 2 d with 10 μg/ml of OVA or OVA-ICs and collected, and then 1×10^6 of the antigen-pulsed cells were intravenously administered to groups of three to six B6 mice. Serum taken from the mice on the indicated days was diluted (1/200), and then examined for OVA-specific IgG1 (left), IgG2a (middle), or IgG2b (right) by ELISA. The results are representative of two independent experiments with similar results. The data are shown as the means of triplicate determinations ± SD. (B) Formation of the germinal center in the spleens of naive B6 mice transferred with SVDC or BMDC pulsed with OVA-ICs. The formation of germinal centers was evaluated by GL7 staining.

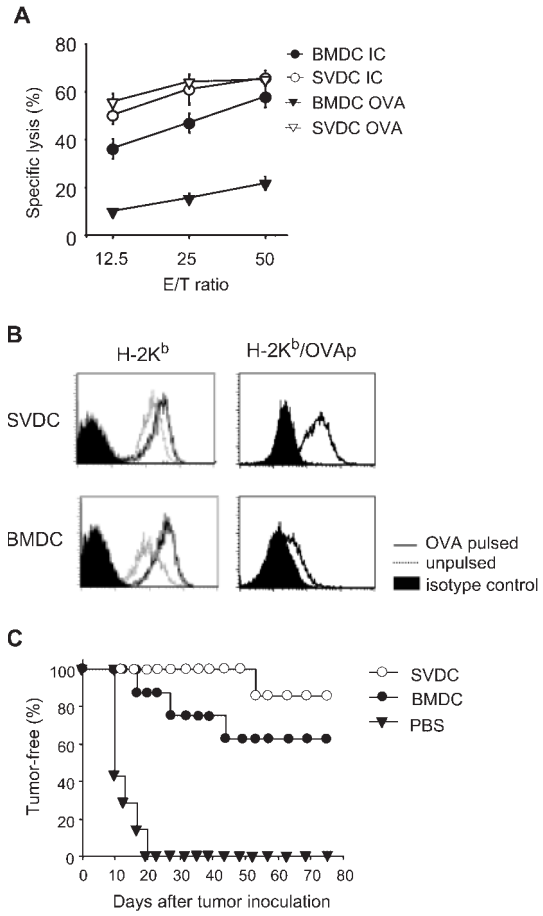

Fig. 5. **Induction of CTL and anti-tumor immunity upon transfer with antigen-pulsed SVDC.** (A) CTL induction by OVA- or OVA-IC–pulsed SVDC and BMDC. Mice (n = 3/group) were immunized intravenously with OVA alone (triangles) or OVA-ICs (circles) pulsed SVDC (open symbols) or BMDC (closed symbols) (1×10^6 cells each) as described under "MATERIALS AND METHODS." Spleen cells of the immunized mice were re-stimulated *in vitro* and CTL generation was assessed using a standard ^{51}Cr-release assay with E.G7 cells as the target. The results are representative of three independent experiments. The data are shown as the means for triplicate samples ± SD. (B) Enhanced expression of H-2K^b/OVA peptide complexes on OVA-pulsed SVDC. OVA-pulsed DCs (solid line) and unpulsed DCs (dotted line) were stained with anti-H-2K^b or mAb 25-D1.16, which is specific for H-2K^b/OVA peptide complexes, with isotype-matched control antibodies (closed histogram). (C) Enhanced tumor rejection induced by adoptive transfer of OVA-pulsed SVDC. Mice were twice immunized intravenously with PBS (inverted solid triangles, n = 7), 5×10^5 of OVA-pulsed BMDC (solid circles, n = 8), or OVA-pulsed SVDC (open circles, n = 7). The mice were inoculated subcutaneously with 1×10^5 E.G7 tumor cells into the left rear leg 7 d after the second immunization. A tumor of < 5 mm in diameter was defined as tumor-free, and the percentage of tumor-free mice was monitored for 75 d.

IC uptake by SVDC contributes to the development of the germinal center in naive mice. Thus, the adoptive transfer of IC-loaded SVDC into naive mice induced efficient antigen presentation to CD4$^+$ T cells to elicit humoral responses as well as BMDC did.

Adoptive Transfer of SVDC Pulsed with ICs Can Efficiently Induce Antigen-Specific CTLs in Naive Mice—The successful induction of humoral responses by IC-loaded SVDC transfer prompted us to examine whether it is also possible to induce MHC class I–mediated priming, which leads to the activation of CTLs. As shown in Fig. 5A, BMDC pulsed with OVA-ICs induced CTL activity against E.G7-OVA target cells, which present OVA peptides on MHC class I molecules, whereas BMDC pulsed with OVA alone could induce only marginal killer activity. Interestingly, the adoptive transfer of SVDC loaded with OVA alone could induce strong killer activity, which overcame the activity obtained with IC-loaded BMDC. IC loading of SVDC did not further augment the killing activity. Based on the observation that SVDC internalize OVA more efficiently than BMDC (Fig. 2A), we examined whether OVA-pulsed SVDC can present OVA peptides or not on MHC class I molecules more efficiently than OVA-pulsed BMDC, by using a monoclonal antibody, 25-D1.16, which recognizes OVA peptide SIINFEKL bound on H-2K^b (*13*). As shown in Fig. 5B, OVA-pulsed SVDC dramatically up-regulated the H-2K^b/OVA peptide complexes compared to OVA-pulsed BMDC, suggesting a major reason for the strong cross-presentation ability of SVDC as to CTLs.

To demonstrate the *in vivo* effect of enhanced CTL induction by OVA-pulsed SVDC on anti-tumor activity, naive mice were vaccinated twice with OVA-pulsed SVDC. Seven days after the second immunization, the mice were confronted with E.G7 tumor cells. As shown in Fig. 5C, the mice immunized with OVA-pulsed SVDC more efficiently rejected tumors than those immunized with OVA-pulsed BMDC. Thus, adoptive transfer of SVDC not only activated the specific helper T cells in naive mice when they were pulsed with ICs, but also significantly augmented the antigen-specific CTL even when SVDC were pulsed with OVA alone.

DISCUSSION

Several groups have succeeded in generating large numbers of functional DCs or Langerhans cells in both the murine or human system by treating DC precursors with GM-CSF alone or in combination with other growth factors including c-kit ligand, IL-4, and TNF-α (*19*, *24–28*). Cytokines secreted in a paracrine (*e.g.*, GM-CSF, TNF-α) or autocrine (TNF-α, IL-1β) manner control DC movement (*29*), survival (*30*), and antigen-presenting activity (*31*, *32*). However, these DCs could be propagated only for a limited time period, *i.e.*, up to 3 mo at most. On the other hand, growth factor-dependent long-term DC lines derived from mouse fetal or newborn skin have been established (*33*, *34*). Although these lines possess the properties of DC precursors and maintain an immature phenotype, they cannot be induced to mature *in vitro* (*33*, *34*). More recently, Winzler *et al.* have shown that MHC class II–positive growth factor–dependent immature DC, termed D1, derived from adult mice spleen, can be driven *in vitro* to proliferate for more than 1 yr of continuous culture (*35*). The proliferation and survival of these immature cells are strictly dependent on the presence of exogenous GM-CSF and fibroblast-derived growth factors. Upon living bacterial or cytokine stimulation, the DC line could mature and induce antigen-specific T cells to produce IL-12 (*35*).

In this study we characterized our immortalized DC line, termed SVDC, established from bone marrrow cells of tsSV40LT mice. The SVDC were able to proliferate at 33°C, a permissive temperature, in the presence of 20 ng/ml GM-CSF at least up to 12 mo in the absence of any additional growth factors without losing the immature phenotype, and were able to efficiently engulf soluble antigens at 33°C, leading to presentation of the antigenic peptides to specific $CD4^+$ T cells *in vitro* and the induction of IL-12 production. Moreover, SVDC could induce the humoral immune response upon antigen-IC loading followed by adoptive transfer into naive mice. To our surprise, SVDC pulsed with OVA alone could induce very strong CTL activity and anti-tumor immunity upon adoptive transfer into naive mice. This augmented ability of cross-presentation of SVDC may be due to the enhanced ability of antigen uptake that leads to facilitated antigen processing and loading of the antigenic peptide onto MHC class I molecules, which is probably an intrinsic characteristic of SVDC. Thus, our present study has provided simple procedures for establishing long-term cultures of myeloid DCs, which retain an immature phenotype, but upon antigenic stimulation, can mature and induce humoral and cellular immune responses, and has demonstrated the therapeutic potential of the adoptive transfer of the DC line pulsed with an antigen alone or antigen-ICs *in vitro* for various immunodeficiencies, infectious diseases, and cancer.

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