

# Enhanced Priming of Antigen-Specific CTLs In Vivo by Embryonic Stem Cell-Derived Dendritic Cells Expressing Chemokine Along with Antigenic Protein: Application to Antitumor Vaccination<sup>1</sup>

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Dendritic cell (DC)-based immunotherapy is regarded as a promising means for anti-cancer therapy. The efficiency of T cell-priming in vivo by transferred DCs should depend on their encounter with T cells. In the present study, we attempted to improve the capacity of DCs to prime T cells in vivo by genetic modification to express chemokine with a T cell-attracting property. For genetic modification of DCs, we used a recently established method to generate DCs from mouse embryonic stem cells. We generated double-transfectant DCs expressing a chemokine along with a model Ag (OVA) by sequential transfection of embryonic stem cells, and then induced differentiation to DCs. We comparatively evaluated the effect of three kinds of chemokines; secondary lymphoid tissue chemokine (SLC), monokine induced by IFN- $\gamma$  (Mig), and lymphotactin (Lptn). All three types of double transfectant DCs primed OVA-specific CTLs in vivo more efficiently than did DCs expressing only OVA, and the coexpression of SLC or Lptn was more effective than that of Mig. Immunization with DCs expressing OVA plus SLC or Mig provided protection from OVA-expressing tumor cells more potently than did immunization with OVA alone, and SLC was more effective than Mig. In contrast, coexpression of Lptn gave no additive effect on protection from the tumor. Collectively, among the three chemokines, expression of SLC was the most effective in enhancing antitumor immunity by transferred DCs in vivo. The findings provide useful information for the development of a potent DC-based cellular immunotherapy. *The Journal of Immunology*, 2004, 172: 776–786.

Dendritic cells (DCs)<sup>3</sup> are potent immunostimulators. In vivo transfer of Ag-bearing DCs has proven efficient in priming T cell responses specific to Ag. DC-based methods are now regarded as being a promising approach for immunotherapy, especially for anti-cancer immunotherapy. DCs pulsed with peptide Ags or genetically modified to present Ags are currently being clinically tested in cases of immunotherapy for subjects with malignant tumors (1–4).

The efficiency of T cell-priming in vivo by injected DCs should depend on their encounter with T cells. When exogenous Ag was injected intracutaneously, ~25% of the DCs capturing the Ag migrated to the T cell area of draining lymph nodes (LN) (5), where they presented Ag to prime naive T cells specific to the Ag. In contrast, when bone marrow cell-derived DCs (BM-DCs) or splenic DCs are transferred exogenously by s.c. or i.p. injection,

the absolute number of the DCs found within the draining LN represented only a small proportion (0.1–1%) (6–8). It has also been reported that almost all of transferred DCs remained at the s.c. immunization site 24 h after transfer (9). Inefficient migration of exogenous DCs to lymphoid organs may lower the frequency of their encounter with T cells. Therefore, it may be possible to improve the efficacy of exogenously transferred DCs to prime immune responses by augmenting their encounter with T cells. For example, if transferred DCs produce chemokines to intensively attract T cells, they may prime immune response efficiently, even though the DCs do not migrate to lymphoid organs.

Several kinds of chemokines with the capacity to attract T cells are produced by different cell types. Secondary lymphoid tissue chemokine (SLC)/CCL21 is produced in T cell regions of LN and spleen and also by high endothelial venules in LN. SLC chemoattracts T cells, NK cells, B cells, and DCs (10–12). Monokine induced by IFN- $\gamma$  (Mig)/CXCL9 is produced by macrophages and binds to the chemokine receptor CXCR3, which mediates the recruitment of predominantly Th1 cells and activated NK cells (13). Lymphotactin (Lptn)/XCL1, produced by activated T cells, has chemoattractive properties on CD4<sup>+</sup> and CD8<sup>+</sup> T cells and on NK cells (14, 15). This chemotactic action of Lptn is mediated through the receptor XCR1.

Recently, we established a novel method for genetic modification of DCs, where we generated DCs from mouse embryonic stem (ES) cells by in vitro differentiation (16). ES cell-derived DCs (ES-DCs) express MHC class II, CD11c, CD80, and CD86. They can strongly simulate MLR and efficiently process and present protein Ag to T cells. Their capacity to do so is comparable to that of BM-DCs. We can readily generate genetically modified DCs by introducing expression vectors driven by a  $\beta$ -actin promoter and subsequent

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; ES, embryonic stem cell; SLC, secondary lymphoid tissue chemokine; Mig, monokine induced by IFN- $\gamma$ ; Lptn, lymphotactin; LN, lymph node; BM-DC, bone marrow cell-derived DC; ES-DC, ES cell-derived DC; neo-R, neomycin resistant; IRES, internal ribosomal entry site.

induction of their differentiation to DCs. By sporadic selection with a selection drug, ES cell clones transfected with genes can be propagated while maintaining the capacity to express gene products after their differentiation to DCs. Therefore, one can use ES cell transfectants as an infinite source for genetically modified DCs.

In the present study, using this method, we generated DCs expressing chemokine along with a model Ag, OVA. We determined whether coexpression of T cell-attracting chemokine with antigenic protein by DCs enhanced the capacity to prime Ag-specific CTLs upon *in vivo* transfer. We also examined the potency of the genetically modified DCs to elicit antitumor immunity against tumor cells expressing OVA. Among T cell-attracting chemokines, we selected SLC, Mig, and Lptn, and comparatively evaluated their effects.

## Materials and Methods

### Mice

CBA and C57BL/6 mice were obtained from CLEA (Tokyo, Japan) or Charles River Breeding Laboratories (Hamamatsu, Japan) and kept under specific pathogen-free conditions. Male CBA and female C57BL/6 mice were mated to produce (CBA crossed with C57BL/6) F<sub>1</sub> (CBF<sub>1</sub>) mice and all *in vivo* experiments were done with the F<sub>1</sub> mice at 6–8 wk of age.

### Cell lines

The ES cell line TT2, derived from (CBA crossed with C57BL/6) F<sub>1</sub> blastocysts (17), were maintained as described (18). The T cell hybridoma RF33.70 (19), recognizing OVA<sub>257–264</sub> in the context of K<sup>b</sup>, and the M-CSF-defective bone marrow-derived stromal cell line, OP9 (20), have been reported. MO4 (21) was generated by transfection of C57BL/6-derived melanoma B16 with the pAc-neo-OVA plasmid, as described (22). The procedure for induction of differentiation of ES cells into DCs has been reported (16), and ES-DCs recovered after a 14-day culture in bacteriological petri dishes were used for *in vivo* and *in vitro* assays.

### Peptide, cytokines/chemokines, and anti-chemokine Ab

The K<sup>b</sup>-binding peptide OVA<sub>257–264</sub>, SIINFELK, were synthesized using the F-MOC method on an automatic peptide synthesizer (PSSM8; Shimadzu, Kyoto, Japan) then purified by HPLC. Recombinant mouse GM-CSF was provided by Kirin Brewery (Tokyo, Japan). Recombinant mouse SLC, Mig, and Lptn, were purchased from DACO JAPAN (Kyoto, Japan). Goat anti-mouse SLC and Mig Abs and biotinylated goat anti-mouse SLC and Mig Abs were also purchased from DACO JAPAN. Rabbit anti-mouse Lptn Ab was purchased from eBioscience (San Diego, CA), and was biotinylated using a MiniBiotin-XX Protein Labeling kit (F-6347; Molecular Probes, Portland, OR).

### cDNA array analysis of chemokine gene expression

BM-DCs were generated from bone marrow cells of CBF<sub>1</sub> mice, as described (23, 24). Total RNA was extracted from BM-DCs on day 12 and ES-DCs on day 14 of culture in bacteriological petri dishes, using RNeasy mini kits (Qiagen, Studio City, CA). Total RNA (3 μg) from each sample was reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) in the presence of [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham Pharmacia Biotech, Piscataway, NJ). The resulting cDNA probes were hybridized to cDNA fragments spotted on GEArray membranes (SuperArray, Bethesda, MD). Hybridization and wash of the membranes were done following the manufacturer's instructions. The intensity of radioactive signaling from the hybridized probes was analyzed on a BAS-2000 (Fujifilm, Tokyo, Japan). The signal from expression of each chemokine gene was normalized to the signal derived from  $\beta$ -actin on the same membrane and expressed as arbitrary units calculated using the formula: Chemokine mRNA arbitrary units = (chemokine signal – background signal)/( $\beta$ -actin signal – background signal) (25).

### Plasmid construction

A cDNA fragment encoding for OVA protein was transferred to pCAG-IP (26), a mammalian expression vector containing the chicken  $\beta$ -actin promoter and an internal ribosomal entry site (IRES)-puromycin *N*-acetyltransferase gene cassette, to generate pCAG-OVA-IP. To obtain pCAGGS-IRES-neo-R, a DNA fragment containing IRES-neomycin-resistant (neo-R) was inserted

into a mammalian expression vector pCAGGS (27). A cDNA fragment coding for chemokine protein was inserted into pCAGGS-IRES-neo-R. SLC cDNA was obtained by RT-PCR using murine spleen cells as the RNA source and PCR primers, AACCTCTAGCCCCGCCAC-CATGGCTCAGAGATGACTCT (forward) and AACCCGGATCCAGGCGGGCTACTACTGGCTATCC (reverse). Mig cDNA was obtained by RT-PCR using murine spleen cells stimulated for 24 h with IFN- $\gamma$  as the RNA source and the PCR primers, AACCTCTAGACCCGCCGCCACCATGAAGTCCGCTGTCTTTTCC (forward) and AACCCGGATCCAGGGTGTCTGTGGTAAAG (reverse). Lptn cDNA was obtained by RT-PCR, using murine spleen cells as the RNA source stimulated for 24 h with PMA and A23187 and the PCR primers AACCTCTAGACCCGCCGCCACCATGAGACTTCTCTCCTGAC (forward) and AACCCGGATCCCTGGAGGCTGTTACCCAGTC (reverse). The design of these primers results in cloning of chemokine cDNA downstream of the Kozak sequence (28). The PCR products were cloned into a plasmid vector (pGEM-T easy; Promega), confirmed by sequencing analysis, and then transferred to the expression vector.

### Transfection of ES cells and generation of ES-DCs expressing chemokine along with OVA

To generate OVA-transfected ES cell clones, TT2 ES cells were introduced with pCAG-OVA-IP by electroporation and selected with puromycin using the reported procedure (16). OVA-transfected ES cell clones were differentiated to ES-DCs, and an ES cell transfectant clone highly expressing OVA after differentiation to DCs (ES-OVA) was selected, based on the capacity to stimulate RF33.70, the OVA-reacting T cell hybridoma. The selected ES cell clone was transfected with one of three kinds of chemokine expression vectors or pCAGGS-IRES-neo-R (mock). Transfected ES cells were cultured on neo-R primary embryonic fibroblasts feeder layers and selected with G418 (500 μg/ml), and drug-resistant colonies were picked up. Double-transfectant ES cell clones producing high amounts of chemokine after differentiation to DCs were selected. To determine chemokine levels in culture supernatants, ELISAs were done as we reported (29).

### T cell hybridoma assay for detection of OVA peptide-K<sup>b</sup> complexes

Graded numbers of ES-DCs as stimulators were seeded into 96-well flat-bottom culture plates together with RF33.70 as responders ( $5 \times 10^4$  cells/well in final volume of 200 μl). After 24 h of culture, the supernatant (50 μl/well) was collected and added to culture of the IL-2-dependent cell line, CTLL-20 ( $5 \times 10^3/100$  μl/well), in 96-well flat-bottom culture plates. After 16 h, [<sup>3</sup>H]thymidine (248 MBq/mmol) was added (37.5 KBq/well) and cells were incubated for a further 8 h. The incorporation of [<sup>3</sup>H]thymidine by CTLL-20 was measured by scintillation counting.

### In vitro survival assay of ES-DCs

ES-DCs recovered from 14-day culture in petri dishes were cultured again in petri dishes (1.2 × 10<sup>5</sup>/90 mm dish) under several conditions. After 7 days, cells were recovered by pipetting, stained with trypan blue and microscopically counted. Some recovered cells were also stained with propidium iodide (10 μg/ml) and analyzed on a flow cytometer (FACScan, BD Biosciences, San Jose CA) to detect dead cells.

### Assay of the migration of DCs in vivo

DCs ( $2 \times 10^6$ ) labeled with 1 μM CFSE (Molecular Probes, Oss, The Netherlands) in serum-free medium for 10 min at 37°C, were *i.p.* transferred into the CBF<sub>1</sub> mouse. After 40 h, 5-μm frozen sections of the spleen were made and examined under a fluorescence microscope (Olympus, Melville, NY) or stained with H&E. <sup>111</sup>In-labeled DCs ( $1 \times 10^6$ ) were *i.p.* transferred into mice. After 40 h, several organs were isolated and the radioactivity in each organ was measured on a gamma counter as described by Eggert et al. (6) and Morse et al. (9). The radioactivity was expressed as the percentage of injection dose per 0.1 gram of tissue, so that the values were adjusted to 0.1 g of tissue to correct for weight differences of each organ.

### Induction of OVA-specific CTLs in vitro and cytotoxicity assay

ES-DCs ( $4 \times 10^5$ /well) or BM-DCs ( $4 \times 10^5$ /well) were cocultured with T cells ( $2.5 \times 10^6$ /well) purified with a nylon wool column from spleen cells of unprimed CBF<sub>1</sub> mice in 24-well culture plates in RPMI 1640 supplemented with 10% FCS. In some experiments, ES-DCs were killed before use by treatment at 70°C for 20 min. BM-DCs were prepared as described (23) then pulsed with OVA peptide (10 μM) for 4 h, washed twice, and used as stimulators. After 5 days of culture, cells were recovered

and used as effector cells in cytotoxicity assay using peptide-pulsed EL-4 cells as target cells, as described (16).

### Induction of OVA-specific CTLs in vivo

Genetically modified ES-DCs, viable or heat-killed, or OVA protein (50  $\mu$ g) were injected i.p. to mice twice at 7-day intervals, and 7 days after the second transfer, the mice were killed and spleen cells were isolated. Whole spleen cells were cultured in vitro in the presence of OVA peptide (0.1  $\mu$ M) for 5 days and OVA-specific CTL activity was analyzed as described (16).

### Tumor prevention experiments

In tumor prevention experiments and survival studies,  $2 \times 10^4$  or  $3 \times 10^3$  genetically modified ES-DCs were transferred i.p. into mice. Transfers were done twice at 7-day intervals, and 7 days after the second transfer, MO4 cells were challenged s.c. in the shaved left flank region. Tumor sizes were determined biweekly in a blinded fashion and survival rate was monitored. Tumor index was calculated as: Tumor index (in millimeters) = square root (length  $\times$  width).

### In vivo depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes

Mice were transferred i.p. twice with  $3 \times 10^3$  ES-DC-OVA/mock or ES-DC-OVA/SLC at 7-day intervals, and 7 days after the second transfer, the mice were challenged s.c. with  $3 \times 10^6$  MO4 cells (day 0). The mice were given a total of six i.p. transfers (days -18, -15, -11, -8, -4, -1) of the ascites (0.1 ml/mouse/transfer) from hybridoma-bearing nude mice. mAbs used were rat anti-mouse CD4 (clone GK1.5) and rat anti-mouse CD8 (clone 2.43). Normal rat IgG (Sigma-Aldrich, St. Louis, MO; 200  $\mu$ g/mouse/transfer) was used as control. Tumor measurements were made 15 days after tumor challenge. Results are expressed as tumor index  $\pm$  SD. Each group included eight mice. Depletion of T cell subsets by treatment with mAbs was confirmed by flow cytometric analysis of spleen cells, which showed a >90% specific depletion.

### Histological analysis of tumor tissues

Freshly excised tumor tissues were immediately frozen and embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN). Serial 5- $\mu$ m sections were made using cryostat and underwent immunochemical staining with mAbs specific to CD4 (L3T4; BD PharMingen, San Diego, CA) or CD8 (Ly-2; BD PharMingen) and N-Histofine Simple Stain Mouse MAX PO (Nichirei, Tokyo, Japan).

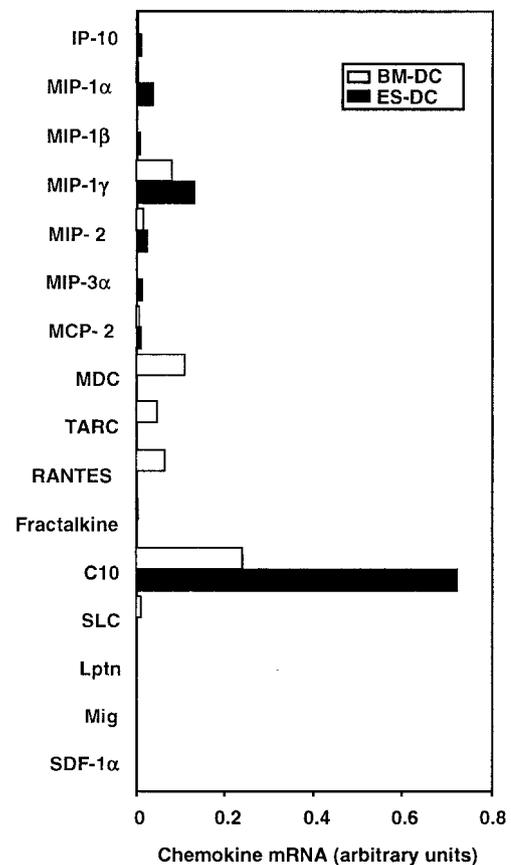
### Statistical analysis

Two-tailed Student's *t* test was used to determine the statistical significance of differences in lytic activity of spleen cell preparations and tumor growth, and between treatment groups. A value of  $p < 0.05$  was considered significant. The Kaplan-Meier plot for survivals was assessed for significance using the Breslow-Gehan-Wilcoxon test. Statistical analyses were made using StatView 5.0 software (Abacus Concepts, Calabasas, CA).

## Results

### Profile of chemokine gene expression in ES-DCs

We recently established a culture method to generate DCs from mouse ES cells. ES-DCs have the capacity to stimulate T cells comparable to BM-DCs (16). At the beginning of the present study, to determine the profile of chemokine gene expression by ES-DCs, we analyzed chemokine mRNAs by cDNA macroarray hybridization analysis, comparing ES-DCs and BM-DCs. The gene expression of DC-derived chemokines and chemokines that chemoattract T cells is shown in Fig. 1. The analysis revealed that chemokine gene expression profile of ES-DCs was somewhat different from that of BM-DCs. However, both DCs expressed C10, and expression of T cell-attracting chemokines produced by cells other than DCs such as SLC, Lptn, Mig, or stromal cell-derived factor 1 $\alpha$  were rarely detected in both types of DCs generated in vitro. Therefore, we presumed augmentation of the immunomod-



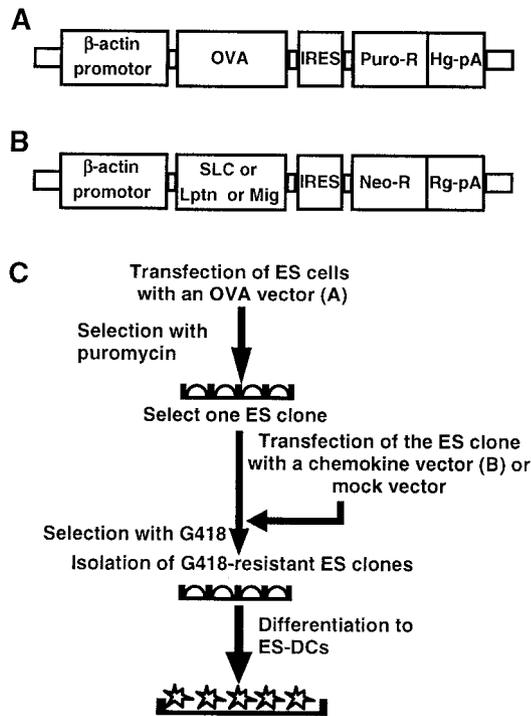
**FIGURE 1.** Profile of chemokine gene expression in ES-DCs and BM-DCs. Radiolabeled cDNA generated from ES-DCs and BM-DCs were hybridized to chemokine gene-specific 44 cDNA fragments spotted on nylon membranes. The hybridization signals were normalized to the signal derived from  $\beta$ -actin on the same membrane. Data for DC-derived chemokines and chemokines with T cell-attracting property are shown.

ulating capacity by in vivo transferred DCs through genetic modification of DCs to express such T cell-attracting chemokines.

### Generation of ES-DCs expressing chemokine along with antigenic protein

Using the expression vector driven by the  $\beta$ -actin promoter and containing the IRES-drug-resistant marker gene (Fig. 2, A and B), we can generate ES cell transfectant clones expressing the gene products after their differentiation to DCs. Using this system, we first prepared an ES cell transfectant clone highly expressing OVA after differentiation to DCs. Subsequently, we introduced chemokine expression vectors or mock vector into the ES cell clone expressing OVA (ES-OVA) (Fig. 2C). We selected one double-transfectant ES cell clone for each chemokine gene or mock vector transfection and generated four kinds of ES-DCs expressing chemokine along with OVA or OVA alone, and designated them ES-DC-OVA/SLC, ES-DC-OVA/Lptn, ES-DC-OVA/Mig, and ES-DC-OVA/mock. Therefore, the four double transfectant ES cell clones used in this study originated from the same ES cell clone transfected with the OVA gene.

We acquired DCs from these ES cell transfectant clones and compared their capacity to stimulate the OVA<sub>257-264</sub>-specific and K<sup>b</sup>-restricted T cell hybridoma, RF33.70. As shown in Fig. 3A, ES-DC-OVA/SLC, ES-DC-OVA/Lptn, ES-DC-OVA/Mig, ES-DC-OVA, and ES-DC-OVA/mock could stimulate RF33.70 with a comparable efficiency. The amounts of chemokine produced by the

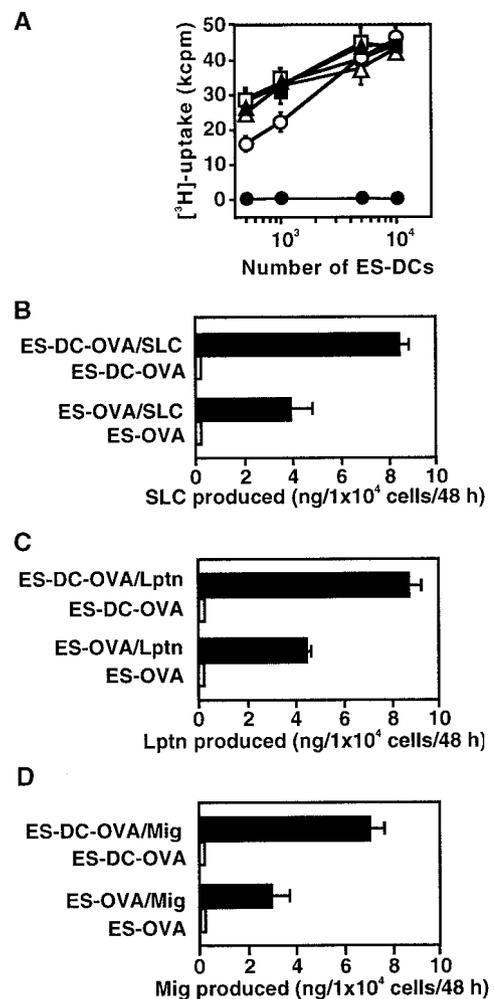


**FIGURE 2.** Generation of ES-DCs expressing chemokine simultaneously with OVA. *A*, Structure of OVA protein expression vector, pCAG-OVA-PI. The expression of this gene is driven by the chicken  $\beta$ -actin promoter. The OVA protein coding sequence is followed by the IRES-puromycin *N*-acetyltransferase gene (Puro-R) and the polyadenylation signal sequence of human growth hormone (Hg-pA). *B*, Structure of chemokine expression vector, pCAGGS-chemokine-IRES-neo-R. Expression of this gene is driven by the chicken  $\beta$ -actin promoter. The chemokine coding sequence is followed by the IRES-neo-R gene (Neo-R) and a polyadenylation signal sequence of rabbit  $\beta$ -globin poly(A) (Rg-pA). *C*, TT2 ES cells were transfected with pCAG-OVA-PI. Puromycin-resistant colonies were picked up and expanded. An ES cell clone highly expressing OVA was selected and transfected with one of three kinds of pCAGGS-chemokine-IRES-neo-R or with a mock vector. G418-resistant colonies were picked up and expanded. One ES clone expressing large amounts of chemokine after DC differentiation was selected for each chemokine and used in the described experiments.

three kinds of chemokine gene-transfected cells used in this study are shown in Fig. 3, *B–D*. Both ES cells and differentiated ES-DCs produced transgene-derived chemokines, and comparable protein amounts of chemokines were produced by the three chemokine gene-transfected ES-DCs. Morphology and surface phenotypes of chemokine gene-transfected ES-DCs were not significantly different from ES-DC-TT2 (DCs derived from parental TT2 ES cells) (data not shown). These results suggest that the forced expression of OVA protein and the chemokines by gene transfer to ES cells do not affect their differentiation to DCs.

#### The migration capacity of ES-DCs in vivo

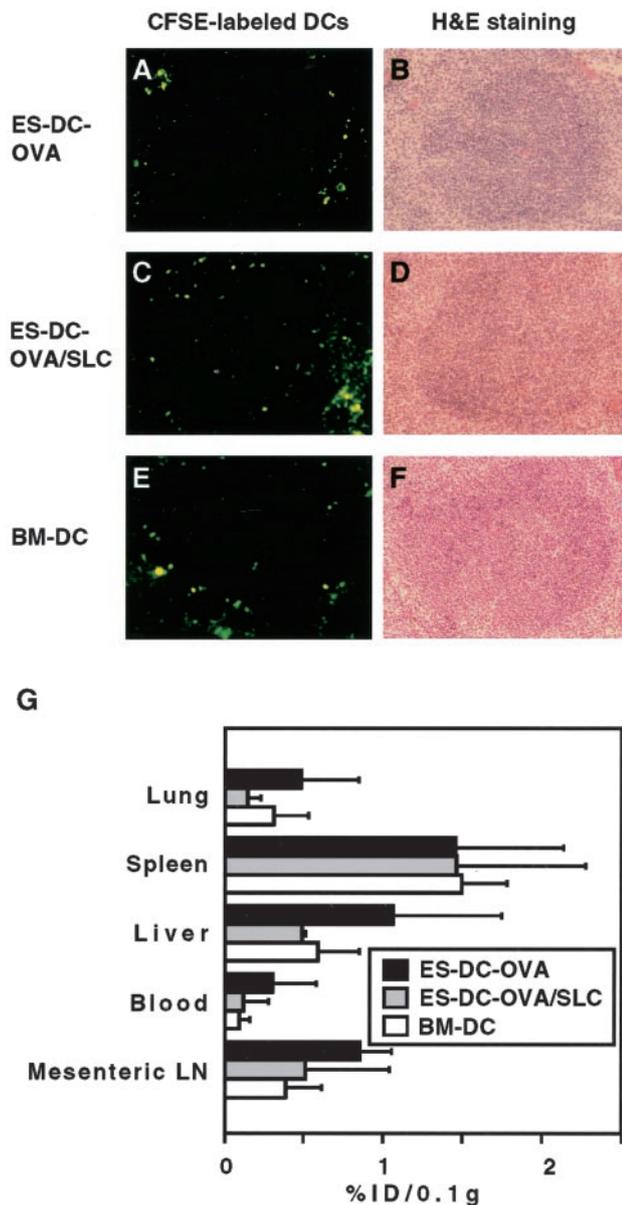
To test the migration capacity of ES-DCs in vivo, we histologically examined the migration of transferred ES-DCs to the spleen. In addition, we tested whether or not the expression of SLC, the chemokine with DC-attracting property, by ES-DCs would affect their in vivo migration. As shown in Fig. 4, *A–F*, CFSE-labeled ES-DC-OVA, ES-DC-OVA/SLC, and BM-DCs migrated to the spleen to the same extent, mostly localizing in the white pulp and the marginal zone (Fig. 4, *B, D, and F*).



**FIGURE 3.** Stimulation of OVA-specific T cell hybridoma and chemokine production by genetically modified ES-DCs. *A*, Stimulation of K<sup>b</sup>-restricted OVA-specific T cell hybridoma, RF33.70, with ES-DC-OVA (■), ES-DC-OVA/mock (□), ES-DC-OVA/SLC (▲), ES-DC-OVA/Lptn (△), ES-DC-OVA/Mig (○), or negative control, ES-DC-TT2 (●) without OVA-expression, was analyzed. Stimulators and RF33.70 were cocultured for 24 h, and IL-2 produced by RF33.70 was quantified by measuring proliferation of CTLL-20 cells. Results were expressed as mean cpm of triplicate cultures  $\pm$  SD. Data are representative of three independent and reproducible experiments. *B–D*, The 48-h culture supernatants of the  $1 \times 10^6$  ES-DC-OVA expressing chemokine or ES-DC-OVA in petri dishes and that of  $1 \times 10^6$  ES-OVA expressing chemokine or ES-OVA on layers of primary embryonic fibroblasts were harvested. The concentrations of chemokine in the supernatants were measured using ELISA. Production of chemokine SLC (*B*), Lptn (*C*), and Mig (*D*) by respective transfectants was quantified. Results are expressed as mean amounts of chemokine per  $1 \times 10^4$  cells of triplicate cultures  $\pm$  SD. Data are representative of two independent and reproducible experiments.

We also investigated the distribution of <sup>111</sup>In-labeled DCs in lymphoid organs after i.p. transfer. The distribution of ES-DCs shown in Fig. 4*G* indicated that ES-DCs and BM-DCs similarly accumulated in the spleen and mesenteric LN 40 h after the transfer, and that expression of SLC by ES-DCs made no significant difference in the migration pattern.

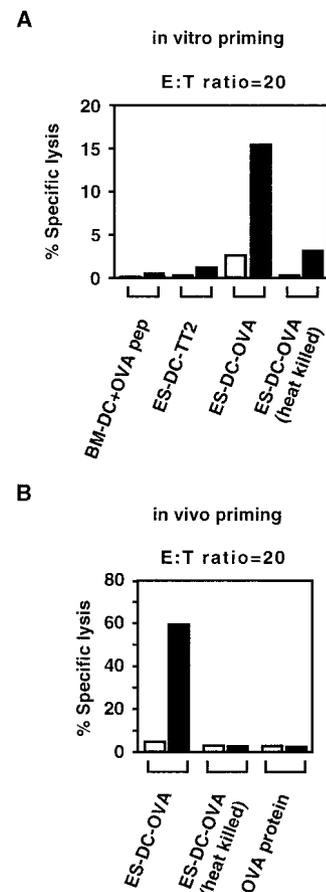
Collectively, the migratory capacity toward lymphoid tissues of ES-DCs is almost comparable to that of BM-DCs, and the SLC produced by ES-DC-OVA/SLC did not prevent them from migrating toward lymphoid tissues.



**FIGURE 4.** The migration capacity of ES-DCs in vivo. *A–F*, DCs ( $2 \times 10^6$ ) were labeled with CFSE and injected i.p. into mice. At 40 h later, frozen sections of spleens were prepared. Injected DCs were ES-DC-OVA (*A* and *B*), ES-DC-OVA/SLC (*C* and *D*), and BM-DCs (*E* and *F*). *A*, *C*, and *E* are fluorescence images of the sections serial to H&E-stained sections shown in *B*, *D*, and *F*, respectively. *G*,  $^{111}\text{In}$ -labeled DCs ( $1 \times 10^6$ ) were injected i.p. into mice, and radioactivity of indicated organs was measured 40 h later. The measured radioactivity in tissues was expressed as percentage of injection dose per 0.1 g tissue (%ID/0.1 g) as described in *Materials and Methods*. Results were expressed as mean %ID/0.1 g + SD ( $n = 3$  per group).

#### Priming of Ag-specific CTLs with genetically modified ES-DCs in vitro and in vivo

We analyzed the capacity of ES-DC-OVA to prime OVA-specific T cells in vitro. ES-DC-TT2, ES-DC-OVA, heat-killed ES-DC-OVA, or BM-DCs prepulsed with OVA peptide were cocultured with splenic T cells derived from unprimed CBF<sub>1</sub> mice. After 5 days, cells were recovered and OVA-specific CTL activity was analyzed. The results shown in Fig. 5*A* indicate that OVA-specific CTLs were primed in vitro by intact ES-DC-OVA but not by ES-DC-TT2, BM-DCs prepulsed with OVA<sub>257–264</sub> peptide, or heat-



**FIGURE 5.** Priming of OVA-specific CTLs with genetically modified ES-DCs. *A*, BM-DCs prepulsed with OVA peptide ( $10 \mu\text{M}$ ), ES-DC-TT2, ES-DC-OVA, or heat-killed ES-DC-OVA were cocultured with splenic T cells of unprimed CBF<sub>1</sub> mice. After 5 days, the resultant cells were assayed for the capacity to kill EL-4 tumor cells either pulsed with  $10 \mu\text{M}$  OVA peptide (■) or left unpulsed (□) at an E:T ratio of 20. *B*, Mice were transferred i.p. twice with ES-DC-OVA ( $2 \times 10^4$ ), alive or heat killed, or OVA protein ( $50 \mu\text{g}$ ) on days  $-14$  and  $-7$ . Spleen cells were harvested from the mice on day 0, pooled for each group (four mice per group), and cultured in the presence of OVA<sub>257–264</sub> ( $0.1 \mu\text{M}$ ) for 5 days. The resultant cells were assayed for the capacity to kill EL-4 tumor cells either pulsed with  $10 \mu\text{M}$  OVA peptide (■) or left unpulsed (□) at an E:T ratio of 20. Results are expressed as mean specific lysis of triplicate assays, and SDs of triplicates were  $<2\%$ . Data are representative of two independent and reproducible experiments.

killed ES-DC-OVA. BM-DCs prepulsed with OVA peptide could prime OVA-specific CTLs in vitro only in the presence of exogenous IL-2, whereas ES-DC-OVA could prime OVA-specific CTLs, regardless of whether or not IL-2 had been added (our unpublished observations).

Furthermore, the capacity of ES-DC-OVA to prime OVA-specific T cells in vivo was analyzed. ES-DC-OVA ( $2 \times 10^4$ ), heat-killed ES-DC-OVA ( $2 \times 10^4$ ), or OVA protein ( $50 \mu\text{g}$ ) were injected i.p. into CBF<sub>1</sub> mice twice within a 7-day interval. Spleen cells were isolated 7 days after the second injection and then cultured in vitro in the presence of OVA<sub>257–264</sub> peptide. After 5 days, cells were recovered and assayed for their capacity to kill EL-4 thymoma cells (H-2<sup>b</sup>) prepulsed with the OVA peptide. The results shown in Fig. 5*B* indicate that CTLs specific to the OVA epitope were primed in vivo with ES-DC-OVA but not with heat-killed ES-DC-OVA or soluble OVA protein.

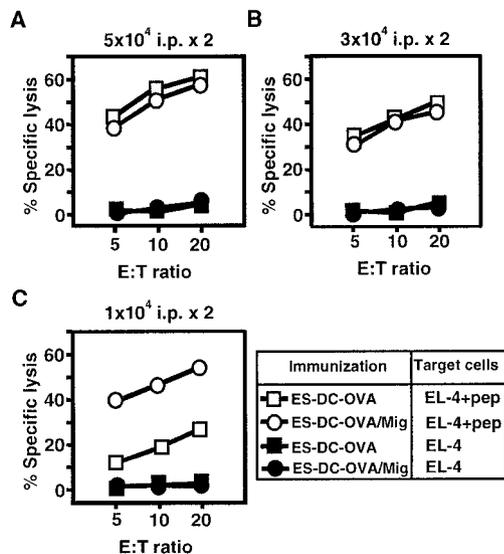
These results demonstrated that live ES-DCs genetically modified to express an antigenic protein have the capacity to prime

Ag-specific CTLs both in vitro and in vivo. There is little possibility that endogenous host DCs, which phagocytosed ES-DCs expressing OVA or OVA protein, played a major role in priming CTLs, based on the result that CTLs were not primed either by injection with heat-killed ES-DC-OVA or by OVA protein.

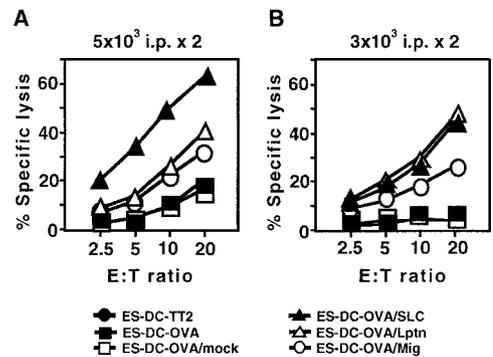
*Efficient priming of OVA-specific CTLs by DCs producing chemokine along with OVA*

We analyzed the capacity of genetically modified ES-DCs expressing Mig along with OVA to prime OVA-specific T cells in vivo. Graded numbers of ES-DC-OVA or ES-DC-OVA/Mig were transferred i.p. to mice twice at a 7-day interval. Spleen cells were isolated 7 days after the second transfer then cultured in vitro in the presence of OVA<sub>257-264</sub> peptide. After 5 days, cells were recovered and assayed for their capacity to kill EL-4 thymoma cells (H-2<sup>b</sup>) prepulsed with the OVA peptide (Fig. 6). When  $5 \times 10^4$  or  $3 \times 10^4$  DCs were transferred twice, a comparable level of OVA-specific CTL activity was primed by ES-DC-OVA and ES-DC-OVA/Mig. In contrast, when  $1 \times 10^4$  DCs were transferred twice, ES-DC-OVA/Mig primed CTL activity to a greater extent than seen with ES-DC-OVA. As we reported, OVA-specific CTLs were not primed by transfer of ES-DC-TT2, even when  $5 \times 10^5$  DCs were transferred twice (16).

We next analyzed effects of expression of the three chemokines on in vivo CTL-priming using the same experimental procedure as previously described except that smaller numbers of DCs were transferred into the mice (Fig. 7). When mice were given  $5 \times 10^3$  ES-DCs twice, all OVA-expressing DCs stimulated OVA-specific CTLs, and the T cell-priming capacity of DCs coexpressing either of the three chemokines was significantly stronger than those expressing OVA alone. Even when only  $3 \times 10^3$  DCs were transferred twice, OVA-specific CTLs were primed by the three kinds of ES-DC-OVA chemokine. Conversely, priming of CTLs by ES-DCs expressing OVA alone was not detected under this condition.



**FIGURE 6.** Priming of OVA-specific CTLs in vivo by immunization with ES-DC-OVA/Mig. Mice were transferred i.p. twice with ES-DC-OVA or ES-DC-OVA/Mig on days -14 and -7 with  $5 \times 10^4$  (A),  $3 \times 10^4$  (B), or  $1 \times 10^4$  (C) ES-DCs. Spleen cells from transferred mice were harvested on day 0, pooled for each group (three mice per group), and cultured in the presence of OVA<sub>257-264</sub> (0.1  $\mu$ M) for 5 days. The resultant cells were assayed for the capacity to kill EL-4 tumor cells either pulsed with 10  $\mu$ M OVA peptide or left unpulsed. Results are expressed as mean specific lysis of triplicate assays, and SDs of triplicates were <2%. Data are representative of three independent and reproducible experiments.



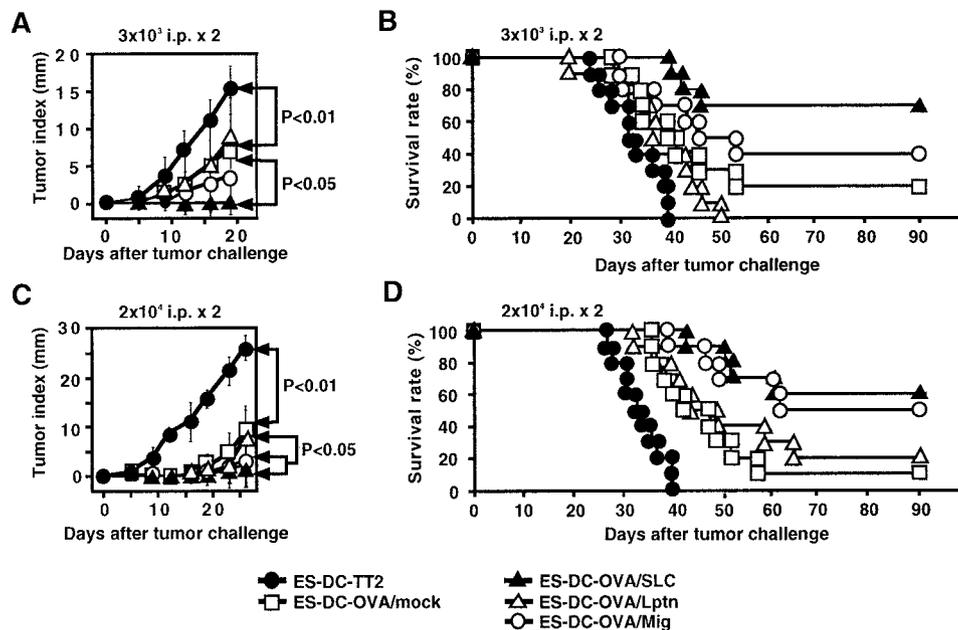
**FIGURE 7.** Enhanced priming of OVA-specific CTLs in vivo by immunization with ES-DCs expressing chemokine along with OVA. Mice were transferred i.p. twice on days -14 and -7 with  $5 \times 10^3$  ES-DCs (A) or  $3 \times 10^3$  ES-DCs (B). ES-DCs expressing chemokine along with OVA were ES-DC-OVA/SLC ( $\blacktriangle$ ), ES-DC-OVA/Lptn ( $\triangle$ ), ES-DC-OVA/Mig ( $\circ$ ). ES-DCs expressing OVA alone as controls were ES-DC-OVA/mock ( $\square$ ) and ES-DC-OVA ( $\blacksquare$ ). DCs differentiated from the parental OVA gene single-transfectant ES cell clone. Spleen cells of the mice were isolated on day 0, pooled for each group (three to seven mice per group), and assayed for the CTL activity using the same procedure as in Fig. 5. For all effectors, specific lysis was <2% when target EL-4 cells were not prepulsed with OVA peptide. Results were expressed as mean specific lysis of triplicate assays, and SDs of triplicates were <2%. Data are representative of three independent and reproducible experiments.

These results clearly demonstrate that coexpression of the chemokines along with Ag in DCs enhances their capacity to prime the Ag-specific CTLs in vivo. The results shown in Fig. 7 also indicate that coexpression of SLC or Lptn in DCs is more effective than that of Mig in the priming of CTLs in vivo.

*Protective effects of immunization with chemokine gene-modified DCs against tumor cell challenge*

We next asked whether coexpression of chemokine with OVA in DCs would enhance their capacity to induce protective immunity against tumor cells expressing OVA. We immunized mice by twice i.p. transfers of DCs at 7-day intervals, and 7 days after the second transfer, the mice were challenged s.c. with  $3 \times 10^5$  MO4 cells, OVA-expressing melanoma cells derived from B16. In case of two transfers of  $3 \times 10^3$  ES-DCs, as shown in Fig. 8A, immunization with ES-DCs expressing OVA alone (ES-DC-OVA/mock) provided significant protection against the MO4 challenge, in comparison with ES-DC-TT2 ( $p < 0.01$ ). Conversely, transfer of ES-DC-TT2 gave no significant protection, compared with no DC transfer (data not shown). Immunization with ES-DC-OVA/SLC provided greater protection than did immunization with ES-DC-OVA/mock ( $p < 0.05$ ). In contrast, protection given by immunization with ES-DC-OVA/Mig or ES-DC-OVA/Lptn was at a comparable level to that provided by ES-DC-OVA/mock. As shown in Fig. 8B, immunization with ES-DC-OVA/mock showed a significant prolongation of survival, compared with immunization with ES-DC-TT2 ( $p < 0.05$ ). Immunization with ES-DC-OVA/SLC resulted in a further prolongation of survival. However, coexpression of Lptn or Mig had no significant additive effect on survival.

In case of twice transfers of  $2 \times 10^4$  ES-DCs, as shown in Fig. 8C, immunization with ES-DC-OVA/mock provided significant protection against MO4 challenge, compared with ES-DC-TT2 ( $p < 0.01$ ). Under this condition, immunization with ES-DC-OVA/SLC and ES-DC-OVA/Mig provided greater protection than that seen with ES-DC-OVA/mock ( $p < 0.05$ ). In contrast, effect of immunization with ES-DC-OVA/Lptn was comparable to that of



**FIGURE 8.** Suppression of tumor growth and prolongation of survival by immunization with ES-DCs expressing chemokine along with OVA. Mice were transferred i.p. twice on day  $-14$  and  $-7$  with  $3 \times 10^3$  (A and B) or  $2 \times 10^4$  ES-DCs (C and D). The mice were challenged s.c. with  $3 \times 10^5$  MO4 tumor cells expressing OVA on day 0. Tumor index (A and C) and survival rate (B and D) were monitored. The differences in tumor index between ES-DC-TT2 and ES-DC-OVA/mock as well as between ES-DC-OVA/mock and ES-DC-OVA/SLC are statistically significant ( $p < 0.01$  and  $p < 0.05$ , respectively) (A). The differences in survival rates between ES-DC-TT2 and ES-DC-OVA/mock as well as between ES-DC-OVA/mock and ES-DC-OVA/SLC are statistically significant ( $p < 0.01$ ) (B). The difference in tumor index between ES-DC-TT2 and ES-DC-OVA/mock is statistically significant ( $p < 0.01$ ) (C). The differences in tumor index between ES-DC-OVA/mock and ES-DC-OVA/Mig as well as between ES-DC-OVA/mock and ES-DC-OVA/SLC are also statistically significant ( $p < 0.05$ ) (C). The differences in survival rate between ES-DC-TT2 and ES-DC-OVA/mock as well as between ES-DC-OVA/mock and ES-DC-OVA/SLC are statistically significant ( $p < 0.01$ ) (D). The difference in survival rate between ES-DC-OVA/mock and ES-DC-OVA/Mig is also statistically significant ( $p < 0.05$ ) (D). A and C, Results are expressed as mean tumor index  $\pm$  SD ( $n = 10$  per group). B and D, Kaplan-Meier plot depicts the survival rate ( $n = 10$  per group).

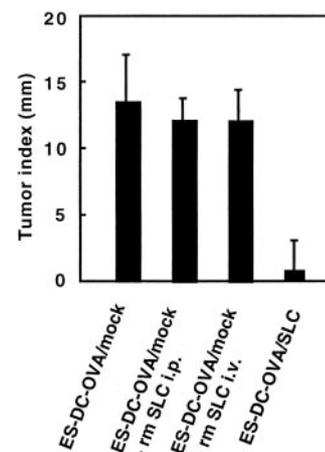
ES-DC-OVA/mock. As shown in Fig. 8D, immunization with ES-DC-OVA/SLC resulted in a longer survival time than that seen with ES-DC-OVA/mock ( $p < 0.01$ ). In addition, ES-DC-OVA/Mig was more effective than ES-DC-OVA/mock ( $p < 0.05$ ), but less effective than ES-DC-OVA/SLC. Immunization with ES-DC-OVA/Lptn again resulted in survival at the same level as seen with ES-DC-OVA/mock. When mice were twice transferred with  $2 \times 10^4$  ES-DCs and challenged with  $3 \times 10^6$  MO4 tumor cells, among the three chemokine-expressing ES-DCs, only immunization with ES-DC-OVA/SLC was more effective than ES-DC-OVA/mock (data not shown).

Collectively, ES-DC-OVA/SLC was always more effective than ES-DC-OVA/mock. Expression of Mig in ES-DC increased survival time under some experimental conditions. In contrast, ES-DC-OVA/Lptn did not elicit more protection than did ES-DC-OVA/mock under the conditions we tested. These results suggest that expression of SLC along with antigenic protein is the most effective among the three chemokines for induction of protective immunity against tumor cells expressing the Ag.

#### No effect of SLC simultaneously injected with ES-DCs

As described, coexpression of SLC along with OVA in ES-DCs enhanced their capacity to induce protective immunity against tumor cells expressing OVA (Fig. 8). To examine the effect of SLC upon simultaneous injection with ES-DCs expressing OVA, we compared immunization with  $2 \times 10^4$  ES-DC-OVA/SLC to immunization with  $2 \times 10^4$  ES-DC-OVA/mock accompanying i.p. or systemic (i.v.) injection of recombinant mouse SLC ( $3 \mu\text{g}$ ). The amount of injected recombinant mouse SLC was much higher than that expected to be produced by injected ES-DC-OVA/SLC after the transfer (Fig. 3B). Transfer of ES-DCs and tumor cell challenge with

$3 \times 10^5$  MO4 cells were done using the same schedule as previously described. The tumor index in millimeters 30 days after MO4 challenge is shown in Fig. 9. In case of cotransfer of recombinant mouse SLC i.p. or i.v. with ES-DC-OVA/mock, tumor indexes were similar



**FIGURE 9.** No effect of simultaneous injection of recombinant mouse SLC together with ES-DC-OVA. Mice were immunized with ES-DC-OVA/mock ( $2 \times 10^4$ /mouse) with or without simultaneous injection of recombinant mouse SLC ( $3 \mu\text{g}$ , i.v. or i.p.). Other mice were immunized with ES-DC-OVA/SLC ( $2 \times 10^4$ /mouse). Transfers of ES-DCs plus SLC were done twice at a 7-day interval, and 7 days after the second transfer, mice were challenged with  $3 \times 10^5$  MO4 cells. The tumor index (in millimeters) 30 days after the MO4 challenge was shown. In mice immunized with ES-DC-OVA/SLC, the tumor index was significantly smaller than the others ( $p < 0.05$ ). Results are expressed as mean tumor index  $\pm$  SD ( $n = 4-6$  per group).

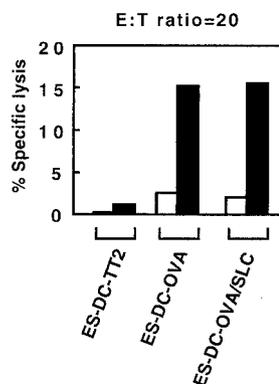
to those in case of immunization with  $2 \times 10^4$  ES-DC-OVA/mock, indicating that coinjection of recombinant mouse SLC was without effect. In contrast, in case of immunization with ES-DC-OVA/SLC, the tumor index was significantly smaller than those in other conditions ( $p < 0.05$ ), such being consistent with the data shown in Fig. 8.

#### No effect of SLC on survival of ES-DCs and on CTL priming activity of ES-DC in vitro

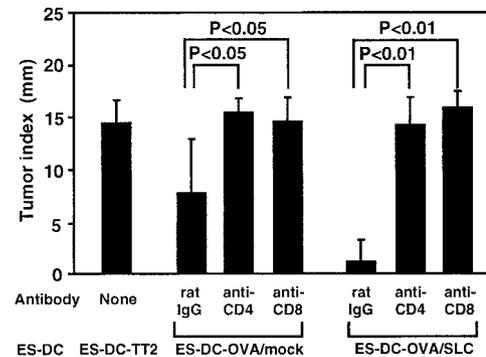
We tested to see whether the SLC would have any effect on the survival of DCs in vitro. ES-DC-OVA/mock and ES-DC-OVA/SLC were cultured for 7 days. Other ES-DC-OVA/mock were cultured in the presence of recombinant mouse SLC (300 ng/ml). Numbers of recovered ES-DCs after the culture were 77.8%, 88.3%, and 77.7% of the starting cells in case of ES-DC-OVA/mock, ES-DC-OVA/SLC, and ES-DC-OVA/mock plus recombinant mouse SLC, respectively. Dead cells were fewer than 1% of the recovered cells under any conditions. These results indicate that the SLC have no significant effect on the survival of DCs in vitro. In addition, there was no difference in the in vitro CTL-priming capacity between ES-DC-OVA and ES-DC-OVA/SLC (Fig. 10). These results suggest that the enhanced CTL-priming by ES-DC-OVA/SLC observed in case of in vivo injection is not due to the direct effect of SLC on ES-DCs.

#### Involvement of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in protection against MO4 induced by ES-DCs expressing OVA

To determine the role of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in protection against tumor cells induced by genetically modified ES-DCs, we depleted mice of CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes by treatment with anti-CD4 or anti-CD8 mAb in vivo, respectively. By this treatment, >90% of CD4<sup>+</sup> and CD8<sup>+</sup> T cells were depleted (data not shown). During this procedure, mice were immunized with ES-DC-OVA/SLC or ES-DC-OVA/mock and challenged with MO4 cells. As shown in Fig. 11, depletion of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells totally abrogated the protective immunity induced by ES-DC-OVA/SLC or ES-DC-OVA/mock. Although some populations of physiological DCs have been reported to express CD4 or CD8 molecules, the number of CD11c<sup>+</sup> splenic DCs did not change with this treatment (data not shown), indicating that the abrogation



**FIGURE 10.** Similar capacity of ES-DC-OVA and ES-DC-OVA/SLC to prime OVA-specific CTLs in vitro. ES-DC-TT2, ES-DC-OVA, and ES-DC-OVA/SLC ( $4 \times 10^5$ /well) were cocultured with nylon wool-purified splenic T cells ( $2.5 \times 10^6$ /well) of unprimed CBF<sub>1</sub> mice in 24-well culture plates. After 5 days, the cells were harvested and assayed for the capacity to kill EL-4 tumor cells either pulsed with 10  $\mu$ M OVA peptide (■) or left unpulsed (□). Results are expressed as mean specific lysis of triplicate assays, and SDs of triplicates were <2%. Data are representative of two independent and reproducible experiments.



**FIGURE 11.** Involvement of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in antitumor immunity induced by ES-DCs. CD4<sup>+</sup> or CD8<sup>+</sup> T cells were depleted in vivo by inoculation of anti-CD4<sup>+</sup> or anti-CD8<sup>+</sup> mAbs during immunization with ES-DC-OVA/mock or ES-DC-OVA/SLC. As control, other mice were given i.p. by transfer of ES-DC-TT2. The mice were challenged s.c. with  $3 \times 10^6$  MO4 tumor cells, and tumor measurements were made 15 days after the tumor cell challenge. In case of immunization with ES-DC-OVA/mock, the differences in tumor index between mice inoculated with rat IgG and those with anti-CD4 mAb as well as between mice inoculated with rat IgG and those with anti-CD8 mAb are statistically significant ( $p < 0.05$ ). In case of immunization with ES-DC-OVA/SLC, the differences in tumor index between mice inoculated with rat IgG and those with anti-CD4 mAb as well as between mice inoculated with rat IgG and those with anti-CD8 mAb are statistically significant ( $p < 0.01$ ). Results were expressed as mean tumor index + SD ( $n = 8$  per group).

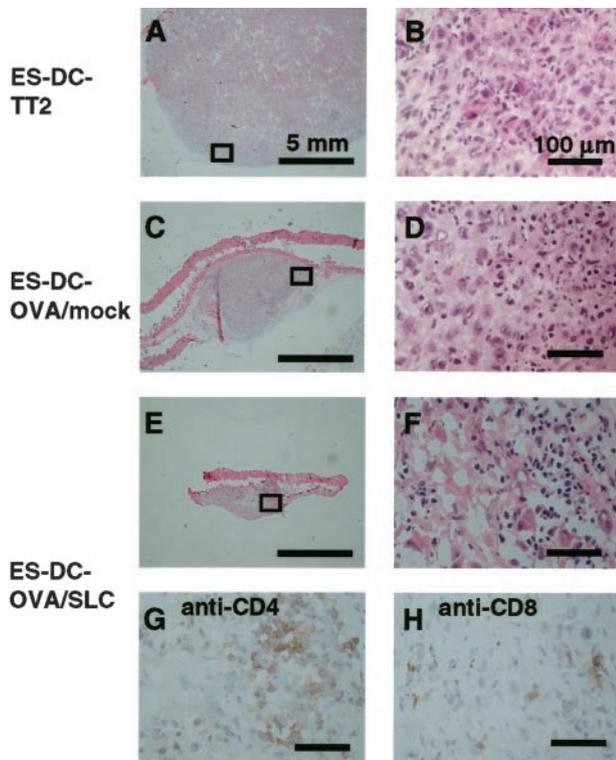
of protective immunity by Ab treatment is due to the depletion of T cells and not due to the effect on endogenous host DCs. These results suggest that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells play critical roles in antitumor immunity induced by OVA-expressing DCs, regardless of whether or not they coexpress SLC.

We histologically investigated the tumor tissues to search for infiltration of lymphocytes. As shown in Fig. 12, A–F, the size of the tumor in mice immunized with ES-DC-OVA/SLC was much smaller than that of mice immunized with ES-DC-OVA/mock or ES-DC without OVA (ES-DC-TT2). There was a large number of inflammatory cells infiltrating into tumor tissues of mice immunized with ES-DCs expressing OVA, particularly in mice immunized with ES-DC-OVA/SLC. The infiltrating cells consisted of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 12, G and H). These results also suggest that the antitumor effect induced by ES-DC expressing SLC along with OVA is mediated by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

## Discussion

In the present study, we attempted to improve the capacity of in vivo transferred DCs to prime T cells by genetic modification to express a chemokine with a T cell-attracting property. Among the chemokines, we comparatively evaluated the effects of three chemokines, SLC, Mig, and Lptn, not produced by DCs under physiological conditions. For the genetic modification of DCs, we used a method to generate DCs from mouse ES cells. By sequential transfection of ES cells with expression vectors for OVA Ag and for chemokines and by subsequent induction of differentiation to DCs, we generated DCs expressing a chemokine along with OVA.

ES-DCs have a migratory capacity toward lymphoid tissues (Fig. 4) and the capacity is almost comparable to that of BM-DCs. ES-DCs expressing OVA could induce the Ag-specific priming of CTLs both in vivo and in vitro (Fig. 5). ES-DCs expressing OVA could prime OVA-specific CTLs in the absence of IL-2 in vitro, whereas stimulation with CD40 ligand (30) or presence of exogenous IL-2 (our unpublished observations) is essential for BM-DCs to prime Ag-specific CTLs in vitro. Therefore, the capacity of



**FIGURE 12.** Infiltration of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells into tumor tissues. Mice were transferred twice with ES-DC-TT2 (A and B), ES-DC-OVA/mock (C and D), or ES-DC-OVA/SLC (E–H). Seven days after the second transfer, mice were challenged with  $3 \times 10^6$  MO4 tumor cells. Twelve days after the tumor cell challenge, frozen sections of tumor tissues were made and stained with H&E (A–F) or immunostained with anti-CD4 (G) or anti-CD8 (H) mAb. F–H, Serial sections are shown. B, D, and F, Enlarged views of the portion indicated in the square of A, C, and E, respectively. Note that size of the tumor in mice immunized with ES-DC-OVA/SLC (E) was much smaller than that of mice immunized with ES-DC-TT2 (A) and ES-DC-OVA/mock (C). Scale bars are 5 mm (A, C, and E) and 100  $\mu$ m (B, D, F, G, and H).

Ag-expressing ES-DCs to induce CTLs specific to the Ag is no way inferior to BM-DCs. Recently, several reports suggested transfer of Ag or peptide-MHC complexes from adoptively transferred DCs to endogenous host DCs (8, 31). Therefore, it is possible that intrinsic host DCs played some role also in priming of CTLs in our system. However, based on the finding that transfer of heat-killed ES-DC-OVA did not induce priming of CTLs (Fig. 5B), we consider that the OVA-specific CTL-priming in our system mainly depends on the direct action of injected ES-DCs expressing OVA.

Among the three chemokines, expression of SLC was the most effective in eliciting protection against OVA-expressing tumor cells (Fig. 8). However, simultaneous injection of recombinant mouse SLC i.p. or i.v. together with an i.p. injection of DCs expressing OVA had no significant additive effect on protection against tumor (Fig. 9). In addition, SLC had no significant effect on the survival of DCs and CTL-priming capacity in vitro (Fig. 10). These results suggest that the enhanced immunizing effect of ES-DC-OVA/SLC observed with in vivo transfer is not due to the effect of SLC on ES-DCs but rather due to attraction of T cells to the site of transferred ES-DCs, and emphasize the significance of the production of the chemokine by DCs.

We consider that antitumor effects induced by transfer of ES-DCs expressing OVA are primarily mediated by CD4<sup>+</sup> and CD8<sup>+</sup> T cells reacting to OVA. This notion is supported by findings that

the antitumor effect was abrogated by depletion of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells by treatment with specific mAbs in vivo (Fig. 11). In addition, immunohistochemical analyses demonstrated obvious infiltration of both CD8<sup>+</sup> and CD4<sup>+</sup> T cells into tumor tissues in mice immunized with ES-DC-OVA/SLC (Fig. 12). The total abrogation of antitumor effects upon challenge with B16 tumor cells or derivative cells not only by depletion of CD8<sup>+</sup> T cells but also by depletion of CD4<sup>+</sup> T cells is consistent with reported data (32–34). In addition to providing aid for activation of CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells may directly attack B16 or MO4 cells that express MHC class II molecules upon stimulation with IFN- $\gamma$  (33).

Expression of Lptn in DCs enhanced CTL priming no less effectively than that of Mig. In contrast, expression of Lptn in DCs did not result in any significant enhancement of protection against tumor challenge. This observation is inconsistent with the report by Cao et al. (35) that showed the effect of expression of Lptn in peptide Ag-pulsed DCs on promoting protective antitumor immunity. The discrepancy between their report and ours may be attributed to retention of OVA-specific activated T cells nearby transferred ES-DC-OVA/Lptn in our experiments. Lptn attracts memory or activated rather than naive T cells (36). We consider that, under our experimental conditions, significant numbers of OVA-specific T cells primed with DCs transferred by the first transfer were particularly attracted toward ES-DCs expressing Lptn transferred by the second transfer, which was given 7 days before the tumor challenge, and the T cells could not efficiently migrate to site of the tumor cell inoculation. Although this speculation has not been experimentally verified, the selective attraction of effector/memory T cells by Lptn could be beneficial when we attempt to down-modulate immune responses by genetically modified ES-DCs, aiming at treatment of autoimmune diseases, and allergy or prevention of transplant rejection.

Although it has been demonstrated that SLC gene-introduced and tumor cell lysate-loaded DCs promoted strong antitumor responses (37), ours is the first study to comparatively evaluate effects of three chemokines. We generated DCs expressing chemokine simultaneously with antigenic protein. For induction of antitumor immunity, gene-based Ag-expression by DC is considered superior to peptide, protein, or cell lysate-loading in DC-based immunization. The expression of genes encoding for entire tumor-specific Ags circumvents the need for identification of specific CTL epitopes within the protein (38). Expression of tumor-specific Ags within DCs provides a continuous and renewable supply of Ags for presentation, as opposed to a single pulse of peptides or tumor cell lysates. In fact, in the current study, transfer of genetically modified ES-DCs ( $3 \times 10^3$  crossed two times) elicited significant CTL responses and protection against tumor challenge. Numerous tumor-associated Ags have been identified by investigators including us (39–41). We are planning to test antitumor effects of the newly identified natural tumor Ags in in vivo experiments using genetically modified ES-DCs expressing the Ags.

As for the methods for gene transfer to DCs, electroporation, lipofection, and virus vector-mediated transfection have been developed. Many clinical trials using DCs transfected with virus-based vectors are now in progress. However, there are several problems related to the presently used strategies, i.e., efficiency of gene transfer, stability of gene expression, potential risk accompanying the use of virus vectors, and immunogenicity of virus vectors. Although improvements have been made in these methods (42, 43), development of more efficient and safer means is needed. For ES cells, efficient methods for gene-transfer and for isolation of appropriate recombinant cell clones have been established. In the present study, we introduced ES cells sequentially with two

expression vectors containing puromycin-resistant and neo-R genes. It should be feasible to generate more than triple-gene transfectant ES-DCs by sequential or simultaneous transfection with multiple expression vectors, or by using an exchangeable gene-trap system (16, 44). Although formation of teratomas accompanying the transfer of ES cell-derived cells may be anticipated (45), we observed no apparent abnormality, including teratoma formation in mice transferred with ES-DCs 300 days before. When we tested our *in vitro* differentiation protocol with ES cell lines other than TT2 cells, we observed that DCs can be generated from all of these lines, which included ES cell lines of 129 and C57BL/6 mice origin. We are now planning to generate DCs expressing immunoregulatory molecules along with antigenic proteins, attempting Ag-specific immunosuppression as well as immunostimulation.

A method was established to generate mouse ES cell lines of an appropriate genetic background by nuclear transfer from allogeneic somatic cells to already established ES cell lines (46, 47). Recently, differentiation of hematopoietic cells from human and monkey ES cells has been reported (48, 49). Generation of DCs from human ES cells should also be feasible. With advances in the ES cell-related technologies, immunomodulation by genetically engineered ES-DCs may be applied to the treatment of autoimmune diseases and allergy, prevention of rejection of transplanted organs, and antitumor immunotherapy.

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