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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: $100 \times$ [(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.

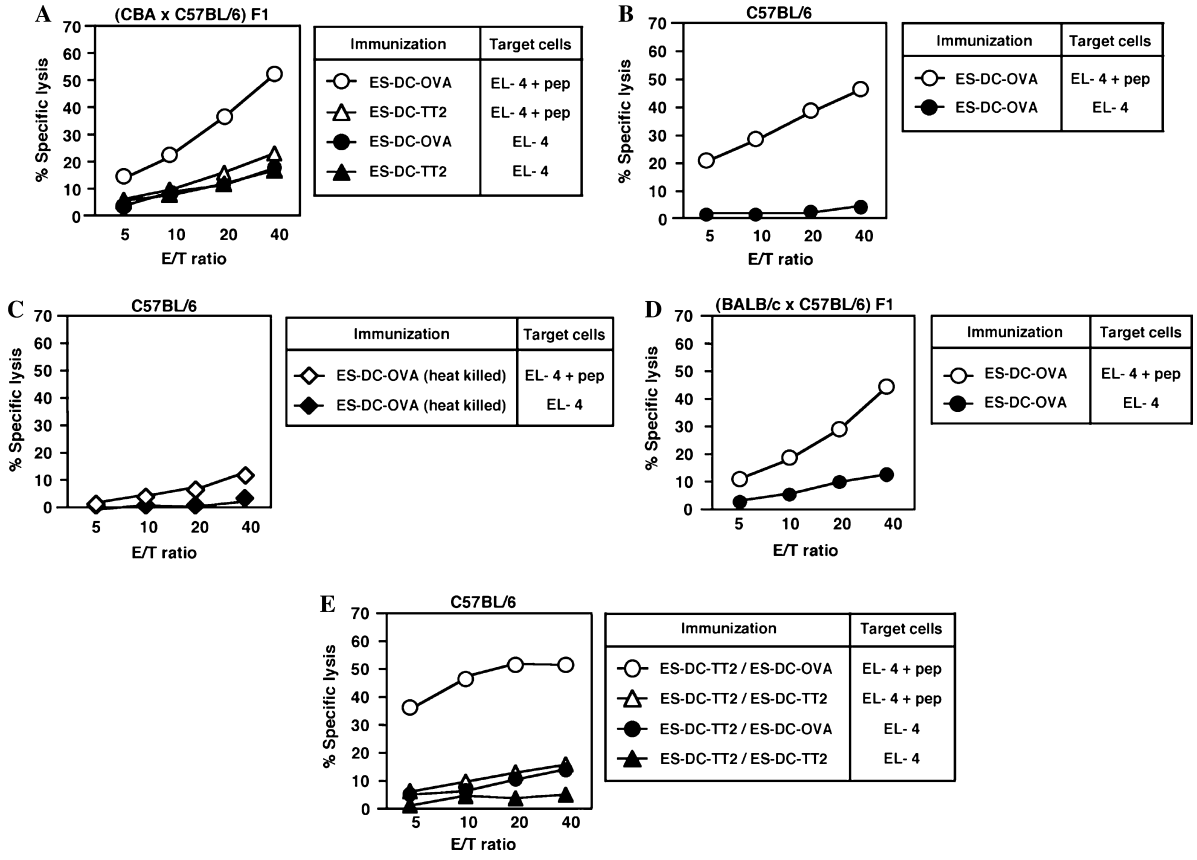

Fig. 1. Stimulation of OVA-specific CTL by ES-DC expressing OVA in syngeneic and semi-allogeneic mice. (CBA × C57BL/6) F1 (A), C57BL/6 (B, C), or (BALB/c × C57BL/6) F1 (D) mice were injected i.p. twice with ES-DC-OVA or ES-DC-TT2 (1×10^5/injection/mouse) on days −14 and −7. In (C), ES-DC-OVA were heat-killed 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

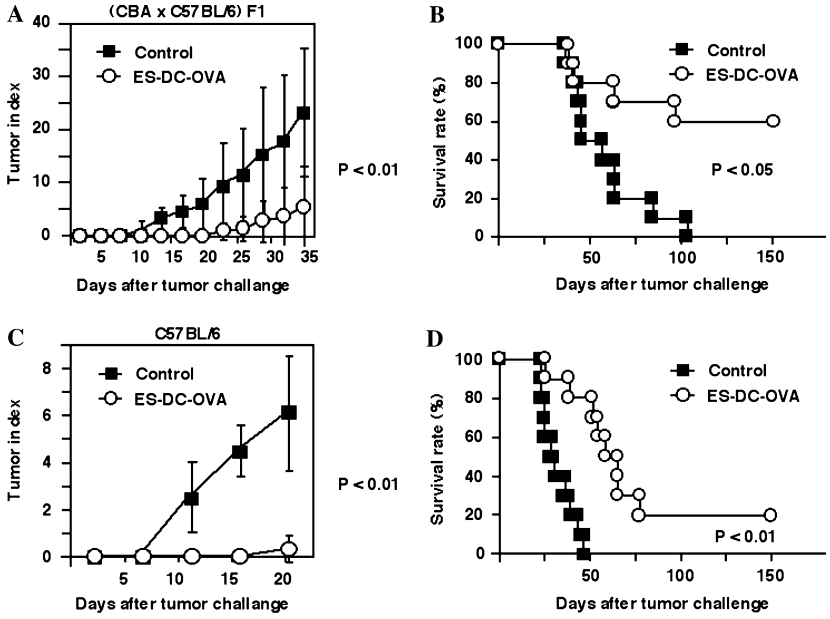

Fig. 2. Induction of protective immunity by ES-DC expressing OVA against OVA-expressing tumor cells in syngeneic and semi-allogeneic mice. (CBA × C57BL/6) F1 mice were injected i.p. twice on days −14 and −7 with ES-DC-OVA (2×10^4/injection/mouse) or medium (RPMI-1640) only as control and were challenged 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.

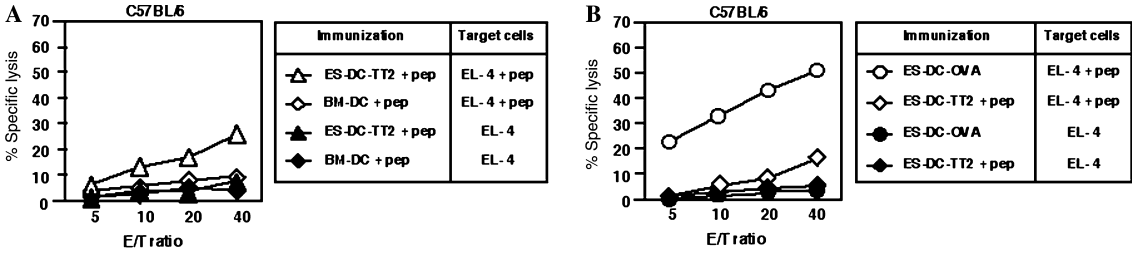

Fig. 3. Priming of OVA-specific CTL by OVA peptide-loaded DC or OVA-expressing ES-DC in semi-allogeneic mice. BM-DC or ES-DC-TT2 (non-transfectant ES-DC) were pulsed with $OVA_{257–264}$ synthetic peptide (10 μM) for 2 h and injected i.p. into C57BL/6 mice (1×10^5/injection/mouse) (A). ES-DC-OVA or OVA 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

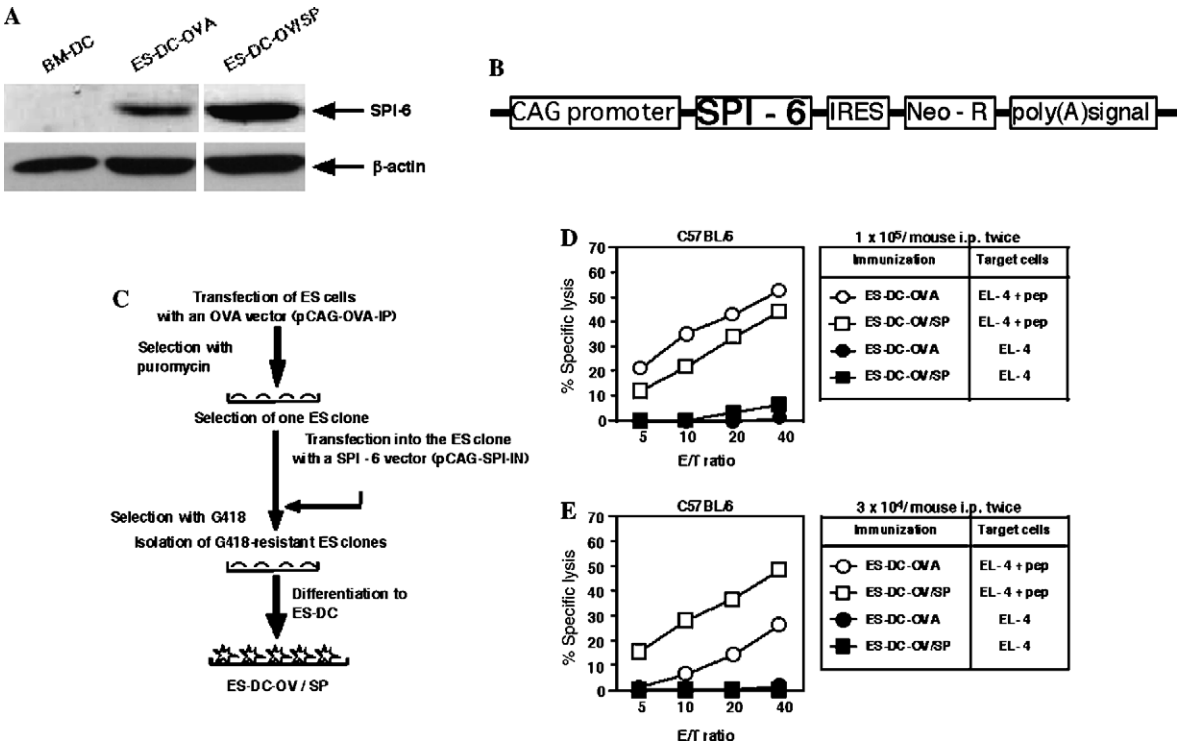

Fig. 4. Expression of SPI-6 in DC and priming of antigen-specific CTL by ES-DC expressing OVA and SPI-6 in semi-allogeneic mice. (A) The levels of expression of SPI-6 in BM-DC, ES-DC-OVA, and ES-DC-OV/SP were analyzed by a Western blotting analysis. The same samples were analyzed also for β-actin expression 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.

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