or stage. Our evaluation of stage IV individuals revealed the SPARC concentrations to be comparatively low in this group compared with those observed in the early stages. This suggested that SPARC may be a useful tumor marker for detecting relatively early-stage melanoma, although the mechanism of secretion of SPARC from melanoma remains to be elucidated. We are now undertaking further studies to answer these questions.

Positive correlation between the serum SPARC level and platelet count have been reported previously (36). In our work, the serum SPARC concentrations closely correlated with platelet counts in healthy donors (data not shown). In addition, no significant difference was observed between the platelet counts of healthy donors and melanoma patients [213,000 ± 9,000/mm^3 versus 216,000 ± 8,000/mm^3 (mean ± SE)]. In this study of SPARC measurements in sera, there were three (4.9%) false-positive cases, although those increased values were lower than those in most of the positive patients with melanoma. We thought the elevated SPARC concentrations in healthy donors might thus have been due to confounding conditions, such as thrombocytosis; however, no thrombocytosis cases were identified. Although we considered whether the influence of the platelets might be suppressed by measuring SPARC in plasma, we also had one false-positive case (1 of 21, 4.8%). The positive rate of increased plasma SPARC did not significantly differ from that of the increased serum SPARC in melanoma patients (36.4% versus 33.0%). Thereby, it is unlikely that increased SPARC levels in sera of melanoma patients are due to secretion of SPARC from platelets.

Melanocytic nevi tissues expressed both SPARC and GPC3 protein (10). The SPARC levels in sera were increased in two of five (40%) patients with large congenital melanocytic nevus, although the GPC3 level did not increase. We have to thus pay close attention to cases, which are GPC3 negative and SPARC positive, because of the risk of making a false-positive diagnosis.

We used sera from Japanese patients only. The incidence of acral lentiginous melanoma in the Japanese population is much higher than that in Caucasians, whereas superficial spreading melanoma and lentigo maligna melanoma are frequent types observed in Caucasians. Some groups have reported that acral lentiginous melanoma differs from other types of melanomas in its clinical, histopathologic, and genetic characteristics (37–40). We compared the positive rate of increased serum SPARC and GPC3 among patients classified by these clinical types. Thus, no significant correlations were observed between the positive rate and melanoma types (data not shown). Therefore, it seems likely that the usefulness of SPARC and GPC3 for diagnosis of melanoma is not restricted to only Japanese patients.

In conclusion, SPARC was found to be a useful tumor marker for melanoma particularly at the early stage of the disease, and the addition of the other two markers (GPC3 and 5-*S*-cysteinyldopa) had added benefit in diagnosis. On the other hand, the serum levels of these three markers are still unknown in a population of patients with atypical nevi syndrome or other high-risk population in this study. Further investigations are needed to consider future applications of serum SPARC and GPC3 for the mass screening of melanoma.

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Cancer prevention with semi-allogeneic ES cell-derived dendritic cells ☆

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Abstract

Dendritic cells (DC) genetically modified to present tumor-associated antigen are a promising means for anti-cancer immunotherapy. By introducing expression vectors into ES cells and subsequently inducing differentiation to DC (ES-DC), we can generate transfectant DC expressing the transgenes. In the future clinical application of this technology, the unavailability of human ES cells genetically identical to the patients will be a problem. However, in most cases, semi-allogeneic ES cells sharing some of HLA alleles with recipients are expected to be available. In the present study, we observed that model tumor antigen (OVA)-expressing mouse ES-DC transferred into semi-allogeneic mice potently primed OVA-reactive CTL and elicited a significant protection against challenge with OVA-expressing tumor. Genetic modification of ES-DC to overexpress SPI-6, the specific inhibitor of granzyme B, further enhanced their capacity to prime antigen-specific CTL in semi-allogeneic recipient mice. These results suggest the potential of ES-DC as a novel means for anti-cancer immunotherapy.

Keywords: Anti-cancer immunotherapy; Embryonic stem cells; Dendritic cells; Serine proteinase inhibitor

Activation of CTL reactive to tumor-associated antigens is crucial for anti-tumor immunotherapy. Dendritic cells (DC) are potent immune-stimulators, and the adoptive transfer of antigen-loaded DC has proven to be an efficient method for priming T cells specific to the antigen. DC-based methods are now regarded as a promising approach for anti-cancer immunotherapy. For loading tumor antigens to DC for anti-cancer immunotherapy, gene-based antigen-expression by DC is considered to be superior to loading antigen as peptide, protein, or tumor cell lysate [1]. For efficient gene transfer to DC, the use of virus-based vectors is required because DC are relatively reluctant to genetic modification. Clinical trials using DC genetically modified with virus vectors, for example, monocyte-derived DC introduced with adenovirus vectors encoding for tumor antigens, are now in progress. Considering the broader medical applications of this method, the drawbacks of genetic modification with virus vectors include the potential risk accompanying the use of virus vectors and legal restrictions related to it. Thus, the development of safer and more efficient means would be desirable.

We recently established a novel method for the genetic modification of DC [2]. In the method, we generated DC from mouse embryonic stem (ES) cells

☆ *Abbreviations:* ES cell, embryonic stem cell; ES-DC, embryonic stem cell-derived dendritic cell; BM-DC, bone marrow-derived dendritic cell; SPI-6, serine proteinase inhibitor 6; PI-9, proteinase inhibitor 9; neo-R, neomycin resistant; IRES, internal ribosomal entry site.

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by in vitro differentiation. The capacity of ES cell-derived DC (ES-DC) to simulate T cells was comparable to that of DC generated in vitro from BM cells (BM-DC). We can readily generate genetically modified ES-DC by introducing expression vectors into ES cells and the subsequent induction of their differentiation into ES-DC. The transfection of ES cells can be done with electroporation using plasmid vectors, and the use of virus-based vectors is not necessary. Once a proper ES cell transfectant clone is established, it then serves as an infinite source for genetically modified DC.

In the future clinical application of this technology, we will face the problem of histoincompatibility between patients to be treated and the ES cells as source of DC. In general, ES cells genetically identical to patients will not be available. However, ES cells sharing some of HLA class I alleles with the patients are expected to be available in most cases. By adoptive transfer of ES-DC derived from such semi-allogeneic ES cells, we will be able to stimulate tumor antigen-specific CTL restricted to the shared HLA molecules. The obstacle to performing anti-cancer immunotherapy by this strategy would be the presence of allogeneic antigen-reactive T cells, which mainly recognize the HLA molecules expressed by ES-DC but not by the recipients. It is anticipated that such allo-reactive T cells, mainly $CD8^+$ CTL, may attack the transferred APC and rapidly eliminate them, based on a previous report [3]. Mouse BM-DC bearing antigens adoptively transferred are rapidly eliminated, if CTL recognizing the antigens already exist in the recipient mice [4]. Thus, the crucial point is whether the transferred ES-DC can activate tumor antigen-specific CTL restricted to the shared MHC class I molecules before they are eliminated by allo-reactive CTL.

In the present study, we addressed this issue using a mouse system. We adoptively transferred OVA-expressing ES-DC to semi-allogeneic mice and examined whether or not they could activate OVA-specific CTL and elicit protective immunity against tumor cells expressing OVA. We introduced an expression vector for SPI-6, a molecule specifically inhibiting the apoptotic effect of granzyme B, to OVA-transfectant ES cells, and generated double transfectant ES-DC expressing OVA and simultaneously overexpressing SPI-6. We then evaluated this strategy for improving the efficiency of ES-DC to prime antigen-specific CTL, by making ES-DC resistant to attack by CTL.

Materials and methods

Mice. CBA, BALB/c, and C57BL/6 mice were purchased from Clea Animal (Tokyo, Japan) or Charles River (Hamamatsu, Japan) and kept under specific pathogen-free conditions. Male CBA and female C57BL/6 mice were mated to produce (CBA × C57BL/6) F1 mice, and male BALB/c and female C57BL/6 mice were mated to produce (BALB/c × C57BL/6) F1 mice. The animal experiments in this study were approved by Animal Experiment Committee of Kumamoto University (permission number A16-074).

Cells. The ES cell line TT2, derived from (CBA × C57BL/6) F1 embryo, was maintained on a feeder cell layer of mouse primary embryonic fibroblasts, as previously described [5]. The OVA-transfectant ES cell clone (TT2-OVA) generated previously by introduction of OVA-expression vector, pCAG-OVA-IP, to TT2 ES cells was maintained with a sporadic selection with puromycin (2 μg/ml) [6]. MO4 was generated by the transfection of C57BL/6-derived melanoma B16 with the pAc-neo-OVA plasmid, as described [7]. The procedure for inducing the differentiation of ES cells to ES-DC has been described previously [2].

Generation of BM-DC. The generation of dendritic cells from mouse BM cells was done according to the reported procedures [8] with some minor modifications. In brief, bone marrow cells were isolated from (C57BL/6 × CBA) F1 mice and cultured in bacteriological petri dishes (3.0×10^6/7.5 ml medium/90 mm dish) in RPMI-1640 medium supplemented with 10% FCS, GM-CSF (500 U/ml), IL-4 (20 ng/ml), and 2-ME (50 μM) [4]. The culture medium was changed by half on day 3, and floating cells harvested by pipetting between 6 and 8 days of the culture were used in the assays.

Analysis of the priming of CTL in vivo. The indicated number of ES-DC or BM-DC was injected i.p. into the mice twice with a 7-day interval. In some experiments, ES-DC were heat-killed by incubation at 70 °C for 20 min before injection. The mice were sacrificed 7 days after the second injection and spleen cells were isolated. After hemolysis, the spleen cells were cultured in RPMI-1640/10% horse serum/2-ME (50 μM) containing $OVA_{257-264}$ peptide (0.1 μM) and recombinant human IL-2 (100 U/ml). Five days later, the cells were recovered and used as effector cells in a cytotoxicity assay. As target cells, EL-4 thymoma cells were labeled with sodium [^{51}Cr]chromate for 1 h and washed. Subsequently, target cells were incubated in 24-well culture plates (1×10^6 cells/well) with or without 10 μM OVA peptide for 3 h, washed, and seeded into 96-well round-bottomed culture plates (5×10^3 cells/well). The effector cells were added to the target cells according to the indicated E/T ratio and incubated for 4 h at 37 °C. At the end of the incubation, supernatants (50 μl/well) were harvested and counted on a gamma counter. The percentage of specific lysis was calculated as: 100 × [(experimental release − spontaneous release)/(maximal release − spontaneous release)]. The spontaneous release and maximal release were determined in the presence of medium alone and PBS-1% Triton X-100, respectively.

Tumor challenge experiments. The indicated number of genetically modified ES-DC was transferred i.p. into (CBA × C57BL/6) F1 or C57BL/6 mice. Such transfer was done twice with a 7-day interval and, 7 days after the second transfer, 2 or 3×10^5 MO4 cells were injected s.c. in the shaved left flank region. The tumor sizes were determined biweekly in a blinded fashion and the survival rate of mice was also monitored. The tumor index was calculated as: tumor index (in millimeters) = square root (length × width).

Western blot analysis. The cell samples were lysed in an appropriate amount of lysing buffer, 150 mM NaCl, 50 mM Tris, pH 7.4, 1% Nonidet P-40, 1 mM sodium orthovanadate (Wako, Osaka, Japan), 1 mM EDTA, plus a protease inhibitor tablet (Amersham, Arlington Heights, IL). The supernatant fluids of the lysates were separated by 10% SDS–PAGE and then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were then blocked with 5% skimmed milk and 0.2% Tween 20 in Tris-buffered saline. Subsequently, the membranes were incubated with the anti-human PI-9 (mouse mAb, Alexis Biochemicals), cross-reactive to mouse SPI-6, or the anti-β-actin (mouse mAb, SIGMA), followed by HRP-conjugated rabbit anti-mouse Ig. The signal was detected using the ECL detection kit (Amersham Bioscience).

Generation of double transfectant ES-DC expressing OVA and overexpressing SPI-6. Mouse SPI-6 cDNA was prepared by RT-PCR amplification from total RNA of mouse spleen cells with PCR primers 5′-gagactcgagcccgccgccaccatgaatactctgtctgaaggaaat-3′ and 5′-gagagcggccgcgtgtctttatggagatgagaacct-3′. The design of these primers results in the cloning of SPI-6 cDNA downstream of the Kozak sequence. The PCR products were subcloned into a pGEM-T-Easy vector (Promega, Madison, WI), and the cDNA insert was then confirmed by a sequencing analysis. The cDNA fragments were cloned into pCAG-INeo, a mammalian expression vector driven by a CAG promoter and containing the internal ribosomal entry site (IRES)-neomycin resistance gene cassette [6], to generate pCAG-SPI-INeo (Fig. 4B). The transfection of ES cells and the induction of the differentiation of ES cells into DC were done as previously described [2].

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

Results

Priming of antigen-specific CTL by adoptive transfer of antigen-expressing ES-DC into syngeneic and semi-allogeneic recipients

We tested whether or not OVA-transfectant ES-DC (ES-DC-OVA) derived from TT2 ES cells (H-$2^{k/b}$), which originated from a CBA (H-2^{k}) × C57BL/6 (H-2^{b}) F1 embryo, could prime OVA-specific CTL upon adoptive transfer into semi-allogeneic C57BL/6 mice. Both the TT2-derived ES-DC and C57BL/6 mice possess the H-2^{b} haplotype, but MHC of the H-2^{k} haplotype expressed in ES-DC is allogeneic to the recipient C57BL/6 mice.

ES-DC-OVA or non-transfectant ES-DC (ES-DC-TT2) were injected i.p. into syngeneic (CBA × C57BL/6) F1 mice (H-$2^{k/b}$) or semi-allogeneic C57BL/6 mice twice with a 7-day interval. The spleen cells were isolated from the mice 7 days after the second injection and cultured in vitro in the presence of a K^{b}-binding $OVA_{257–264}$ peptide. After 5 days, the cultured spleen cells were recovered and assayed for their capacity to kill EL-4 thymoma cells (H-2^{b}) pre-pulsed with the OVA peptide. The results shown in Figs. 1A and B indicate that OVA-specific, H-2^{b}-restricted CTL was induced in both (CBA × C57BL/6) F1 and C57BL/6 mice injected with ES-DC-OVA but not in those injected with ES-DC-TT2. Although the results suggest that ES-DC-OVA primed OVA-specific CTL before they were killed by H-2^{k}-reactive T cells, it was also possible that the OVA protein released from ES-DC-OVA killed by allo-reactive CTL was taken up by endogenous APC and presented to prime OVA-specific CTL. To assess this possibility, we heat-killed ES-DC-OVA before injection into C57BL/6 mice. As shown in Fig. 1C, injection of heat-killed ES-DC-OVA did not result in priming of OVA-specific CTL. These results indicate that priming of OVA-specific CTL was mostly mediated by the direct presentation of OVA epitope by ES-DC-OVA, but not by cross-presentation by endogenous host APC. Thus, antigen-expressing ES-DC injected into semi-allogeneic mice can prime CTL specific to the antigen before they are killed by allo-reactive T cells. In addition, ES-DC-OVA primed OVA-specific CTL also in (BALB/c × C57BL/6) F1 mice (H-$2^{d/b}$) (Fig. 1D).

We next examined the priming of OVA-specific CTL by semi-allogeneic ES-DC under the condition by which the allo-reactive CTL that could attack the ES-DC had been pre-activated. We injected ES-DC-TT2 without an expression of OVA into C57BL/6 mice and 7 days later injected ES-DC-OVA. Under this condition, the first injected ES-DC-TT2 activated H-2^{k}-reactive CTL, and the ES-DC-OVA injected 7 days later would be attacked more rapidly by the once primed H-2^{k}-reactive CTL than in the former condition. The spleen cells were isolated 7 days after the second injection, and the CTL activity was analyzed by the same procedure as described above. As shown in Fig. 1E, a substantial priming of K^{b}-restricted OVA-specific CTL was also observed under this condition. This result indicates that, even in the presence of pre-activated allo-reactive CTL, antigen-expressing ES-DC is able to prime the antigen-specific CTL.

Induction of protective immunity against OVA-expressing tumor cells by ES-DC expressing OVA in semi-allogeneic recipients

We next asked whether CTL primed by ES-DC-OVA adoptively transferred into semi-allogeneic mice could protect the recipient mice from a subsequent challenge with tumor cells expressing OVA antigen. ES-DC-OVA were i.p. transferred into (CBA × C57BL/6) F1 mice or C57BL/6 mice twice with a 7-day interval and 7 days after the second transfer, the mice were inoculated s.c. with MO4, OVA-expressing B16 melanoma cells originating from a C57BL/6 mouse. As shown in Figs. 2A and B, the transfer of ES-DC-OVA into syngeneic (CBA × C57BL/6) F1 mice elicited a significant degree of protection against the challenge with MO4 in comparison to the transfer of ES-DC-TT2, and these findings were consistent with our previous report [6]. The transfer of ES-DC-OVA provided protection also in the semi-allogeneic C57BL/6 mice (Figs. 2C and D). These results suggest that the anti-cancer cellular vaccination with ES-DC genetically engineered to express tumor antigens is effective not only in syngeneic but also in semi-allogeneic recipients.

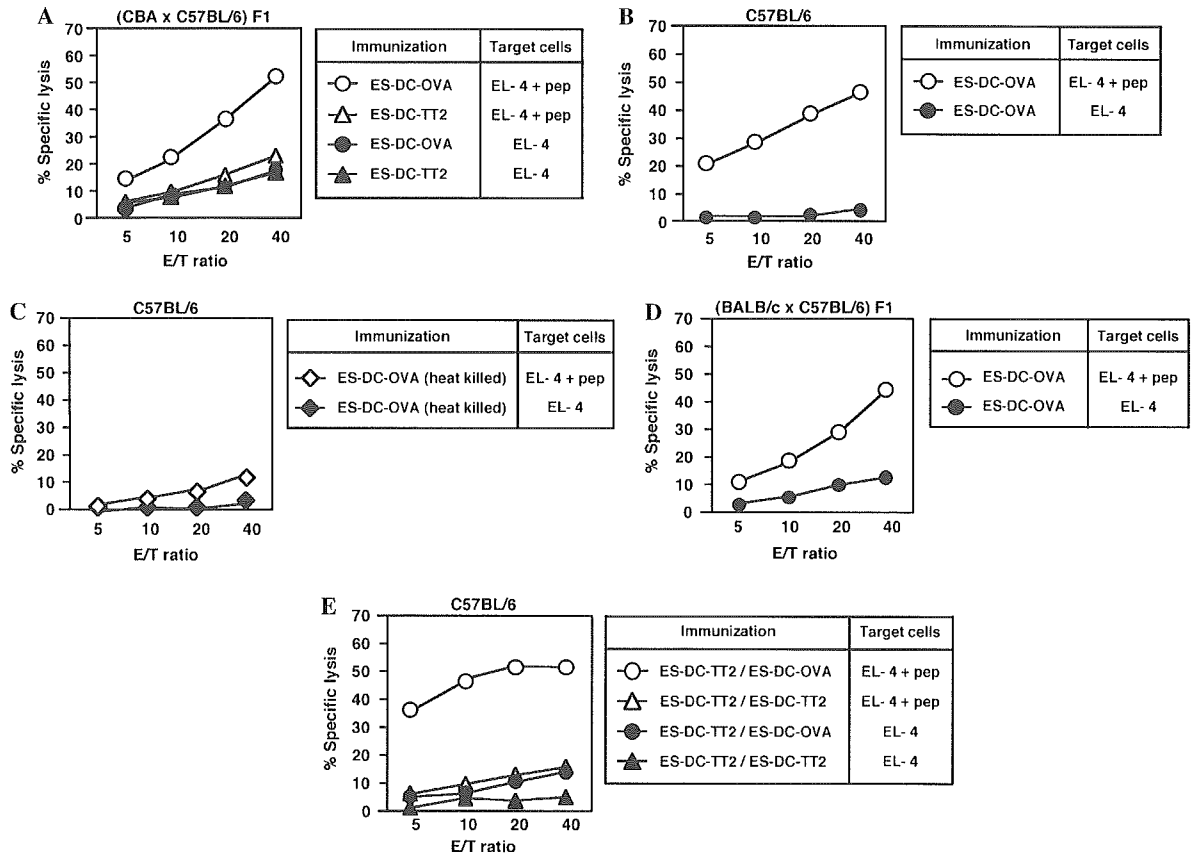

Fig. 1. Stimulation of OVA-specific CTL by ES-DC expressing OVA in syngeneic and semi-allogeneic mice. (CBA × C57BL/6) F1 (A), C57BL/6 (B, C), or (BALB/c × C57BL/6) F1 (D) mice were injected i.p. twice with ES-DC-OVA or ES-DC-TT2 (1×10^5/injection/mouse) on days −14 and −7. In (C), ES-DC-OVA were heat-killed before injection. C57BL/6 mice were injected with ES-DC-TT2 on day −14 and injected with ES-DC-OVA on day −7 (E). Spleen cells from the ES-DC-injected mice were harvested on day 0, pooled for each group (three mice per group), and cultured in the presence of $OVA_{257-264}$ peptide (0.1 μM) for 5 days. Next, the cells were harvested and assayed for their activity to kill EL-4 tumor cells either pulsed with 10 μM OVA peptide or left unpulsed. The results are expressed as the mean specific lysis of triplicate assays and SEM of triplicates were less than 2%.

Genetic modification of ES-DC to express antigen is superior to the loading of antigenic peptide to BM-DC in the priming of antigen-specific CTL in semi-allogeneic mice

The above described results were somewhat unexpected, considering the results of a previous study with peptide antigen-loaded BM-DC [4]. In that study, the presence of CTL in the recipient mice recognizing certain antigens presented by transferred DC severely diminished the priming of CTL specific to another antigen presented by the same DC. A possible reason for the substantial priming of antigen-specific CTL observed in our present experiments is that ES-DC is superior to BM-DC in priming antigen-specific CTL upon transfer to semi-allogeneic mice. Another possible reason is that, as a means for loading the antigen to DC to simulate CTL, the genetic modification of DC to produce antigenic protein is more efficient than the loading of antigenic peptide to DC.

To address the former possibility, we compared the efficiency of priming of OVA-specific CTL by the transfer of BM-DC and ES-DC. We isolated BM cells from (CBA × C57BL/6) F1 mice and generated BM-DC, which were genetically identical to ES-DC-TT2. BM-DC or ES-DC-TT2 were pre-pulsed with $OVA_{257-264}$ synthetic peptide (10 μM) for 2 h and injected i.p. into C57BL/6 mice. The injections were done twice with a 7-day interval, and 7 days after the second injection the spleen cells were isolated and the priming of OVA-specific CTL was analyzed by the method described above. The degree of priming of OVA-specific CTL by peptide-loaded BM-DC was very slight. $OVA_{257-264}$ peptide-loaded ES-DC-TT2 primed OVA-specific CTL more efficiently than the BM-DC did (Fig. 3A). These results indicate that ES-DC is superior to BM-DC in priming antigen-specific CTL upon loading with antigen and transfer to semi-allogeneic mice. However, the magnitude of priming of OVA-specific CTL by OVA peptide-loaded ES-DC-TT2 was lower than that primed

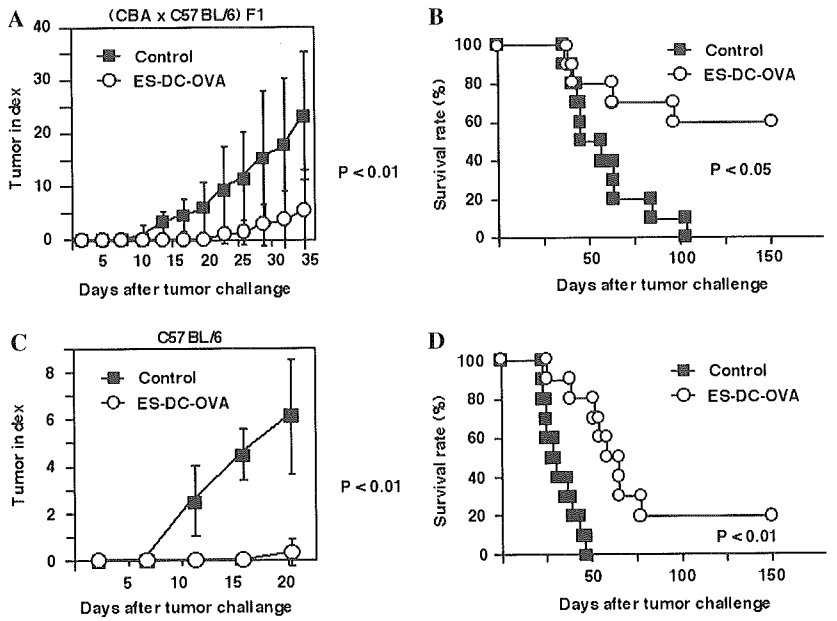

Fig. 2. Induction of protective immunity by ES-DC expressing OVA against OVA-expressing tumor cells in syngeneic and semi-allogeneic mice. (CBA × C57BL/6) F1 mice were injected i.p. twice on days −14 and −7 with ES-DC-OVA (2×10^4/injection/mouse) or medium (RPMI-1640) only as control and were challenged s.c. with MO4 tumor cells (3×10^5/mouse) on day 0 (A,B). C57BL/6 mice were injected with ES-DC-OVA (3×10^4/injection/mouse) and challenged with MO4 (2×10^5/mouse) by the same schedule (C,D). Growth of tumor (A,C) and survival of mice (B,D) were monitored. The tumor size was indicated as tumor index, square root of (length × width) in mm, ± SEM. The measurement of tumor sizes was stopped at the time point when one mouse of either of the mouse groups died (at day 35 in A and at day 20 in C). The differences in the tumor index and survival rate between ES-DC-OVA and control were significant ($P < 0.01$ in A, $P < 0.05$ in B, $P < 0.01$ in C, and $P < 0.01$ in D). For each experimental group, 10 mice were used.

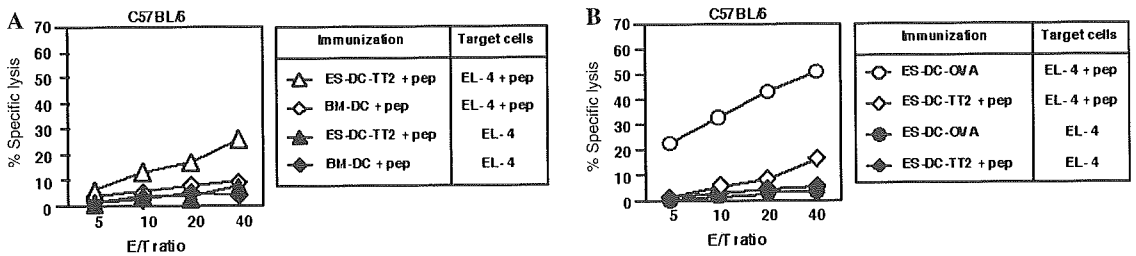

Fig. 3. Priming of OVA-specific CTL by OVA peptide-loaded DC or OVA-expressing ES-DC in semi-allogeneic mice. BM-DC or ES-DC-TT2 (non-transfectant ES-DC) were pulsed with $OVA_{257-264}$ synthetic peptide (10 μM) for 2 h and injected i.p. into C57BL/6 mice (1×10^5/injection/mouse) (A). ES-DC-OVA or OVA peptide-pulsed ES-DC-TT2 were injected i.p. into C57BL/6 mice (1×10^5/injection/mouse) (B). Injections were done twice on days −14 and −7. Spleen cells from the mice were harvested on day 0, and cytotoxic activity of OVA-specific CTL was analyzed as in Fig. 1.

by ES-DC-OVA, expressing transgene-derived OVA (Fig. 3B). The latter possibility mentioned above may thus also be true in that the genetic modification of DC to express antigen is superior to loading the antigenic peptide to DC in the priming of antigen-specific CTL.

Enhanced priming of antigen-specific CTL by ES-DC overexpressing SPI-6

As shown in Fig. 3A, ES-DC was superior to BM-DC in priming CTL in semi-allogeneic recipient mice. One possible reason for this was that ES-DC might be relatively resistant to attack by CTL and can survive for a longer period of time after transfer and thus primed OVA-specific CTL more efficiently than BM-DC did.

SPI-6 is a specific inhibitor of granzyme B, the major mediator of cytotoxic activity of CTL, and has been presumed to make DC resistant to attack by CTL during stimulation of CTL. As shown in Fig. 4A, SPI-6 was scarcely detected in BM-DC. On the other hand, ES-DC showed an evident expression of SPI-6. Thus, the substantial priming of OVA-specific CTL by ES-DC-OVA in semi-allogeneic mice may be attributed, at least in part, to the higher expression level of SPI-6. To verify the hypothesis that SPI-6 protected ES-DC from the cytotoxicity of allo-reactive CTL and resultingly enabled

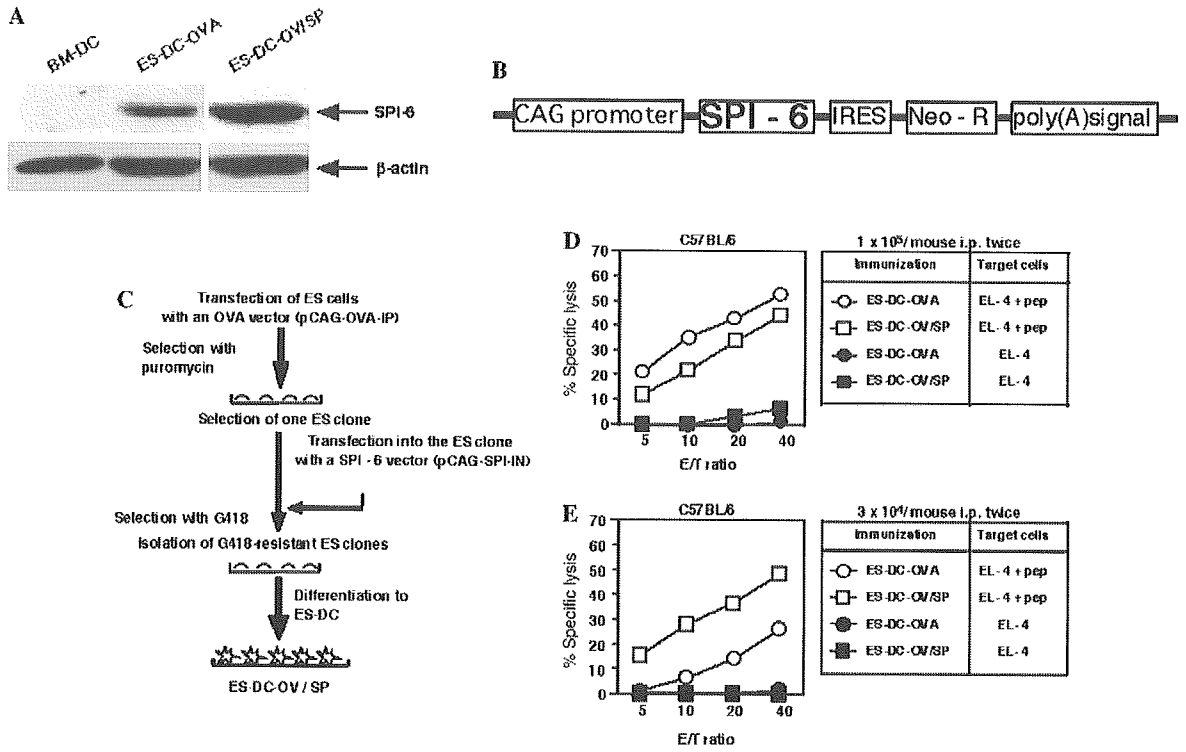

Fig. 4. Expression of SPI-6 in DC and priming of antigen-specific CTL by ES-DC expressing OVA and SPI-6 in semi-allogeneic mice. (A) The levels of expression of SPI-6 in BM-DC, ES-DC-OVA, and ES-DC-OV/SP were analyzed by a Western blotting analysis. The same samples were analyzed also for β-actin expression as control. (B) Structure of SPI-6 expression vector, pCAG-SPI-IN. (C) Schematic depiction of the generation of double transfectant ES-DC expressing OVA and overexpressing SPI-6. (D,E) ES-DC-OVA or ES-DC-OV/SP were injected i.p. to C57BL/6 mice (1×10^5/injection/mouse in D and 3×10^4/injection/mouse in E). Injections were done twice on days −14 and −7. Spleen cells were harvested from the mice on day 0, and activity of OVA-specific CTL was analyzed as shown in Fig. 1.

ES-DC to prime OVA-specific CTL more efficiently, we decided to generate double transfectant ES-DC expressing OVA and simultaneously overexpressing SPI-6. We made an expression vector for SPI-6 and introduced it into the OVA-gene transfectant ES cells (Fig. 4B). We then subjected the double transfectant ES cells to an induction culture for ES-DC, thereby generating ES-DC-OV/SP (Fig. 4C). As shown in Fig. 4A, ES-DC-OV/SP expressed a higher level of SPI-6 than ES-DC-OVA did. We compared ES-DC-OVA and ES-DC-OV/SP in their capacity to prime OVA-specific CTL in semi-allogeneic mice. The two clones of transfectant ES-DC were injected i.p. into C57BL/6 mice twice and the priming of OVA-specific CTL was analyzed. As shown in Fig. 4D, when 1×10^5 ES-DC were used for one injection, the degree of CTL-priming by ES-DC-OV/SP was similar to or somewhat lower than that primed by ES-DC-OVA. On the other hand, when lower number of ES-DC (3×10^4) were injected, ES-DC-OV/SP primed OVA-specific CTL more efficiently than ES-DC-OVA did (Fig. 4E). It is presumed that, when the lower number of ES-DC were transferred, the survival period of the injected ES-DC influenced more greatly the efficiency of priming OVA-specific CTL. Thus, the data shown in Figs. 4D and E suggest that an overexpression of SPI-6 in ES-DC improved the efficiency of priming OVA-specific CTL, and the effect was evident when a lower number of ES-DC were transferred for immunization.

Discussion

In recent years, a number of tumor-associated antigens have been identified, by the aid of genetic approaches such as expression cloning with tumor-reactive CTL, serological analysis of recombinant cDNA expression libraries (SEREX), or cDNA microarray analysis [9–13]. These antigens are potentially good targets for anti-cancer immunotherapies. To establish truly effective anti-cancer immunotherapy, development of a means for potently polarizing the immune system toward these tumor-associated antigens is essential. Anti-tumor immunotherapy with DC loaded with HLA-binding peptides derived from tumor antigens has been clinically tested in many institutions [14]. In most cases, DC are generated by the culture of monocytes obtained from peripheral blood of the patients. Apheresis, a procedure which is sometimes invasive for patients with cancer, is necessary to obtain a sufficient

number of monocytes as a source for DC. In addition, the culture to generate DC should be done separately for each patient and for each treatment, and thus the presently used method is labor-intensive and also expensive.

As a means for loading of tumor antigen to DC, genetic modification of DC to express antigenic protein has several advantages in comparison to the loading of peptide antigen to DC. The expression of genes encoding for tumor-specific antigens circumvents the need for identification of specific CTL epitopes within the protein. The expression of tumor antigens within DC provides a continuous and renewable supply of antigens for presentation, as opposed to a single pulse of peptides or tumor cell lysates. In most cases, adenovirus vector is used for the genetic modification of human monocyte-derived DC. However, there are several problems related to the use of adenovirus vectors, i.e., the efficiency of gene transfer, the stability of gene expression, and the potential risk accompanying the use of virus vectors. In addition, use of virus-based vectors outside of isolated laboratories is prohibited by law in many countries.

As we showed in both our previous and the present report, we can use ES cell transfectants as an infinite source of genetically modified DC. If the ES cell-based method can be clinically applied, then the repeated isolation of monocytes from patients is not necessary. In addition, we will be able to generate genetically engineered DC without the use of virus vectors, because the genetic modification of ES cells can be done with the introduction of plasmid DNA by electroporation. Furthermore, it is feasible to generate multiple gene-transfectant ES-DC with enhanced capacity to elicit anti-tumor immunity, by the sequential transfection with multiple expression vectors as demonstrated in our present and previous reports [6,15].

Considering clinical application, one drawback of the ES-DC method is the unavailability of human ES cells genetically identical to the patients to be treated. Based on previous studies, the stimulation of antigen-specific CTL by antigen-bearing, semi-allogeneic APC is considered to be difficult. The efficiency of priming antigen-specific CTL by adoptively transferred BM-DC presenting the antigen significantly decreased if the DC were targets of a pre-existing CTL [4]. APC transferred to MHC-incompatible mice were rapidly eliminated by allo-reactive CTL of the recipient mice [3]. However, the results of the present study revealed that adoptively transferred mouse ES-DC expressing OVA stimulated OVA-specific CTL not only in syngeneic (CBA × C57BL/6) F1 mice but also in semi-allogeneic C57BL/6 and (BALB/c × C57BL/6) F1 mice (Fig. 1). The OVA-specific CTL, and probably also the OVA-specific helper T cells, were primed by OVA-expressing ES-DC and protected the recipient C57BL/6 mice from subsequent challenge with tumor cells bearing OVA (Fig. 2). These results thus show the promise of prevention of cancer with ES-DC.

As shown in Fig. 4A, upon loading with $OVA_{257-264}$ peptide and transfer into semi-allogeneic C57BL/6 mice, ES-DC primed $OVA_{257-264}$-specific CTL more potently than BM-DC did, thus suggesting that ES-DC was superior to BM-DC in priming antigen-specific CTL in semi-allogeneic conditions. ES-DC-OVA, ES-DC genetically engineered to express OVA, was further more potent than OVA peptide-loaded ES-DC in the priming of OVA-specific CTL (Fig. 4B). Thus, the substantial priming of antigen-specific CTL by ES-DC-OVA in semi-allogeneic mice may be partly due to the efficient CTL-priming capacity of ES-DC and also due to the method of loading of antigen, namely genetic modification.

The level of expression of SPI-6 in ES-DC was higher than that in BM-DC (Fig. 4). SPI-6, the mouse homologue of human protease inhibitor 9 (PI-9), is a specific inhibitor of granzyme B, the major mediator of cytotoxicity of CTL [16–18]. SPI-6 is expressed in CTL, DC, and mast cells and it has been hypothesized to protect these cells from granzyme B-mediated apoptosis during immune responses [19–22]. It has recently been reported that the co-administration of expression vector for SPI-6 with a DNA vaccine for tumor antigen enhanced the vaccination potency, possibly because the expression of the vector-derived SPI-6 made antigen-presenting DC resistant to cytotoxic activity of CTL [23]. Thus, an evident intrinsic expression of SPI-6 in ES-DC may be one reason for that the capacity of OVA-expressing ES-DC to stimulate CTL in semi-allogeneic recipient mice was more potent than that of BM-DC. To address this possibility, we introduced OVA-transfectant ES cells with an expression vector for SPI-6, and thus generated double transfectant ES-DC expressing OVA and overexpressing SPI-6, ES-DC-OV/SP. ES-DC-OV/SP primed OVA-specific CTL more efficiently than ES-DC-OVA did when lower number (3×10^4/injection) of cells was transferred for immunization. It is thus suggested that the overexpression of SPI-6 by genetic modification of ES-DC prolonged their survival after transfer to semi-allogeneic mice and enhanced the priming of OVA-specific CTL (Fig. 4C).

Bcl-2 and Bcl-xL are anti-apoptotic proteins which block the apoptosis induced by various apoptotic signals, and they are reported to be involved in the control of the lifespan of DC [24–26]. We also examined the level of expression of Bcl-2 and Bcl-xL in BM-DC and ES-DC. Both ES-DC and BM-DC expressed Bcl-2 and Bcl-xL, and ES-DC expressed lower level of Bcl-2 and higher level of Bcl-xL than BM-DC did (data not shown). It is possible that an overexpression of Bcl-2 or Bcl-xL by genetic modification of ES-DC may also have an ability to enhance the efficiency to prime antigen-specific CTL in vivo.

For the efficient induction of cytotoxic effector function of $CD8^+$ T cells, $CD4^+$ helper T cells are known to play a crucial role. They produce cytokines such as IL-2 and IFN-γ, which directly stimulate CTL, and make DC more potent in activation of T cells, via CD40–CD40–ligand interactions. After the adoptive transfer of semi-allogeneic ES-DC presenting tumor antigen, a large number of allo-reactive $CD4^+$ T cells of the recipients may be activated by MHC class II molecules expressed on ES-DC and provide potent help for priming of antigen-reactive CTL. Therefore, while the expression of allogeneic MHC class I by transferred ES-DC may reduce the efficiency of the induction of anti-tumor immunity, allogeneic MHC class II expressed by ES-DC may confer considerable advantages for induction of anti-tumor immunity.

In order to realize the future clinical application of ES-DC, we recently established a method to generate ES-DC from non-human primate, cynomolgus monkey, ES cells, and also for genetic modification of them (manuscript in preparation). We believe that this method should be applicable to human ES cells, although some modifications might be necessary. It is expected that human ES cells sharing some of the HLA alleles with patients are available in most cases. Although HLA genes are highly polymorphic, a few prevalent alleles exist in each locus of HLA gene for each ethnic group in general. For example, the gene frequency of HLA-A*0201, A*0206, A*2402, and A*2601 in Japanese population is 0.11, 0.10, 0.36, and 0.10, respectively [27]. This indicates that more than 90% of the Japanese people possess at least one of these four alleles in the HLA-A locus. So far, a number of human ES cells have been established, and most of the human ES cells probably have HLA alleles dominant in the ethnic group to which the donors belong. We can thus expect that human ES cell lines sharing some of the HLA alleles with patients to be treated will be available in most cases.

In the future, antigen-specific anti-tumor immunotherapy by the in vivo transfer of human ES-DC expressing tumor antigen may well be achieved. The overexpression of PI-9, the human homologue of mouse SPI-6, by genetic modification is a promising way to enhance the effect of the cellular vaccination using human ES-DC semi-allogeneic to the recipients. We believe that the present study paves the way for the future clinical application of anti-cancer immunotherapy utilizing ES-DC.

Acknowledgments

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DNA vaccination of HSP105 leads to tumor rejection of colorectal cancer and melanoma in mice through activation of both CD4+ T cells and CD8+ T cells

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We report that HSP105, identified by serological identification of antigens by recombinant expression cloning (SEREX), is overexpressed in a variety of human cancers, including colorectal, pancreatic, thyroid, esophageal, and breast carcinoma, but is not expressed in normal tissues except for the testis. The amino acid sequences and expression patterns of HSP105 are very similar in humans and mice. In this study, we set up a preclinical study to investigate the usefulness of a DNA vaccine producing mouse HSP105 whole protein for cancer immunotherapy *in vivo* using BALB/c and C57BL/6 mice, Colon26, a syngeneic endogenously HSP105-expressing colorectal cancer cell line, and B16.F10, a melanoma cell line. The DNA vaccine was used to stimulate HSP105-specific T-cell responses. Fifty percent of mice immunized with the *HSP105* DNA vaccine completely suppressed the growth of subcutaneous Colon26 or B16.F10 cells accompanied by massive infiltration of both CD4+ T cells and CD8+ T cells into tumors. In cell transfer or depletion experiments we proved that both CD4+ T cells and CD8+ T cells induced by these vaccines play critical roles in the activation of antitumor immunity. Evidence of autoimmune reactions was not present in surviving mice that had rejected tumor cell challenges. We found that HSP105 was highly immunogenic in mice and that the *HSP105* DNA vaccination induced antitumor immunity without causing autoimmunity. Therefore, HSP105 is an ideal tumor antigen that could be useful for immunotherapy or the prevention of various human tumors that overexpress HSP105, including colorectal cancer and melanoma. (*Cancer Sci* 2005; 96: 695–705)

Colorectal cancer (CRC) and melanoma are common and serious malignancies, for which surgery remains the main treatment, although the success of the treatment depends on the stage of the disease. Although adjuvant systemic chemotherapy or chemoradiation can confer a limited but significant survival advantage, novel and more effective therapies are needed. Identification of tumor associated antigens (TAA) expressed by CRC or melanomas remains one of the goals for designing novel immunological treatments for these tumors. Ideal targets for immunotherapy are gene products that are silenced in normal tissues except immune privilege tissue such as testis tissue, and that are overexpressed in cancer cells.

More than 2000 candidate TAA have been identified by using the serological identification of antigens by recombinant expression cloning (SEREX) method. We have also reported TAA identified by using this method.[1–4] We earlier found that HSP105 (often called HSP110), as identified by SEREX was overexpressed specifically in a variety of human cancers, including colorectal, pancreatic, thyroid, esophageal, and breast carcinoma, but was not expressed in normal tissues except for testis tissue.[1,5] We recently found that HSP105 was also overexpressed in melanoma (unpublished data). If HSP105 can induce strong antitumor immunity, it may be a potential candidate as a target antigen for cancer immunotherapy. In the present study, we set up a preclinical study to investigate the usefulness of a *HSP105*-DNA vaccine, using BALB/c and C57BL/6 mice, the syngeneic endogenously HSP105-expressing CRC cell line Colon26, and the melanoma cell line B16.F10. Using these models, we analyzed both the antitumor effects and side-effects, including autoimmunity of the *HSP105* DNA vaccination.

The pioneering studies of Srivastava and colleagues led to the proposal that several HSP, including HSP70, HSP90 and gp96, bind antigenic peptides and deliver these peptides (through receptor-mediated endocytosis of the HSP) into the antigen-processing pathway of the antigen presenting cell (APC) for presentation on major histocompatibility complex (MHC) class I molecules. This HSP-involved pathway has been demonstrated to evoke potent antiviral and antitumor immune responses.[6] However, many researchers have identified MHC class I-presented peptide epitopes derived from HSP. HSP are

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Abbreviations: C26 (C20), Colon26 clone 20; CRC, colorectal cancer; CTL, cytotoxic T lymphocytes; HE, hematoxylin and eosin; HSP105, heat shock protein 105; APC, antigen presenting cell; mAb, monoclonal antibody; MHC, major histocompatibility complex; SEREX, serological identification of antigens by recombinant expression cloning; TAA, tumor associated antigens.

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rich sources of MHC-bound peptides, and the expression of these peptides increases as a result of cellular stresses.[7]

Recently, Subjeck and colleagues tested a vaccine using the chaperoning properties of HSP110 as Srivastava and colleagues had done before them.[8,9] They reported that HSP110 overexpression increases the immunogenicity of murine CT26 colon tumors.[10] *HSP110* cloned from CHO cells[11] and *HSP105* cloned from mice[12] and humans[13] are homologs. We show here that this HSP105 is highly immunogenic for stimulating tumor immunity against mouse CRC and melanoma. Furthermore, both $CD4^+$ T cells and $CD8^+$ T cells induced by the *HSP105* DNA vaccination play critical roles in the activation of antitumor immunity. These findings indicate that HSP105 itself could be considered a valuable TAA for the immune-based therapy of various tumors overexpressing HSP105, including CRC and melanoma.

Materials and Methods

Cell lines and mice

A subline of the BALB/c-derived CRC cell line Colon26, C26 (C20),[14] was provided by Dr Kyoichi Shimomura (Fujisawa Pharmaceutical Co., Japan). B16.F10 was kindly provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). These cell lines were maintained *in vitro* in RPMI-1640 medium supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere. Female 7-week-old BALB/c mice ($H\text{-}2^d$) and C57BL/6 mice ($H\text{-}2^b$), purchased from Charles River Japan (Yokohama, Japan), were kept in the Center for Animal Resources and Development (CARD) of Kumamoto University, and handled in accordance with the animal care policy of Kumamoto University.

Histological and immunohistochemical analysis

Immunohistochemical detections of HSP105, CD8 and CD4 were carried out as described elsewhere.[1,5,15–18] The primary antibody used in this study, rabbit polyclonal antihuman HSP105 was purchased from Santa Cruz (Santa Cruz, CA, USA). Hematoxylin and eosin (HE) staining and standard methods were used for histological analysis. We purchased Human Normal Organs and Cancer Multi Tissue Slide, BC4, from SuperBioChips Laboratories (Seoul, Korea) for immunohistochemical analysis.

Construction of a mouse *HSP105* expression plasmid DNA

Plasmid pcDNA105, which expresses mouse *HSP105* whole protein was generated as described elsewhere.[12] To construct this plasmid, the mouse *HSP105* full-length cDNA derived from the pB105-1 plasmid was subcloned into *Eco*RV–*Xba*I sites of the mammalian expression vector pcDNA3 (Invitrogen, Osaka, Japan). The pCAGGS expression vector was kindly provided by Dr Junichi Miyazaki (Osaka University, Japan) and this vector induces strong gene expression when injected into muscle.[19] We constructed a pCAGGS-*HSP105* plasmid by inserting mouse *HSP105* cDNA into the *Eco*RI site of the pCAGGS expression vector, which carries the CAG (cytomegalovirus immediate-early enhancer/chicken β-actin hybrid) promoter, and prepared the plasmid using a Qiagen EndoFree plasmid Mega kit (Qiagen GmbH, Hilden, Germany). We used the empty pCAGGS plasmid as a control.

DNA vaccination

We immunized mice twice by intramuscular injection into the anterior tibialis muscle. Booster immunization was carried out at 7 days after the primer immunization. The groups of mice were given the following vaccines: (i) saline group: given with 100 μL saline; (ii) control vector group: given 50 μg pCAGGS plasmids lacking inserts and diluted in 100 μL saline; (iii) *HSP105* DNA vaccine group: given 50 μg of pCAGGS-*HSP105* plasmid diluted in 100 μL saline.

In vivo tumor challenge

Subcutaneous tumors were established by the injection of 3×10^4 C26 (C20) cells or 1×10^4 B16.F10 cells suspended in 100 μL Hanks' Balanced Salt Solution (Gibco, Grand Island, NY, USA) medium into the right flank of BALB/c or C57BL/6 mice 7 days after the last vaccination. Tumor incidence and volume were assessed twice weekly using calipers until the mice died. Tumor area was calculated as a product of width and length. The results are presented as mean area of tumor ± SE; however, individual tumor area is presented for some experiments.

In vivo depletion of $CD4^+$ T cells and $CD8^+$ T cells

Each mouse was given a total of six intraperitoneal transfers (days −18, −15, −11, −8, −4, −1) of ascites (0.1 mL per mouse per transfer) from hybridoma-bearing nude mice. The mAbs used were rat antimouse CD4 (clone GK1.5) and rat antimouse CD8 (clone 2.43). Normal rat IgG (Sigma, St. Louis, MO, USA; 200 μg per mouse per transfer) was used as a control. The depletion of T cell subsets by treatment with mAbs was confirmed by flow cytometric analysis of spleen cells, which showed a > 90% specific depletion.

Cell transfer *in vivo*

We purified $CD8^+$ T cells, $CD4^+$ T cells, and natural killer (NK) cells from spleen cells using the magnetic cell sorting system with antimouse CD8α (Ly-2) mAb, antimouse CD4 (L3T4) mAb, antimouse NK (DX5) mAb, and these $CD8^+$ T cells, $CD4^+$ T cells, and NK cells were used for adoptive transfer into BALB/c mice. To investigate tumor growth in a homeostatic lymphocyte proliferation model, we intravenously injected 1.5×10^7 whole spleen cells or 3×10^6 $CD8^+$ T cells, $CD4^+$ T cells, NK cells, or $CD8^-$ $CD4^-$ NK^- cells 3 days after sublethal irradiation (5 Gy). Subsequently, we subcutaneously inoculated BALB/c mice with C26 cells (3×10^4) 3 days after irradiated mice inoculated with cells.

Statistical analysis

We analyzed all data using the StatView statistical program for Macintosh (SAS, Cary, NC, USA) and evaluated statistical significance using the unpaired *t*-test. The overall survival rate was calculated using the Kaplan–Meier method, and statistical significance was evaluated using Wilcoxon's test.

Results

Similar tissue and cancer-specific expression of HSP105 in mice and humans

We have previously reported that HSP105 is overexpressed in a variety of human cancers, including colorectal, pancreatic, esophageal, thyroid, and breast cancer, whereas HSP105 is

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expressed at low levels in many normal tissues, except for testis tissue.[1,5] In the present study, we carried out an immunohistochemical analysis of HSP105 using various human and mouse tissues (Fig. 1). Human HSP105 is overexpressed in almost all CRC cells, melanoma cells (unpublished data), and normal testis tissue, but there is no expression or only a low-level expression of HSP105 in normal liver, brain, spleen, lung, and kidney tissue (Fig. 1a). Mouse HSP105 is also overexpressed in liver metastasis of the murine colorectal adenocarcinoma cell line C26 (C20), lung metastasis of the murine melanoma cell line B16.F10 and normal testis tissue, but there is no expression or only low-level expression in normal liver, cerebrum, cerebellum, spleen, lung, and kidney tissue (Fig. 1b). Another group reported that HSP105/110 is expressed in neurons in the cerebrum and Purkinje cells in the cerebellum,[20] we found the same pattern in the present study, but the level of expression in the neurons and Purkinje cells was much weaker than that in CRC and testis tissue (Fig. 1a,b). As a result, the expression levels of HSP105 protein in human colorectal, pancreatic, esophageal, thyroid, and breast cancers, melanoma, C26 tumors, and B16.F10 tumors were evidently much higher than those in all normal adult tissues, including brain, but not testis in both humans and mice. Because the expression pattern of HSP105 is very similar in humans and mice, we are able to analyze both the antitumor effects and side-effects (including autoimmunity) of HSP105 vaccination using this mouse model of CRC and melanoma.

HSP105 DNA induced rejection of C26 and B16.F10 tumor challenge in mice

We investigated the effects of *HSP105* DNA vaccination using a subcutaneously injected C26 (Fig. 2a–d) and B16.F10 (Fig. 2e–h) tumor model. Mice were divided into three groups: mice inoculated with (i) saline; (ii) pCAGGS, and (iii) pCAGGS-*HSP105*. No mice died during the vaccination period.

Subcutaneous inoculation of C26 cells (3×10^4) into the right flank was given 7 days after the last vaccination (Fig. 2a–d). In groups (i) and (ii), subcutaneous tumors appeared in some mice 10 days after inoculation. Measurement of tumor size was continued until 24 days after inoculation with the tumor cells, when one mouse died. The mean tumor size on day 24 in group (iii) mice (26.4 ± 10.8 mm^2) was significantly smaller than that in the other two groups (105.0 ± 15.7, and 86.0 ± 8.3 mm^2, respectively; $P < 0.05$; Fig. 2a). Six of the 10 mice (60%) in group (iii) did not have subcutaneous tumors on day 24 (Fig. 2b). All mice in groups (i) and (ii) had subcutaneous tumors within 13 days, and died within 41 days of inoculation with the tumor cells (Fig. 2c,d). Five of the 10 mice (50%) in group (iii) completely rejected the 3×10^4 C26 cells during the 108 days after the inoculation (Fig. 2c,d). A statistically significant difference in survival time was found between group (iii) and groups (i) and (ii) ($P < 0.05$).

Subcutaneous inoculation of B16.F10 cells (1×10^4) into the right flank was carried out 7 days after the last vaccination (Fig. 2e–h). Measurement of tumor size was continued until 30 days after inoculation with the tumor cells, when one mouse died. Mean tumor size on day 30 in group (iii) mice (103.9 ± 49.8 mm^2) was significantly smaller than that in the other two groups (272.1 ± 69.7, and 361.6 ± 50.3 mm^2, respectively; $P < 0.05$; Fig. 2e). Six of eight mice (75%) in group (iii) did not have subcutaneous tumors on day 30 (Fig. 2f). All mice in groups (i) and (ii) had subcutaneous tumors within 41 days, and died within 65 days of inoculation with the tumor cells (Fig. 2g,h). Four of eight mice (50%) in group (iii) completely rejected the 1×10^4 B16.F10 cells during the 100 days after the inoculation (Fig. 2g,h). A statistically significant difference in survival time was found between group (iii) and groups (i) and (ii) ($P < 0.05$). Therefore, the *HSP105* DNA vaccine has the potential to prevent the growth of tumors expressing HSP105.

We also subcutaneously inoculated five surviving group (iii) mice that completely rejected the first challenges with C26 cells with further (3×10^4) C26 cells. These mice also rejected the second challenge with C26 cells, even at 108 days after the first challenge (data not shown). These results demonstrate that the effects of vaccination in group (iii) continued for a long time, and that the vaccination prevented the recurrence of HSP105-expressing tumors.

Expression of HSP105 protein and infiltration of CD4$^+$ T cells and CD8$^+$ T cells in the injection sites

To observe HSP105 expression and infiltrating cells in muscles injected with the *HSP105* DNA vaccine, we carried out intramuscular immunizations with pCAGGS DNA into the right anterior tibialis muscle, and with pCAGGS-*HSP105* DNA into the left anterior tibialis muscle of four mice. After 48 h, we killed the mice and evaluated the muscles by histological and immunohistochemical analysis (Fig. 3). In HE-stained sections, there were some transverse sections of injection sites that included many cells in both the pCAGGS- and pCAGGS-*HSP105*-immunized muscles. But only in the transverse sections of the injection sites in pCAGGS-*HSP105*-immunized muscles could we observe many cells expressing HSP105 at a high level, and also a considerable number of both CD4$^+$ T cells and CD8$^+$ T cells. Although we did not immunohistochemically stain the dendritic cells in these traverse sections, we did find some dendritic cell-like large cells.

Infiltration of CD4$^+$ T cells and CD8$^+$ T cells into the C26 tumor after vaccination

To observe the antitumor effects of *HSP105 DNA*-vaccination, we evaluated the tumor using immunohistochemical staining of CD8 and CD4. Figure 4a shows the tumor inoculation sites from two *HSP105* DNA-immunized mice, a saline-inoculated mouse, and a pCAGGS-immunized mouse that did not reject the tumor challenge. There were few lymphocytes in the tumors removed from both the saline-inoculated mouse and the pCAGGS immunized mouse, but there were many CD4$^+$ T cells and considerable numbers of CD8$^+$ T cells making contact with the tumor cells and surrounding the tumors removed from the two *HSP105* DNA-immunized mice. These layers of CD4$^+$ T cells surrounding the tumor were thick in the case of *HSP105 DNA* vaccinated mice. Furthermore, there were a considerable number of CD8$^+$ T cells and CD4$^+$ T cells that had infiltrated into the tumor (Fig. 4a).

Vaccination with *HSP105* DNA did not induce damage of normal tissues

HSP105 expression in normal adult mice is limited to several tissues, and HSP105 expression levels in these tissues are

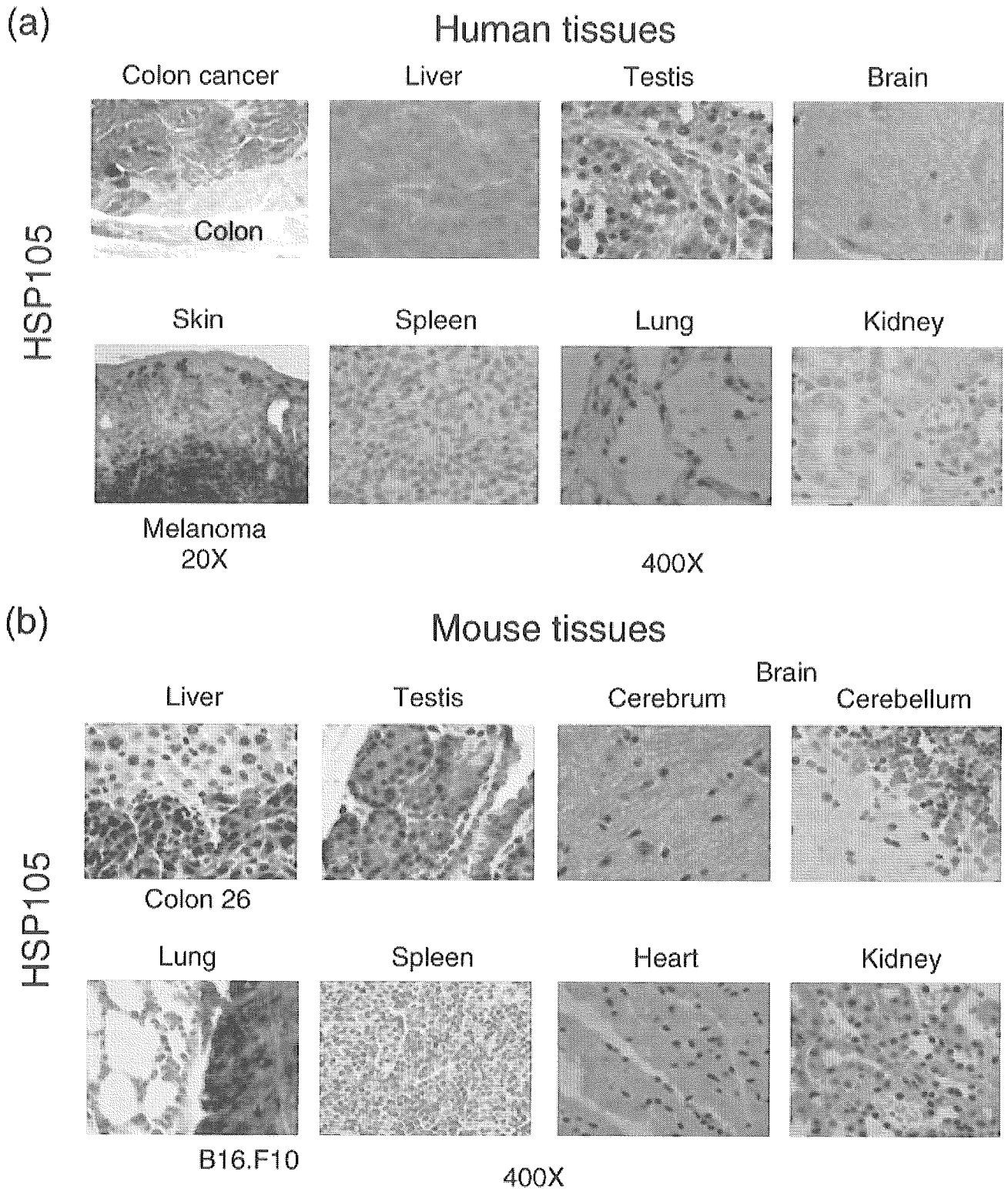

Fig. 1. Expression of the HSP105 protein, a candidate for immunotherapy for CRC and melanoma, in human and mouse tissues and cells. Expression of HSP105 protein detected by immunohistochemical analysis in various (a) human and (b) mouse tissues. Objective magnification was 400× or 20×.

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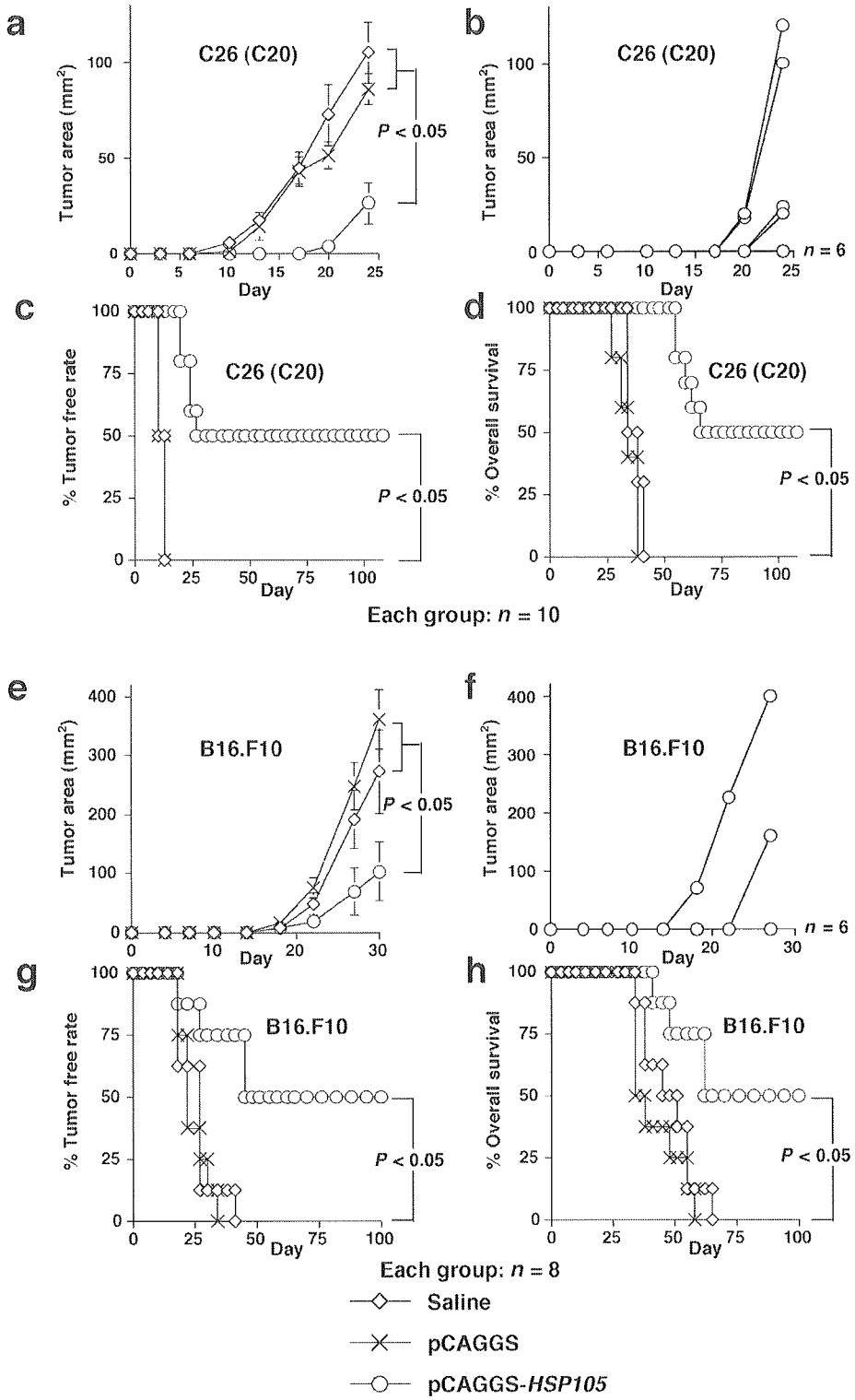

Fig. 2. Vaccination with *HSP105* DNA suppressed the growth of (a–d) C26 and (e–h) B16.F10 tumors in mice. Each group consisted of 10 (a–d) or eight (e–h) mice. (a,b,e,f) Suppression of the growth of HSP105-expressing C26 (a,b) or B16.F10 (e,f) tumors inoculated subcutaneously into mice vaccinated with *HSP105* DNA. The tumor area was calculated as the product of width and length. The result is presented as mean area of tumor ± SE, and we evaluated statistical significance using the unpaired *t*-test (a,e). Growth curves of 10 and eight individual tumors in the mouse group treated with pCAGGS-*HSP105* are presented in (b) and (f), respectively. (c,d,g,h) Percentage tumor free rate (c,g) and percentage overall survival (d,h) were calculated using the Kaplan–Meier method, and the statistical significance of differences between groups was evaluated using Wilcoxon's test.

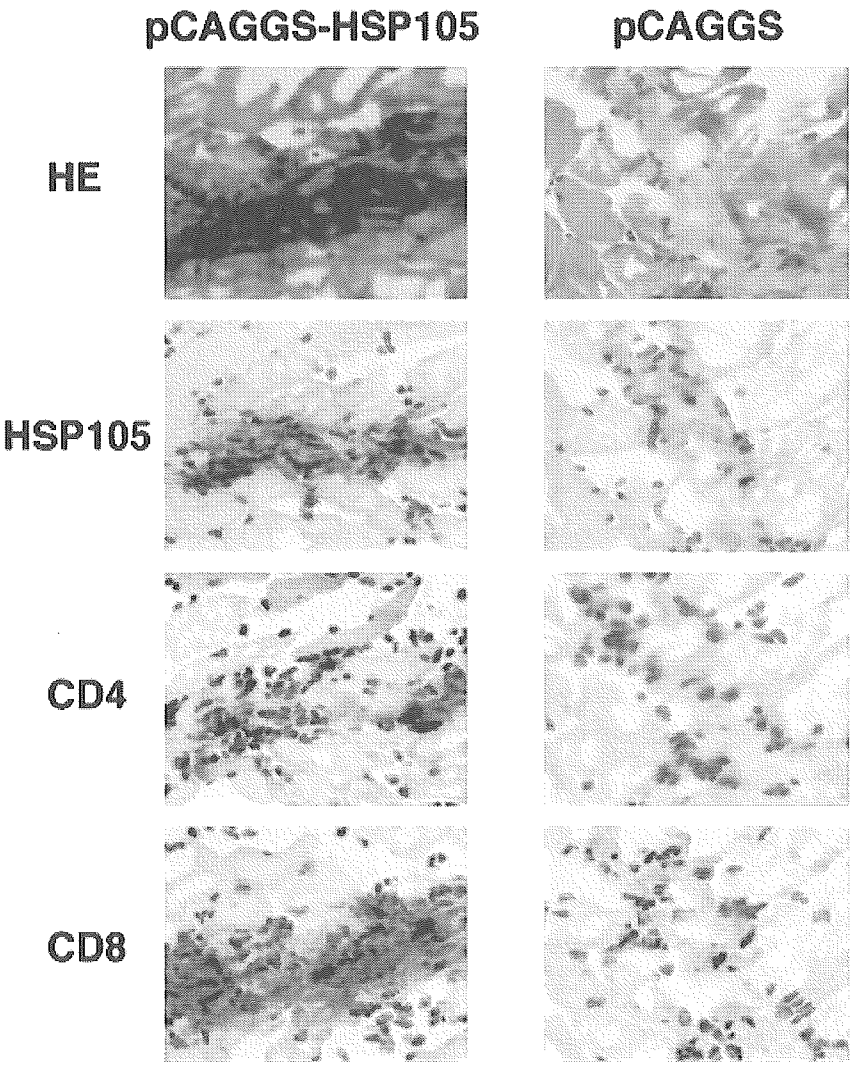

Fig. 3. Expression of HSP105 protein and infiltration of $CD4^+$ T cells and $CD8^+$ T cells in the *HSP105* DNA vaccine-injected sites. To observe HSP105 expression and infiltrating cells in muscles injected with the *HSP105* DNA vaccine, we carried out intramuscular immunizations with pCAGGS-DNA into the right anterior tibialis muscle, and with pCAGGS-*HSP105* DNA into the left anterior tibialis muscle in four mice. After 48 h, we killed the mice and studied their muscle tissue by using HE staining and histological analysis, and immunohistochemical analysis of HSP105, CD4, and CD8. Representative results are shown. Objective magnification was 400×.

lower than those in C26 (C20) tumor cells, which suggests a low risk of damage to normal tissue as a result of immune responses to the HSP105 antigen. To evaluate the risk of autoaggression by immunization against self-HSP105, the tissues of mice immunized with *HSP105* DNA were histologically examined. All mice were apparently healthy, and without abnormalities, suggesting autoimmunity for, for example, dermatitis, arthritis, or neurological disorders. The brain, liver, lung, heart, kidney, and spleen tissues of *HSP105*-immunized mice were critically scrutinized and compared with those of normal mice. These tissues had normal structure and cellularity for each of the two groups examined, and pathological changes caused by immune response, such as infiltrations of $CD8^+$ or $CD4^+$ T cells, or tissue destruction and repair, were not present (Fig. 4b). Although $CD4^+$ T cells and $CD8^+$ T cells infiltrated into the C26 tumor (Fig. 4a), infiltration of $CD4^+$ T cells or $CD8^+$ T cells was not observed in any of the normal adult tissues examined (Fig. 4b). These results indicate that T cells stimulated with the *HSP105* DNA vaccine do not recognize normal cells that express HSP105 at physiological levels.

Anti-C26 tumor adoptive immunity elicited by injection with $CD4^+$ T cells or $CD8^+$ T cells from *HSP105* DNA-vaccinated mice

Antitumor responses could be augmented by homeostatic T cell proliferation in the periphery, involving the expansion of T cells recognizing MHC/tumor antigenic peptide ligands.[21–23] To ascertain that the tumor rejections induced by *HSP105* DNA vaccination were mediated through the activation of $CD8^+$ T cells or $CD4^+$ T cells, in a homeostatic lymphocyte proliferation model, we subcutaneously inoculated BALB/c mice with C26 cells (3×10^4) 6 days after sublethal irradiation (5 Gy). We intravenously injected 1.5×10^7 whole spleen cells or 3×10^6 $CD8^+$ T cells, $CD4^+$ T cells, NK cells, or $CD8^-$ $CD4^-$ NK^- cells derived from each untreated or *HSP105* DNA-vaccinated mouse on day 3 before the tumor inoculation (Fig. 5a). Measurements of tumor size were continued for 22 days after inoculation with the tumor cells (Fig. 5b). Each group consisted of four mice. Inoculation with whole spleen cells or $CD8^+$ T cells, $CD4^+$ T cells, NK cells, or $CD8^-$ $CD4^-$ NK^- cells derived from untreated mice, and with NK cells, or $CD8^-$ $CD4^-$ NK^- cells derived from *HSP105* DNA-vaccinated

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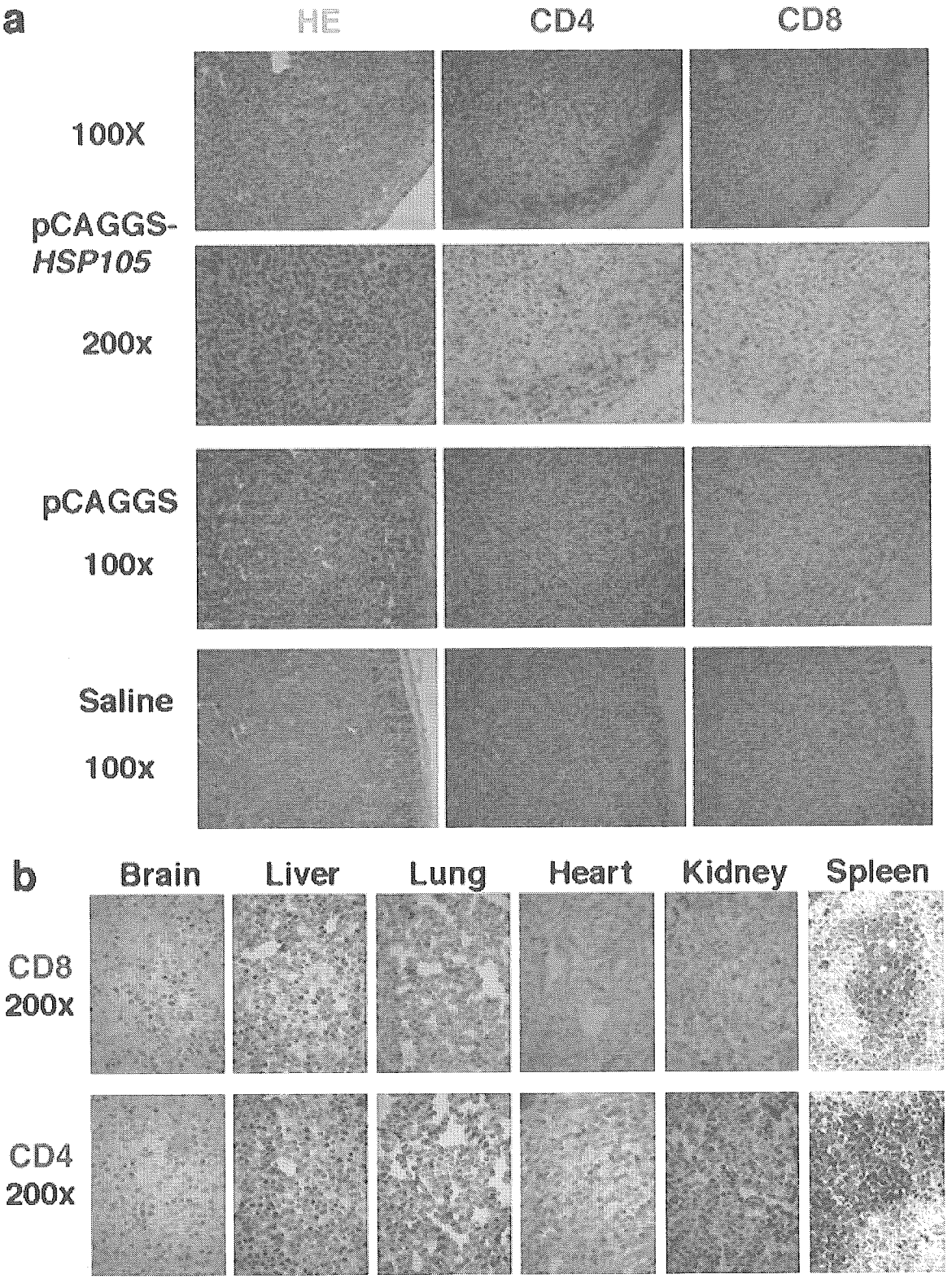

Fig. 4. Vaccination with *HSP105* DNA induced infiltration of both $CD4^+$ T cells and $CD8^+$ T cells into C26 tumors, but not into normal tissues. (a) Subcutaneous C26 tumors removed from two *HSP105* DNA-immunized mice, a saline-inoculated mouse, and a pCAGGS-immunized mouse that did not reject the tumor challenges were analyzed using immunohistochemical staining with anti-CD4 mAb and anti-CD8 mAb. (b) Normal tissues of mice vaccinated with *HSP105* DNA were histologically and immunohistochemically examined. Objective magnification was 200×. The spleen was used as a positive control for staining of both CD4 and CD8.

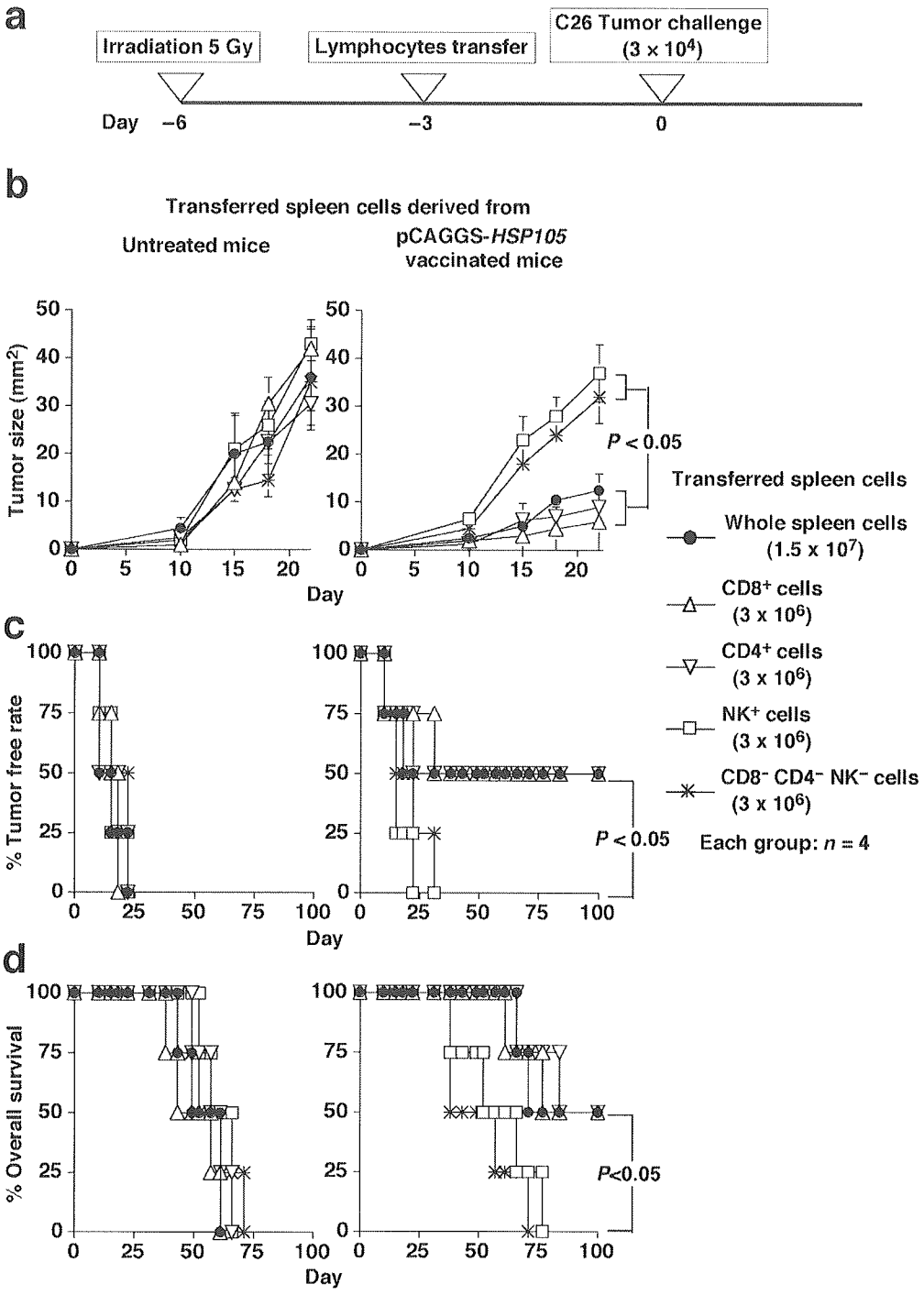

Fig. 5. Injection of either CD4⁺ T cells or CD8⁺ T cells sensitized with *HSP105* DNA vaccine into sublethally irradiated mice elicited effective antitumor adoptive immunity. (a) Experimental protocol; each group consisted of four mice. (b) Suppression of the growth of HSP105-expressing C26 tumors inoculated subcutaneously into mice transferred with each group of spleen cells. Tumor area was calculated as the product of width and length. The result is presented as the mean area of tumor ± SE, and we evaluated the statistical significance using the unpaired *t*-test. (c,d) Percentage tumor free rate (c) and percentage overall survival (d) were calculated using the Kaplan–Meier method, and the statistical significance of differences in survival time between groups was evaluated using Wilcoxon's test.

mice did not cause the mice to reject challenges with C26 cells (3×10^4). Conversely, two of the four mice (50%) that were treated with whole spleen cells, CD8⁺ T cells, or CD4⁺ T cells derived from *HSP105* DNA-vaccinated mice completely rejected challenges with C26 cells (3×10^4; Fig. 5b–d). Thus, sublethally irradiated lymphopenic mice transfused with CD4⁺ T cells or CD8⁺ T cells derived from *HSP105* DNA-vaccinated mice displayed tumor growth inhibition. These results suggest that both CD4⁺ and CD8⁺ T cells play critical roles in antitumor immunity induced by immunization with

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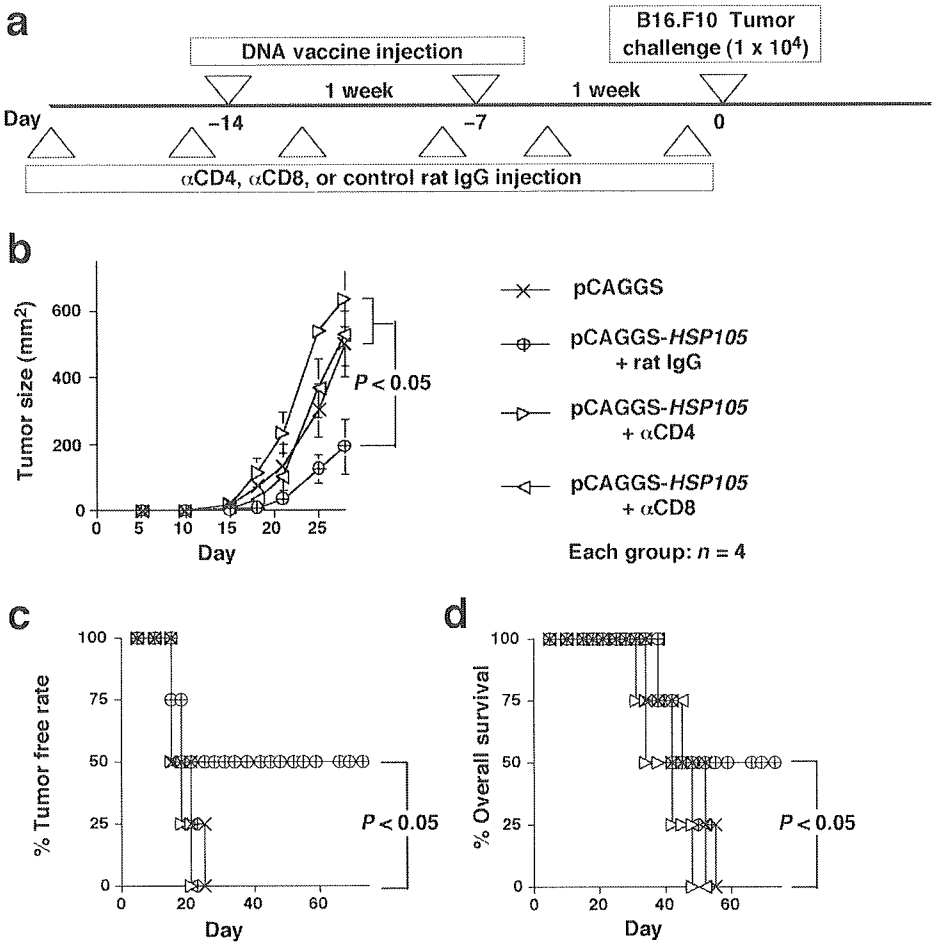

Fig. 6. Involvement of both CD4⁺ T cells and CD8⁺ T cells in protection against B16.F10 induced by vaccination with *HSP105* DNA. (a) Experimental protocol for *in vivo* depletion of CD4⁺ T cells and CD8⁺ T cells. Each group consisted of four mice. (b) Suppression of the growth of HSP105-expressing B16.F10 tumors inoculated subcutaneously into mice vaccinated with *HSP105* DNA. Tumor area was calculated as the product of width and length. Data are presented as mean area of tumor ± SE, and we evaluated the statistical significance using the unpaired *t*-test. (c,d) Percentage tumor free rate (c) and percentage overall survival (d) were calculated using the Kaplan–Meier method, and the statistical significance of differences in survival time between groups was evaluated using Wilcoxon's test.

the *HSP105* DNA-vaccine. The mice shown in Figure 5 were killed more than 100 days after lymphocyte transfer, respectively. All mice were apparently healthy and without abnormalities, suggesting autoimmunity for, for example, dermatitis, arthritis, or neurological disorders. The brain, liver, lung, heart, kidney, and spleen tissues of *HSP105* DNA-immunized mice were critically scrutinized and compared with those of normal mice. These tissues had normal structures and cellularity for each of the two groups examined, and pathological changes caused by immune response, such as CD8⁺ or CD4⁺ T lymphocyte infiltration or tissue destruction and repair, were not present, as shown in Figure 4b. These results indicate that T cells stimulated with HSP105 do not recognize normal cells that express HSP105 at physiological levels.

Involvement of both CD4⁺ T cells and CD8⁺ T cells in protection against B16.F10 induced by *HSP105* DNA-vaccination

To determine the role of CD4⁺ T cells and CD8⁺ T cells in the protection against B16.F10 tumor cells induced by *HSP105* DNA-vaccination, we depleted mice of CD4⁺ T cells or CD8⁺ T cells by treatment with anti-CD4 or anti-CD8 mAb *in vivo*. More than 90% of CD4⁺ T cells or CD8⁺ T cells were depleted (data not shown). During this procedure, mice were immunized with DNA vaccine and challenged with B16.F10 cells (Fig. 6a). Depletion of either CD4⁺ T cells or CD8⁺ T cells almost totally abrogated the protective immunity induced by immunization with *HSP105* DNA vaccine (Fig. 6b–d). These results suggest that both CD4⁺ T cells and CD8⁺ T cells play critical roles in antitumor immunity induced by immunization with *HSP105* DNA vaccine.

Discussion

Advances in molecular biology and tumor immunology have paved the way for identification of a large number of genes encoding TAA and antigenic peptides recognized by tumor-reactive CTL, hence peptide-based cancer immunotherapy has been the focus of much research.[24–26] However, current clinical trials for peptide-based immunotherapy have rarely resulted in tumor regression.[27] The immunogenicity of these