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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.
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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.

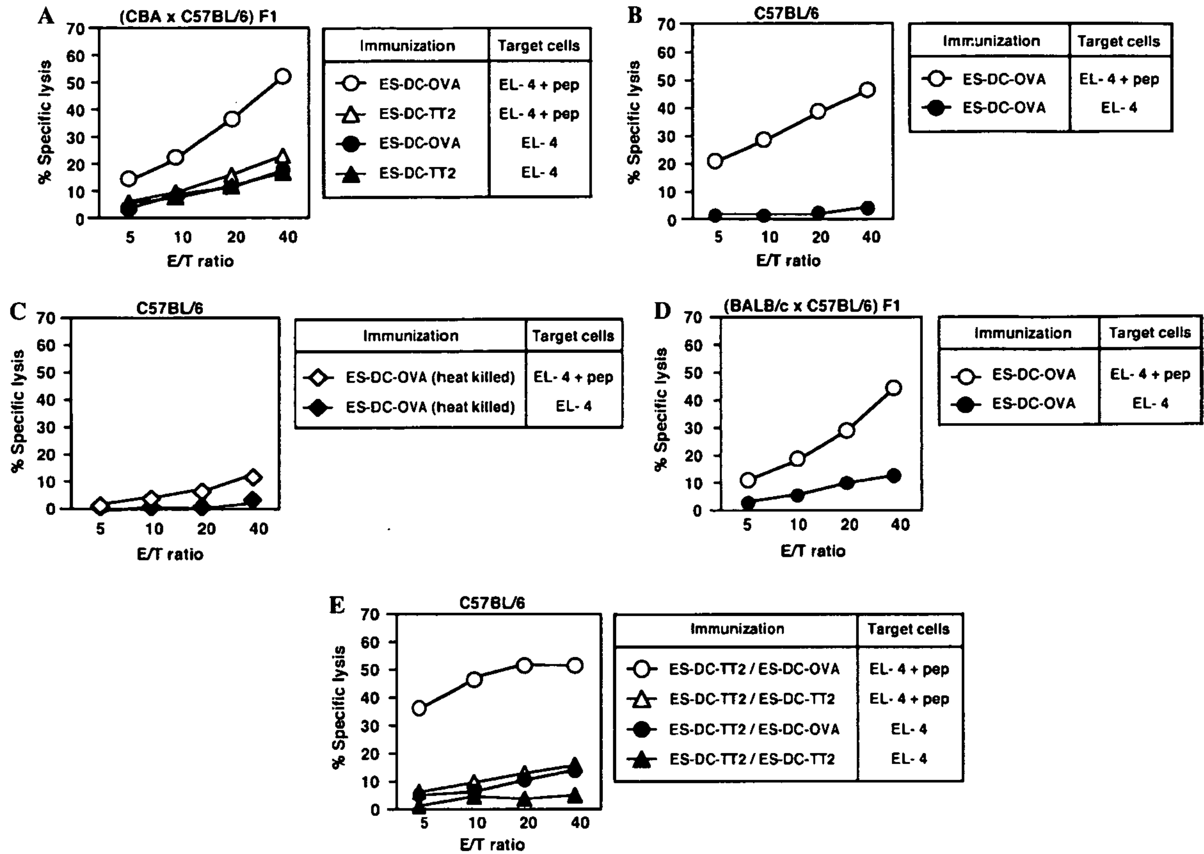

Fig. 1. Stimulation of OVA-specific CTL by ES-DC expressing OVA in syngeneic and semi-allogeneic mice. (CBA × C57BL/6) F1 (A), C57BL/6 (B, C), or (BALB/c × C57BL/6) F1 (D) mice were injected i.p. twice with ES-DC-OVA or ES-DC-TT2 (1×10^5/injection/mouse) on days −14 and −7. In (C), ES-DC-OVA were heat-killed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

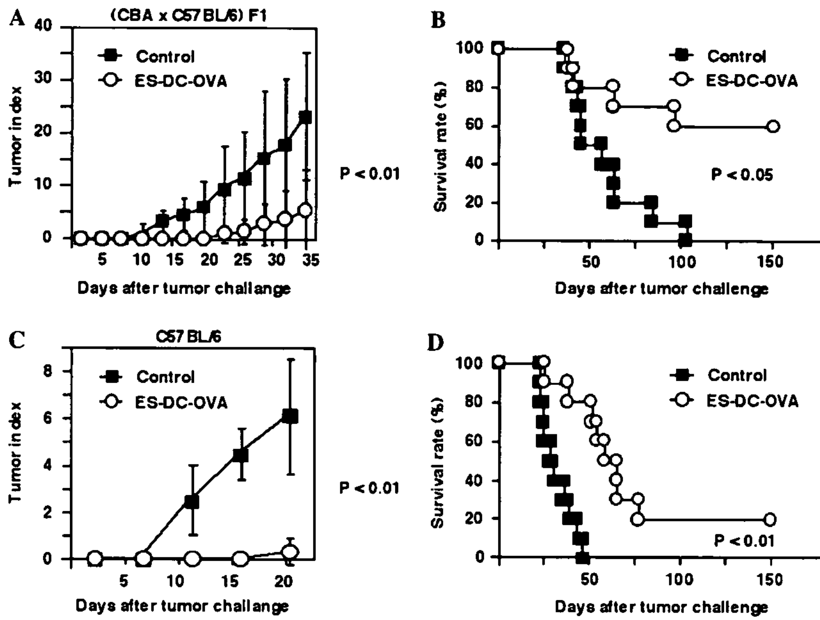

Fig. 2. Induction of protective immunity by ES-DC expressing OVA against OVA-expressing tumor cells in syngeneic and semi-allogeneic mice. (CBA × C57BL/6) F1 mice were injected i.p. twice on days −14 and −7 with ES-DC-OVA (2×10^4/injection/mouse) or medium (RPMI-1640) only as control and were challenged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

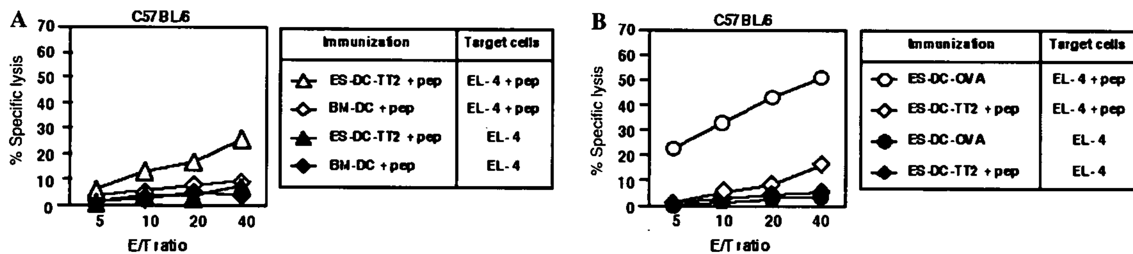

Fig. 3. Priming of OVA-specific CTL by OVA peptide-loaded DC or OVA-expressing ES-DC in semi-allogeneic mice. BM-DC or ES-DC-TT2 (non-transfectant ES-DC) were pulsed with $OVA_{257-264}$ synthetic peptide (10 μM) for 2 h and injected i.p. into C57BL/6 mice (1×10^5/injection/mouse) (A). ES-DC-OVA or OVA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

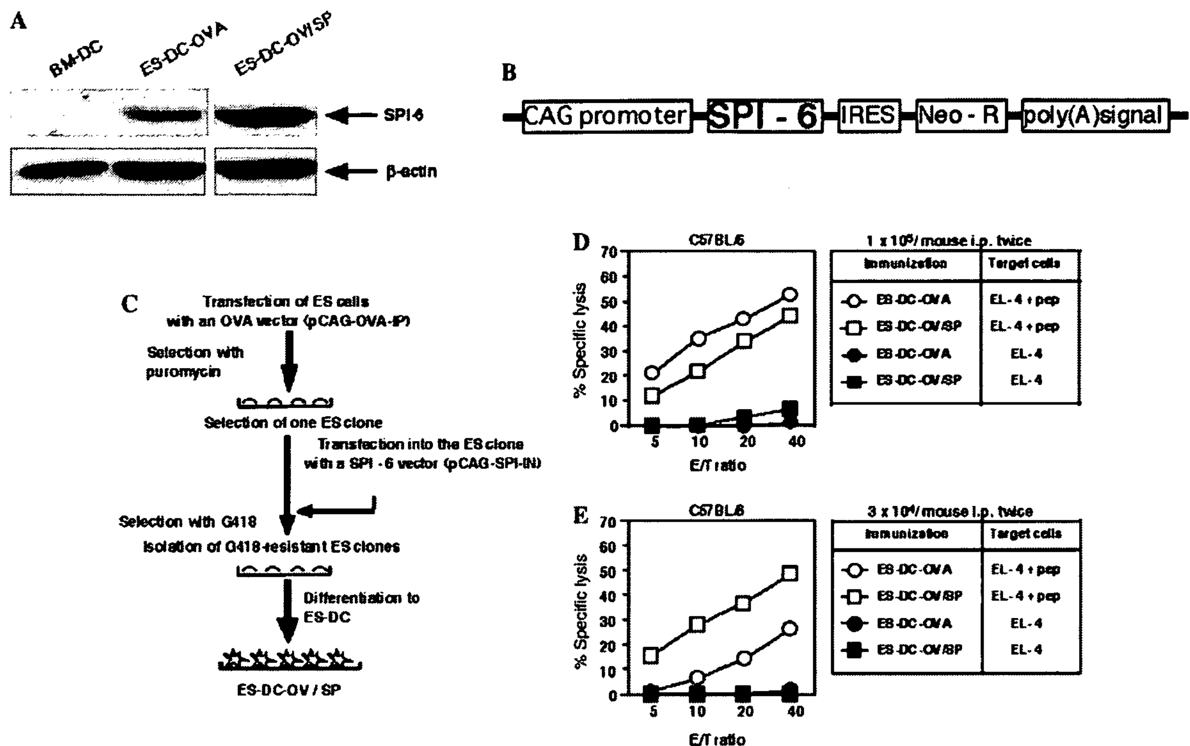

Fig. 4. Expression of SPI-6 in DC and priming of antigen-specific CTL by ES-DC expressing OVA and SPI-6 in semi-allogeneic mice. (A) The levels of expression of SPI-6 in BM-DC, ES-DC-OVA, and ES-DC-OV/SP were analyzed by a Western blotting analysis. The same samples were analyzed also for β-actin expression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

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Brief report

Direct recognition and lysis of leukemia cells by WT1-specific CD4$^+$ T lymphocytes in an HLA class II–restricted manner

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Wilms tumor gene 1 product (WT1) has been recognized as an attractive target antigen of immunotherapy for various malignancies including leukemia. Because tumor-associated antigen-specific CD4$^+$ T lymphocytes undoubtedly play an important role in the induction of an antitumor immune response, we attempted to generate WT1-specific CD4$^+$ T lymphocytes in vitro and examined their antileukemia functions. A CD4$^+$ T-cell line, designated NIK-1, which proliferated and produced Th1 cytokines specifically in response to stimulation with the WT1-derived peptide, $WT1_{337-347}$ LSHLQMHSRKH, in an HLA-DP5–restriced manner was established. NIK-1 exhibited cytotoxicity against HLA-DP5–positive, WT1-expressing leukemia cells but did not lyse HLA-DP5–negative, WT1-expressing leukemia cells or HLA-DP5–positive, WT1–negative cells. NIK-1 did not inhibit colony formation by normal bone marrow cells of HLA-DP5–positive individuals. This is the first report to describe WT1-specific and HLA class II–restricted CD4$^+$ T lymphocytes possessing direct cytotoxic activity against leukemia cells. (Blood. 2005;106:1415-1418)

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Introduction

Because Wilms tumor gene 1 product (WT1) is expressed in most cases of acute leukemia but not in normal tissues, it would be an attractive target antigen for immunotherapy against various malignancies including leukemia.[1-4] Recently, we and other investigators have succeeded in generating CD8$^+$ cytotoxic T lymphocytes (CTLs) that recognize WT1-derived peptides in vitro.[5-9] These WT1-specific CTLs efficiently lysed leukemia cells and solid tumor cells, but not normal cells, in an HLA class I–restricted manner. On the basis of these findings, clinical trials of cancer vaccine using WT1 peptides have been initiated.[10,11]

Increasing evidence from both murine and human studies indicates that tumor-associated antigen-specific CD4$^+$ T lymphocytes play a central role in orchestrating the host immune response against malignancies and infectious diseases.[12] Because identification of epitopes on WT1 recognized by CD4$^+$ T lymphocytes is essential for development of effective cellular immunotherapy for malignaicies targeting WT1, we attempted to generate WT1-derived peptide-specific CD4$^+$ T lymphocytes and examined their antileukemia functions.

Study design

Approval for the present study was obtained from the Institutional Review Board of Ehime University School of Medicine. Informed consent was obtained according to the Declaration of Helsinki.

On the basis of the amino acid sequence of WT1, a comprehensive panel of 43 20-mer peptides with 10 overlapping amino acids were synthesized. The WT1 peptide–specific CD4$^+$ T-cell lines were generated as reported previously.[13] Briefly, peripheral blood mononuclear cells (PBMCs) were stimulated 3 times with synthetic peptides at a concentration of 10 μg/mL. Cells showing a positive response to a WT1 peptide were cultured continuously in interleukin-2 (IL-2)–containing culture medium, and mitomycin C–treated autologous PBMCs and WT1 peptide were added to the wells every 1 to 2 weeks.

Chromium-51 release cytotoxicity assays were performed as described previously.[14] In some experiments, the target cells were incubated with an anti–HLA-DR monoclonal antibody (mAb) (L243), an anti–HLA-DQ mAb (HU-11), or anti–HLA-DP mAb (B7/21) at an optimal concentration for 30 minutes before adding the effector cells. Cold-target inhibition assays were performed as described previously.[15]

WT1 mRNA expression levels in cells were determined by quantitative reverse-transcription polymerase chain reaction (PCR) and calculated relative to that in the human leukemia cell line K562 as described previously.[16]

The effect of WT1-specific T lymphocytes on the growth of normal bone marrow cells was examined by performing the colony-forming assays as described previously.[5]

Results and discussion

A CD4$^+$ T-cell line, designated NIK-1, which proliferated specifically in response to stimulation with one of the 20-mer WT1

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Table 1. WT1-specific and HLA-DP5–restricted cytotoxicity by NIK-1: experiment 1

Target cells	% cytotoxicity, at E/T ratio		
	10:1 ratio	5:1 ratio	2.5:1 ratio
Auto LCL (HLA-DP5–positive)			
Without WT1 peptide	10.3	6.3	3.1
With WT1 peptide	70.2	63.2	49.1
Allo LCL no. 1 (HLA-DP5–positive)			
Without WT1 peptide	8.9	7.3	3.4
With WT1 peptide	73.9	62.1	50.1
Allo LCL no. 2 (HLA-DP5–positive)			
Without WT1 peptide	5.3	5.1	4.1
With WT1 peptide	63.2	56.1	47.3
Allo LCL no. 3 (HLA-DP5–negative)			
Without WT1 peptide	1.0	0.5	0.0
With WT1 peptide	2.1	0.8	0.1
Allo LCL no. 4 (HLA-DP5–negative)			
Without WT1 peptide	2.1	0.0	0.1
With WT1 peptide	4.1	0.8	0.0

Cytotoxicity of NIK-1 against autologous (auto) and various allogeneic (allo) LCL loaded or unloaded with WT1 peptide. E/T ratio indicates effector-target ratio.

peptides ($WT1_{336-355}$ KLSHLQMHSRKHTGEKPYQC) was established. More than 99% of NIK-1 cells were $CD3^+CD4^+CD8^-$. NIK-1 appeared to produce large amounts of T helper-1 (Th1) cytokines, such as interferon-γ, IL-2, and IL-12, upon stimulation with WT1 peptide in the presence of autologous PBMCs (data not shown). In addition to the proliferative response, NIK-1 showed strong cytotoxicity against a WT1 peptide–loaded autologous B-lymphoblastoid cell line (LCL) (Table 1). The restriction element of NIK-1 appeared to be HLA-DP, because the cytotoxicity and proliferative response of NIK-1 against WT1 peptide were significantly inhibited by adding anti–HLA-DP mAb. Because the donor was an HLA-DP5 homozygote, the cytotoxicity of NIK-1 against WT1 peptide–loaded *HLA-DP5* gene–transfected L cells was examined. As expected, NIK-1 exerted cytotoxicity against WT1 peptide–loaded HLA-DP5–positive L cells but not against $HLA-DP9^+$ L cells (Table 2). In addition, NIK-1 showed cytotoxicity against WT1 peptide–loaded HLA-DP5–positive but not HLA-DP5–negative allogeneic cells (Table 1), demonstrating that NIK-1 is the HLA-DP5–restricted $CD4^+$ T-cell line.

Table 2. WT1-specific and HLA-DP5–restricted cytotoxicity by NIK-1: experiment 2

Target cells	% cytotoxicity, at 10:1 E/T ratio
Auto LCL (HLA-DP5–positive)	
Without WT1 peptide	10.5
With WT1 peptide	63.6
With WT1 peptide and anti–HLA-DR mAb	59.9
With WT1 peptide and anti–HLA-DQ mAb	62.5
With WT1 peptide and anti–HLA-DP mAb	11.6
L-DP5 (HLA-DP5–positive)	
Without WT1 peptide	5.7
With WT1 peptide	68.3
With WT1 peptide and anti–HLA-DR mAb	62.6
With WT1 peptide and anti–HLA-DQ mAb	64.7
With WT1 peptide and anti–HLA-DP mAb	8.9
L-DP9 (HLA-DP5–positive)	
Without WT1 peptide	2.5
With WT1 peptide	4.2

Cytotoxicity of NIK-1 against autologous LCL, *HLA-DP5* gene–transduced L cells, and *HLA-DP9* gene–transduced L cells loaded or unloaded with WT1 peptide in the presence or absence of anti-HLA-DR, anti-HLA-DQ, or anti-HLA-DP mAb. E/T ratio indicates effector-target ratio.

Table 3. WT1-specific and HLA-DP5–restricted cytotoxicity by NIK-1: experiment 3

WT1 peptide loaded	% cytotoxicity, at 10:1 E/T ratio
None	3.5
KLSHLQMHSRKHTGEKPYQC	73.5
KLSHLQMHSRKHTGEKPYQ	68.5
KLSHLQMHSRKHTGEKPY	73.1
KLSHLQMHSRKHTGEKP	58.6
KLSHLQMHSRKHTGEK	70.2
KLSHLQMHSRKHTGE	71.5
KLSHLQMHSRKHTG	71.2
KLSHLQMHSRKHT	73.1
KLSHLQMHSRKH	72.7
KLSHLQMHSRK	32.5
KLSHLQMHSR	7.3
KLSHLQMHS	7.5
LSHLQMHSRKHTGEKPYQC	63.9
SHLQMHSRKHTGEKPYQC	32.4
HLQMHSRKHTGEKPYQC	6.6
LQMHSRKHTGEKPYQC	6.9
QMHSRKHTGEKPYQC	4.9
MHSRKHTGEKPYQC	6.4
HSRKHTGEKPYQC	5.9
SRKHTGEKPYQC	7.2
RKHTGEKPYQC	3.5
KHTGEKPYQC	7.8
HTGEKPYQC	7.7
LSHLQMHSRKH	72.1

Cytotoxicity of NIK-1 against autologous LCL loaded and unloaded with various WT1 peptides. E/T ratio indicates effector-target ratio.

We next examined the fine epitope on WT1 recognized by NIK-1. Experiments using deletion peptides clearly demonstrated that the minimal amino acid sequence recognized by NIK-1 is $WT1_{337-347}$ LSHLQMHSRKH (Table 3).

Because NIK-1 showed WT1 peptide–specific cytotoxicity, we addressed the question of whether NIK-1 can lyse leukemia cells. Because most leukemia cell lines are HLA class II negative, only one HLA-DP5–positive leukemia cell line expressing WT1 was available. As shown in Table 4, NIK-1 exerted strong cytotoxicity against HLA-DP5–positive WT1-expressing leukemia cell lines but not against HLA-DP5–negative leukemia cell lines or HLA-DP5–positive or HLA-DP5–negative lymphoma cell lines that are negative for WT1 expression. Similarly to the cytotoxicity against cell lines, HLA-DP5–positive but not HLA-DP5–negative freshly isolated leukemia cells were lysed efficiently by NIK-1. Cytotoxicity of leukemia cells mediated by NIK-1 appeared to be restricted by HLA-DP5, because only HLA-DP5–positive leukemia cells were lysed by NIK-1 and the cytotoxicity of leukemia cells mediated by NIK-1 was inhibited by adding anti–HLA-DP mAb (Table 5).

To further confirm that the cytotoxicity of NIK-1 against leukemia cells was mediated by the specific recognition of endogenously processed WT1, we performed cold-target inhibition experiments. As shown in Table 6, the addition of WT1 peptide–loaded autologous LCL decreased the cytotoxicity of NIK-1 against leukemia cells, whereas the addition of peptide-unloaded autologous LCL had no effect on the cytotoxicity. These data strongly suggest that WT1 is naturally processed in leukemia cells and recognized by WT1-specific $CD4^+$ CTLs in the context of HLA-DP5.

We then addressed the issue of whether NIK-1 recognizes WT1 peptide expressed on normal bone marrow progenitor cells and

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Table 4. Direct recognition and lysis of leukemia cells by NIK-1: experiment 1

Target cells	WT1 expression level	HLA-DP5 expression	% cytotoxicity, at E/T ratio		
			20:1 ratio	10:1 ratio	5:1 ratio
Cell lines					
C2F8; leukemia	2.3×10^0	+	38.8	34.4	31.2
MEG01; leukemia	8.4×10^{-1}	−	3.6	1.8	1.2
KAZZ; leukemia	1.1×10^0	−	2.1	2.7	0.9
IZU; lymphoma	1.1×10^{-5}	+	1.5	0.5	1.0
IKE; lymphoma	8.6×10^{-5}	+	0.7	1.8	0.5
Daudi; lymphoma	3.8×10^{-5}	−	2.0	0.3	0.4
Freshly isolated leukemia cells					
From donor no. 1 (AML M1)	3.5×10^{-1}	+	45.1	34.2	21.0
From donor no. 2 (AML M2)	2.7×10^{-1}	+	33.6	24.3	18.9
From donor no. 3 (AML M2)	8.6×10^{-2}	+	31.2	18.9	13.1
From donor no. 4 (AML M1)	6.5×10^{-1}	−	2.7	2.1	0.6
From donor no. 5 (AML M2)	7.2×10^{-1}	−	1.4	1.8	1.0
From donor no. 6 (ALL L2)	5.8×10^{-1}	−	0.0	0.3	0.0

Cytotoxicity of NIK-1 against various cell lines and freshly isolated leukemia cells. E/T ratio indicates effector-target ratio.

suppresses their growth. As shown in Table 7, after coculture with NIK-1 in the absence of WT1 peptide, the numbers of granulocyte-macrophage colony-forming units (CFU-GMs) and erythroid burst-forming units (BFU-Es) generated from bone marrow cells of 2 HLA-DP5–positive individuals were almost the same as those generated from bone marrow cells cultured alone. However, the numbers of CFU-GMs and BFU-Es decreased significantly when HLA-DP5–positive bone marrow cells were pretreated with WT1 peptide and then cocultured with NIK-1. As expected, NIK-1 had no effect on colony formation by HLA-DP5–negative bone marrow cells that had been pretreated with WT1 peptide or left untreated.

In the present study, we demonstrated for the first time the generation of WT1-specific CD4+ T lymphocytes that can recognize directly leukemia cells in an HLA class II–restricted manner. It is well known that induction of the CD8+ CTL response requires cognate CD4+ T-lymphocyte help.[17] CD4+ T lymphocytes recognize major histocompatibility complex (MHC) class II–binding peptides on antigen-presenting cells, such as dendritic cells (DCs), and their interaction may result not only in activation and priming of CD4+ T lymphocytes but also in activation of the DCs themselves.[18] Consequent to this mutual activation, DCs prime and activate CD8+ CTLs specific for tumor-associated antigens. On the basis of this scenario, it is expected that WT1-specific CD4+ T lymphocytes may be effective for efficient induction of WT1-specific CD8+ CTLs in vivo.

The other interesting finding of this study is that WT1 peptide–specific CD4+ T lymphocytes exerted strong cytotoxicity against WT1-expressing leukemia cells in an HLA class II–restricted manner. In general, endogenous antigens are degraded in the cytoplasm to oligopeptides and bind to newly synthesized MHC class I molecules. On the other hand, exogenous antigens are processed into peptides capable of binding to MHC class II molecules. However, it has recently been shown that the MHC class II pathway can process and present endogenous antigens as well as exogenous antigens. For example, virus-infected cells are recognized by CD4+ T lymphocytes in a viral antigen–specific and MHC class II–restricted manner in vitro and in vivo.[19,20] It has also been reported that tumor cells transfected with syngeneic MHC class II genes could present endogenously synthesized tumor-associated protein-derived peptides in the context of MHC class II molecules to CD4+ T lymphocytes.[21] Taken together with previous data, our present findings strongly suggest that leukemia cells can process and present endogenously synthesized WT1 protein to CD4+ T lymphocytes in the context of HLA class II molecules.

Table 5. Direct recognition and lysis of leukemia cells by NIK-1: experiment 2

Target cells	% cytotoxicity, at 10:1 E/T ratio
C2F8 cells	
No anti-HLA mAb	27.6
With anti-HLA-DR mAb	28.0
With anti-HLA-DQ mAb	30.3
With anti-HLA-DP mAb	3.1
Freshly isolated leukemia cells	
From donor no. 1 (AML M1)	
No anti-HLA mAb	45.1
With anti-HLA-DR mAb	43.2
With anti-HLA-DQ mAb	41.6
With anti-HLA-DP mAb	5.6
From donor no. 2 (AML M2)	
No anti-HLA mAb	33.6
With anti-HLA-DR mAb	31.7
With anti-HLA-DQ mAb	35.1
With anti-HLA-DP mAb	5.6

Cytotoxicity of NIK-1 against leukemia cell line and freshly isolated leukemia cells in the presence or absence of anti-HLA- DR, anti-HLA-DQ, or anti-HLA-DP mAb. E/T ratio indicates effector-target ratio.

Table 6. Direct recognition and lysis of leukemia cells by NIK-1: experiment 3

Hot target cells and cold target cells	% cytotoxicity, at cold-hot target cell ratio			
	0	5:1	10:1	20:1
C2F8 cells				
WT1 peptide–loaded autologous LCL	31.3	22.5	14.3	9.6
Peptide-unloaded autologous LCL	31.3	30.5	29.7	28.1
Freshly isolated leukemia cells from donor no. 1 (AML M1)				
WT1 peptide–loaded autologous LCL	42.1	26.4	15.2	8.8
Peptide-unloaded autologous LCL	42.1	41.3	39.3	40.2

Cytotoxicity of NIK-1 against leukemia cells in the presence or absence of WT1 peptide–loaded autologous LCL or peptide-unloaded autologous LCL at an effector–hot target cell ratio of 10:1.

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Table 7. Direct recognition and lysis of leukemia cells by NIK-1: experiment 4

Donor	Colony formation	
	CFU-GM	BFU-E
HLA-DP5–positive donor no. 1		
Without NIK-1	56 ± 5	86 ± 8
With NIK-1	59 ± 7	93 ± 10
With NIK-1 and WT1 peptide	29 ± 3	37 ± 7
HLA-DP5–positive donor no. 2		
Without NIK-1	136 ± 14	167 ± 24
With NIK-1	138 ± 8	170 ± 23
With NIK-1 and WT1 peptide	66 ± 14	62 ± 13
HLA-DP5–negative donor no. 3		
Without NIK-1	86 ± 15	96 ± 18
With NIK-1	88 ± 7	100 ± 10
With NIK-1 and WT1 peptide	85 ± 15	103 ± 8

The colony formation by normal bone marrow cells cocultured with or without NIK-1 cells in the presence or absence of WT1 peptide.

In summary, we have demonstrated WT1-specific CD4+ T lymphocytes capable of producing Th1 cytokines and exerting direct cytotoxicity against leukemia cells in an HLA class II–restricted manner. Because most types of leukemic cells are positive for HLA class II expression,[22] WT1-specific CD4+ CTLs may play an important role in the antileukemia response through cytotoxic activity as well as helper function for CD8+ CTL induction. On the basis of this concept, we are planning a clinical trial of WT1 peptide vaccination using a combination of peptides derived from epitopes recognized by CD4+ T lymphocytes as well as CD8+ T lymphocytes.

Acknowledgments

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解　説

樹状細胞の移入による免疫抑制療法*

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Key Words：dendritic cell(DC), autoimmune disease, transplantation, ES cell-derived DC(ES-DC)

はじめに

自己免疫疾患の治療法として，現在の診療ではステロイド，免疫抑制剤，非ステロイド性消炎鎮痛剤(NSAIDs)が日常的に用いられている．しかし，全般的免疫抑制に伴う日和見感染症や投与に伴う種々の副作用，無効・再発症例などの問題は少なくない．このため，疾患特異的な免疫制御や副作用の少ない治療法が求められており，生物製剤療法，遺伝子治療などさまざまな試みがなされている．近年，プロフェッショナル抗原提示細胞である樹状細胞(dendritic cell；DC)が，末梢での免疫制御において重要な役割を担っていることが明らかになってきた．この性質を応用して，特殊な培養法やさまざまな免疫抑制分子を遺伝子導入することにより免疫抑制能を強化した樹状細胞を用いた抗原特異的な免疫抑制療法が，研究されている．本稿では，樹状細胞の移入による抗原特異的免疫抑制法の応用の試みについて現況を概説する．

樹状細胞の生理的機能

生体内の各組織に分布する樹状細胞は体外より侵入した微生物などの異物を認識・貪食して，異物蛋白質由来のペプチドを主にMHCクラスII分子とともに細胞表面に提示する．この過程で樹状細胞は成熟し，種々の共刺激分子や細胞接着分子の発現が増強し，T細胞を強力に活性化する能力を獲得する(図1)．抗原特異的なレセプターを有するT細胞が，成熟樹状細胞により活性化されることにより，免疫反応が開始される．このような樹状細胞の特性を生かして，腫瘍免疫や感染免疫を誘導する細胞ワクチン療法が盛んに行われている．

一方で生体の各組織を構成する細胞の恒常的な細胞死に伴って，それらの細胞が有する多くの自己抗原が，樹状細胞に取り込まれる．この場合，樹状細胞は成熟刺激を受けないために，共刺激分子の発現が低い未熟樹状細胞のままにとどまり，この未熟樹状細胞上のMHC・自己ペプチド複合体を認識したT細胞は不活性化(アナジー)される．これらのことから，未熟樹状細胞が末梢での免疫寛容(peripheral tolerance)の維持において中心的役割を果たしていると考えられている[1]．また近年，免疫寛容の維持に積極的にかかわっているregulatory T細胞(制御性T細胞)も，樹状細胞の存在下で増殖することがわかってきた．

抑制性樹状細胞の調整

ヒトおよびマウスの系において，末梢血の単球，骨髄細胞，あるいは末梢血中の造血幹細胞などより，*in vitro*で樹状細胞を分化・誘導する培養法が開発されている．上記のように，生体内において樹状細胞は，その環境によりT細胞応答を抑制する活性を獲得することがあり，*in*

* Therapeutic immunoregulation by transfer of dendritic cell.

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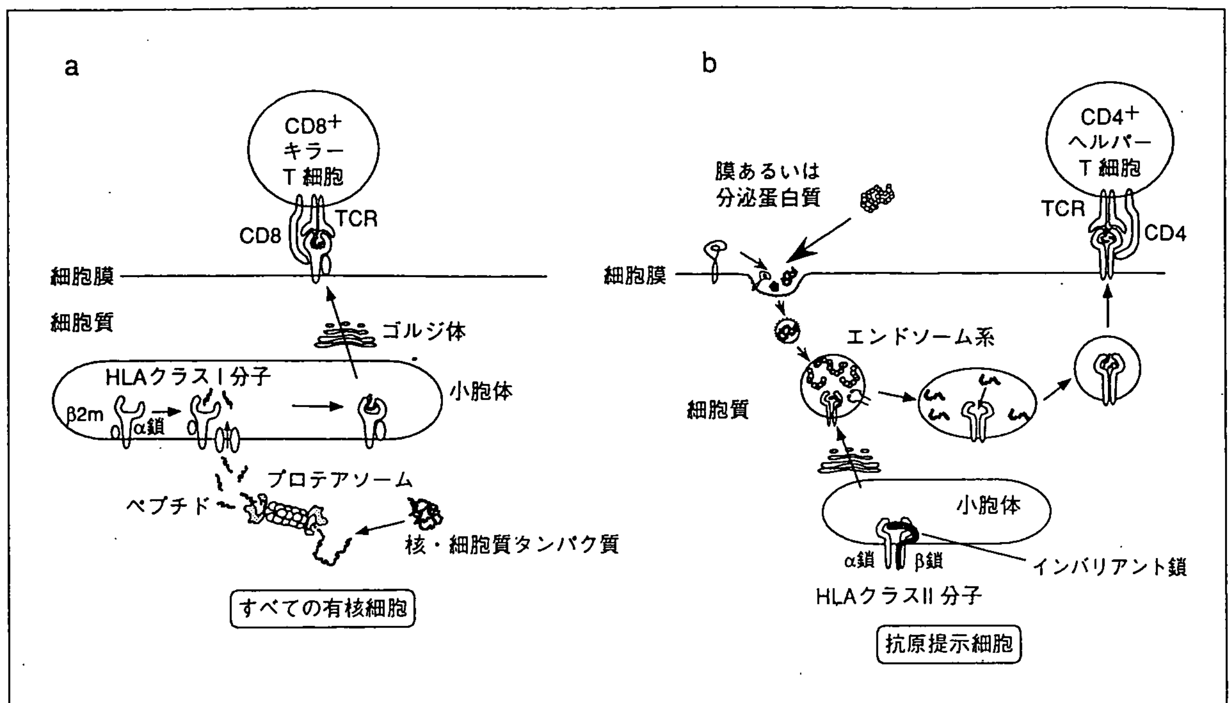

図1　抗原のプロセシングとHLAによるT細胞への抗原提示
a：HLAクラスI分子による抗原の提示：すべての有核細胞において，核あるいは細胞質に存在する自己蛋白質や細胞に感染したウイルスなどの非自己蛋白質に由来するペプチドは，HLAクラスI分子により細胞表面に提示される．これらの蛋白質は，細胞質に存在する蛋白質分解酵素複合体であるプロテアソームにより，ペプチドへと分解され，小胞体内腔へと輸送される．小胞体の内腔で，HLAクラスI分子の重鎖－β2ミクログロブリン(β2m)－ペプチドの複合体が形成され細胞表面へ輸送されて，CD8⁺キラーT細胞へ提示される．
b：HLAクラスII分子による抗原の提示：樹状細胞などの抗原提示細胞は，細胞外あるいは細胞膜に存在する蛋白を取り込み，これをエンドソーム中に存在するカテプシンなどの蛋白分解酵素により限定して分解してオリゴペプチドを産生する．一方，HLAクラスII分子は，小胞体において合成されエンドソームへ輸送される．その後，エンドソーム系の小胞においてHLAクラスII分子と抗原ペプチドが会合し，細胞表面へと輸送されてCD4⁺ヘルパーT細胞へ提示される．

表1　これまでに報告されている樹状細胞(DC)移入療法の概要

樹状細胞(DC)移入療法	免疫抑制効果	参考文献
未熟DCの移入療法	ヒト*in vivo*でのインフルエンザペプチドに対する反応抑制	2)
培養法の調整による抑制性DCの作成		
低濃度GM-CSFによる培養	マウスの同種異系(アロ)心移植の生着延長	3)
TGF-βとIL-10を用いた培養	マウスの異種あるいはアロGVHDの抑制	4)
TNF-αによる成熟刺激	EAEの発症抑制	5)
遺伝子導入による抑制性DCの作成		6)
IL-10	マウスのアロ腸管移植の生着	7)
FasL	マウスのアロ心移植の生着延長	8)
遺伝子改変ES細胞由来の樹状細胞の誘導	EAEの発症抑制(本文参照)	11)

*vitro*でそのような環境を模すことにより抑制性樹状細胞を作製できると考えられる．これまでに，表1に示すような培養方法による抑制性樹状細胞の誘導が報告されている．

1．未熟樹状細胞の移入による抗原特異的免疫抑制[2)]

未熟樹状細胞は，T細胞にアナジーあるいは細胞死を誘導する活性を有していると考えられている．そこでヒトの末梢血から*in vitro*で樹状細胞を分化誘導し，成熟刺激を加えない“未熟”な状態でモデル抗原としてインフルエンザウイルスに由来するペプチドをパルスし，人体に戻すという試みが行われた．この結果，投与する前には認められていた，このペプチドに対するT

細胞応答のみを特異的に抑制できることが示された．

2．特殊な培養法による抑制性樹状細胞の誘導

骨髄などから樹状細胞を誘導する際にはGM-CSF (granulocyte-macrophage colony stimulating factor)が必要であるが，通常用いるよりも低濃度のGM-CSFで誘導したり，あるいは通常濃度のGM-CSFで誘導した骨髄由来の樹状細胞を抗炎症性サイトカインであるTGF-βとIL-10を培養終末に添加することにより，CD80やCD86, CD40などの共刺激分子の発現が低い“未熟な”樹状細胞ができることが明らかになった．これらの樹状細胞を用いた*in vitro*での実験においてアロMLR (mixed lymphoid reaction)を抑制し，さらに*in vivo*においてもこの樹状細胞をマウスに投与することにより，免疫寛容能を誘導した系統に特異的に移植片の長期間の生着や異種あるいはアロのGVHDの抑制が観察された[3)4)]．

また*in vitro*でマウスの樹状細胞の成熟を誘導する場合には，TNF-α，抗CD40抗体，LPSなどで刺激を与えることが多いが，TNF-αのみで刺激した樹状細胞はMHC-クラスⅡや共刺激分子は高発現するにもかかわらず，IL-12などの産生が少ない．このように不完全な成熟を示した樹状細胞(semi-mature DC)は，*in vivo*において抗原特異的に免疫応答を抑制し，実験的自己免疫性脳脊髄炎(experimental autoimmune encephalomyelitis；EAE)の発症を抑制することが報告されている．この*in vivo*での反応は，不完全成熟樹状細胞によりIL-10を産生するCD4陽性T細胞が誘導されることと関連があるようである[5)]．

遺伝子改変による樹状細胞の機能修飾

免疫応答を抑制することが報告されているさまざまな分子〔IL-10, FasL, IDO (indoleamine 2, 3-dioxygenase)，など〕を，遺伝子銃(gene gun)やアデノウイルスあるいはレトロウイルスなどを用いて遺伝子導入して，樹状細胞に発現させることにより，免疫抑制能を強化した免疫寛容誘導性(tolerogenic)樹状細胞を作製する研究が報告されている[6)]．

IL-10遺伝子を導入した樹状細胞は，MHC-クラスⅡや共刺激分子の発現が低く，アロ反応性T細胞の活性化を抑制する．さらにこのDCで前処置したマウスはアロにおける腸管移植の生着が認められた．これは，IL-10が樹状細胞の成熟を抑制することにより免疫抑制作用を発現するものと考えられる．また，IL-10がTh1-Th2のバランスをTh2へシフトさせることも関連していると考えられる[7)]．

正常細胞や癌細胞，活性化T細胞などをアポトーシスに導くことが知られているFasL(CD95L)遺伝子を骨髄由来単球(BM-Mono)や骨髄由来樹状細胞に導入し，FasLを高発現した樹状細胞が作製されている．これらの樹状細胞は，*in vitro*と*in vivo*においてアロ抗原に特異的な免疫応答を抑制した．さらに，この樹状細胞に抗原(卵白アルブミン：OVA)を貪食させたものを用いて，*in vivo*においてOVA抗原特異的な免疫抑制を誘導することが可能であった．これは，樹状細胞に反応するアロ抗原特異的あるいは非自己抗原(OVA)特異的T細胞が，樹状細胞により活性化され，これに伴いFasの発現が高まることでFasLへの感受性が増し，アポトーシスが誘導されるためと考えられている[8)]．

このほかにも，さまざまな免疫制御分子をコードする遺伝子が樹状細胞に導入され，アポトーシス，アナジー，Th1/Th2バランスの制御を介して，アロ抗原あるいは非自己抗原に特異的に免疫寛容を誘導したモデルが示されている．

ES細胞由来の樹状細胞を用いた免疫制御

樹状細胞に遺伝子改変を行う方法として，これまでは，レトロウイルスあるいはアデノウイルスなどのウイルスベクターを用いる方法が主流であった．しかし，ウイルスベクターを用いる方法には，遺伝子導入の効率と安定性，さらにはベクターシステムのもつ潜在的な危険性などの問題が伴う．そこで，われわれはウイルスベクターを使用しなくても，電気穿孔法により容易に遺伝子導入が可能なES細胞に着目し，これに遺伝子導入した後に樹状細胞に分化誘導する方法を開発した．これまでに，マウスES細胞よりOP9(マウス骨髄のストローマ細胞)とGM-CSFを用いて，樹状細胞(ES-DC)を分化誘導する方法を確立している．このES-DCは，貪食能や抗原提

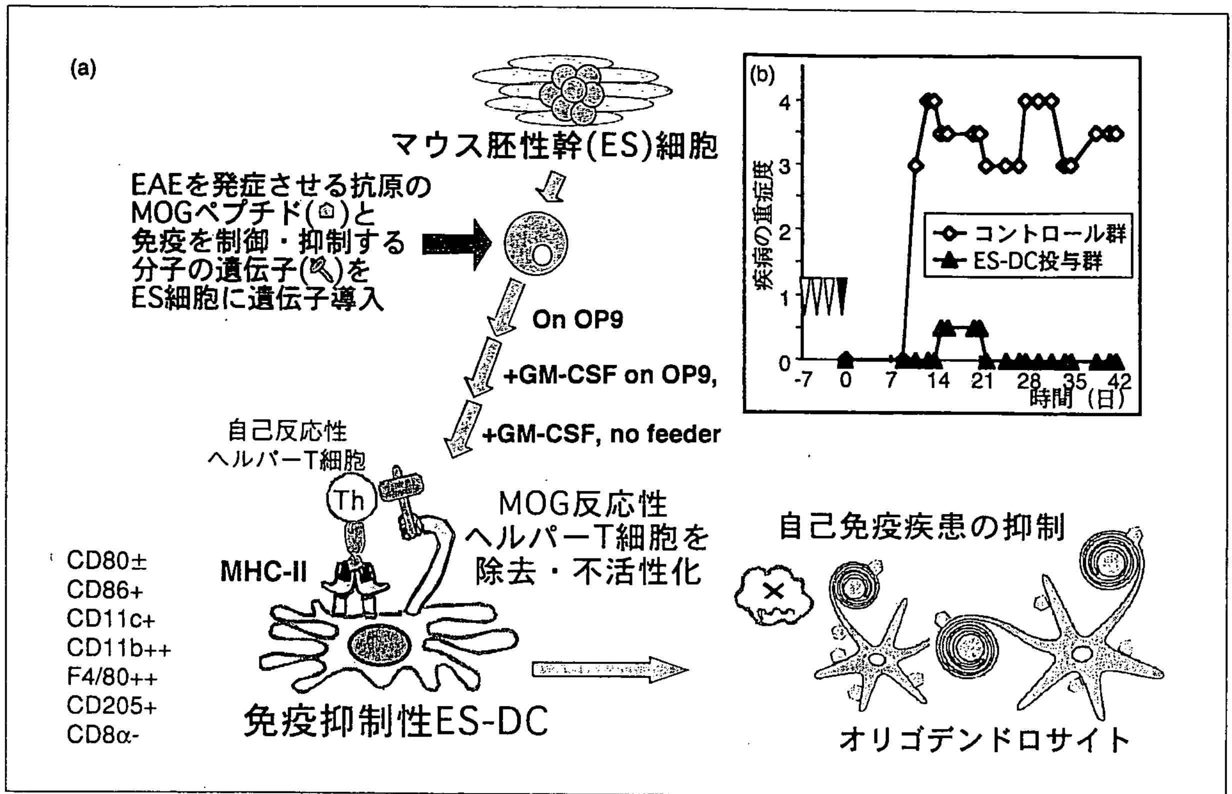

図2　遺伝子改変ES-DCによるEAE(実験的自己免疫性脳脊髄炎)の発症予防
a：EAEを発症させる抗原として知られているMOGペプチドをコードする遺伝子と，TRAILあるいはPD-L1の遺伝子をES細胞に遺伝子導入し，これを樹状細胞(ES-DC)へ分化誘導した．
b：この遺伝子改変ES-DC(▲)を1×10⁶ずつ3回前投与することにより，コントロール群(◇)に比較して，MOGペプチドで誘導されるEAEの発症が抑制された．(▽はES-DCの前投与，▼はEAEの誘導を示す．)

示能などをもち，機能的樹状細胞であることが確かめられている．さらに，ES細胞に任意の抗原遺伝子を遺伝子導入し，これを樹状細胞に分化誘導したところ，*in vitro*において，このES-DCはMHC上に抗原ペプチドを提示していることが確認された．そして，この抗原遺伝子導入ES-DCをマウスへ投与することにより，抗原特異的にT細胞を活性化できた[9)10)]．

この手法を応用して，ES細胞にEAEを誘導するミエリン蛋白質の一種であるMOG(myelin oligodendrocyte glycoprotein)由来のペプチドをコードする遺伝子と，活性化T細胞にアポトーシスを誘導するTRAILやアナジーを誘導するPD-L1の遺伝子を導入し，それを樹状細胞へ分化誘導し，MOGペプチド+MHCクラスⅡとTRAILあるいはPD-L1を細胞表面に高発現したES-DCを作成することができた．この遺伝子改変ES-DCを投与することにより，MOGペプチドで誘導されるEAEの発症を抑制することができ，さらにこのES-DCを投与しても無関係な抗原(KLH)に対する免疫応答への影響は観察されず，抗原特異的免疫抑制が誘導されていることを示した(図2)[11)]．現時点ではヒトES細胞の利用についての倫理的問題が論議されているが，将来的に倫理的・技術的な諸問題を解決できれば，ヒトES-DCの免疫療法への応用が期待される．

おわりに

自己免疫疾患に対する樹状細胞を用いた治療法は，まだ研究段階にあり，樹状細胞を用いた抗原特異的な免疫制御療法については，現時点では，主にモデル抗原を用いた動物実験により有効性やその安全性の確認を行っている段階にある．しかしながら，原因に対する治療法が確立されていない自己免疫疾患に対する治療法開発への新たな戦略のひとつとして，今後のさらなる研究開発が期待される．われわれは，抗原特異的な免疫制御療法の開発は，免疫学に課せ

られた重要な課題として解決すべき問題のひとつであると考えている．

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抗原提示細胞をターゲットとした免疫抑制療法

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近年，プロフェッショナル抗原提示細胞である樹状細胞が，末梢での免疫制御において重要な役割を担っていることが明らかになってきた．この性質を応用して，特殊な培養法やさまざまな免疫抑制分子を遺伝子導入することにより免疫抑制能を強化した樹状細胞を用いた抗原特異的な免疫抑制療法が，研究・開発されている．われわれも遺伝子改変したマウスES細胞より分化誘導した樹状細胞（ES-DC）を用いて，抗原特異的免疫抑制の誘導に成功した．

はじめに

自己免疫疾患の治療法として，現在の診療ではステロイド，免疫抑制薬，非ステロイド性抗炎症薬（non-steroidal anti-inflammatory drugs：NSAIDs）が日常的に用いられている．しかし，投与に伴う種々の副作用や無効症例，再発症例などの問題は少なくない．このため，疾患特異的な免疫制御や副作用の少ない治療法が求められており，生物学的製剤療法，遺伝子治療などさまざまな試みがなされている．自己免疫疾患の本態は，自己抗原に対する免疫寛容が何らかの原因で破綻しているものと考えられており，疾患の標的となっている抗原に対する免疫応答を特異的に抑制する治療法が考案されている．その1つとして，機能を人為的に修飾した抗原提示細胞の移入による病的リンパ球の排除や不活性化が検討されている．本稿では，樹状細胞を用いた抗原特異的免疫抑制法を中心に，その自己免疫疾患治療への応用の試みについて現況を概説する．

Key Words

樹状細胞（DC）
免疫寛容
抗原特異的免疫抑制
ES細胞由来樹状細胞（ES-DC）
実験的自己免疫性脳脊髄炎（EAE）

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1．樹状細胞の生理的機能

生体内の各組織に分布する樹状細胞は体外より侵入した微生物などの異物を認識・貪食して，異物蛋白質由来のペプチドをおもにMHCクラスII分子とともに細胞表面に提示する．この過程で樹状細胞は成熟し，種々の共刺激分子や細胞接着分子の発現が増強し，T細胞を強力に活性化する能力を獲得する（図❶）．抗原特異的なレセプターを有するT細胞が，成熟樹状細胞により活性化され，免疫反応が開始される．このような樹状細胞の特性を生かして，腫瘍免疫や感染免疫を誘導する細胞ワクチン療法が盛んにおこなわれている．

一方で，生体の各組織を構成する細胞の恒常的な細胞

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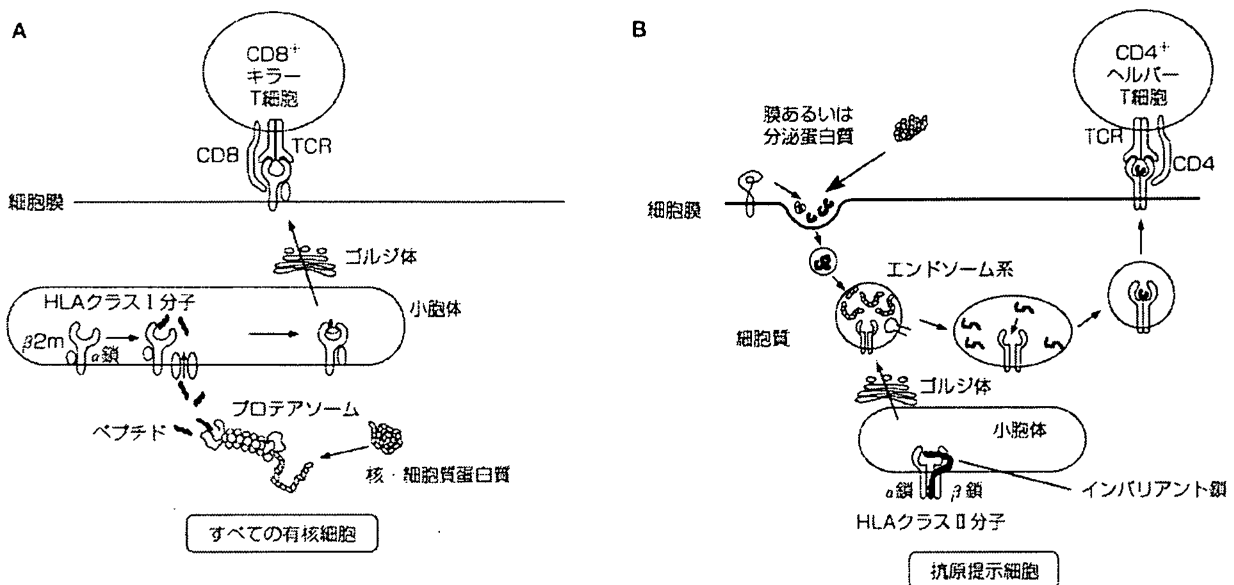

図❶ 抗原のプロセシングと HLA による T 細胞への抗原提示

A：HLA クラスI分子による抗原の提示：すべての有核細胞において，核あるいは細胞質に存在する自己蛋白質や細胞に感染したウイルスなどの非自己蛋白質に由来するペプチドは，HLA クラスI分子により細胞表面に提示される．これらの蛋白質は，細胞質に存在する蛋白質分解酵素複合体であるプロテアソームにより，ペプチドへと分解され，小胞体内腔へと輸送される．小胞体の内腔で，HLA クラスI分子の重鎖-β2 ミクログロブリン(β2M)-ペプチドの複合体が形成され細胞表面へ輸送されて，CD 8+キラーT 細胞へ提示される．

B：HLA クラスII分子による抗原の提示：樹状細胞などの抗原提示細胞は，細胞外あるいは細胞膜に存在する蛋白を取り込み，これをエンドソーム中に存在するカテプシンなどの蛋白分解酵素により限定分解してオリゴペプチドを産生する．一方，HLA クラスII分子は，小胞体において合成されエンドソームへ輸送される．その後，エンドソーム系の小胞において HLA クラスII分子と抗原ペプチドが会合し，細胞表面へと輸送されて CD 4+ヘルパーT 細胞へ提示される．

死（アポトーシス）に伴って，それらの細胞が有する多くの自己抗原が，樹状細胞に取り込まれる．この場合，樹状細胞は成熟刺激を受けないために，共刺激分子の発現が低い未熟樹状細胞のままに留まり，この未熟樹状細胞上の MHC・自己ペプチド複合体を認識した T 細胞は不活性化（アナジー）される．また一部の未熟樹状細胞は，FasL を発現して自己反応性 T 細胞を積極的に除去しているという報告もある．これらのことから，未熟樹状細胞が自己反応性 T 細胞を除去，不活性化することにより，末梢での免疫寛容 (peripheral tolerance) の維持において中心的役割を果たしていると考えられている[1)2)]．また近年，免疫寛容の維持に積極的にかかわっている制御性 T 細胞 (regulatory T cell) も，樹状細胞の存在下で増殖することがわかってきた[3)]．

そこで，これらの樹状細胞の性質を利用して，さまざまな方法により免疫抑制機能を強化した樹状細胞を作成し，抗原とともに投与することにより，抗原特異的な免疫抑制を誘導する研究が試みられている（図❷）．

2．抑制性樹状細胞の調整

ヒトおよびマウスの系において，末梢血の単球，骨髄細胞，あるいは末梢血中の造血幹細胞などより，*in vitro* で樹状細胞を分化・誘導する培養法が開発されている．上記のように，生体内において樹状細胞は，その環境により T 細胞応答を抑制する活性を獲得することがあり，*in vitro* でそのような環境を模すことにより抑制性樹状細胞を作製できると考えられる．これまでに，表❶に示すような培養方法による抑制性樹状細胞の誘導が報告されている．

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