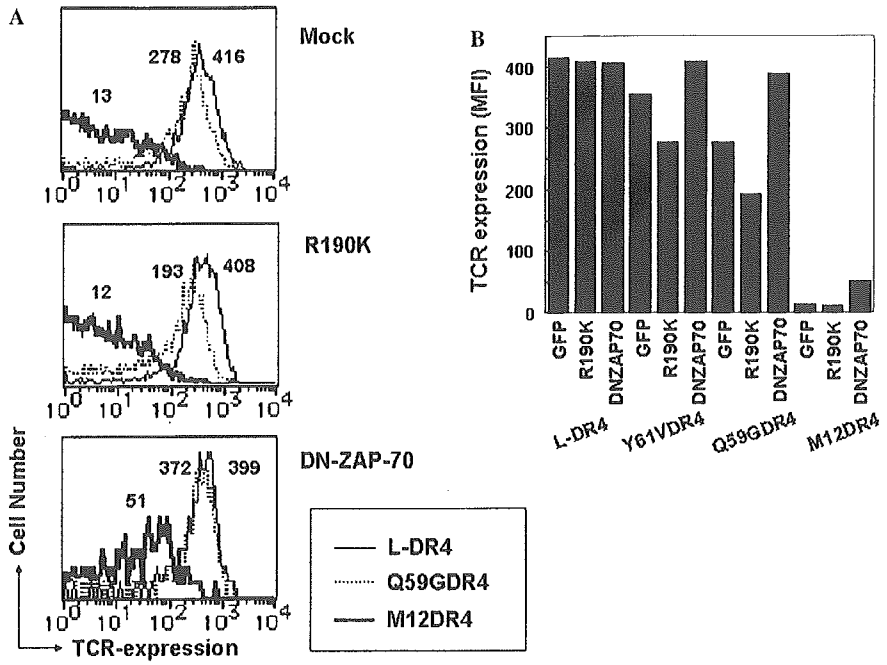

Fig. 5. Inhibition of TCR down-modulation in T cells stimulated with each L cell transfectant by DN-ZAP-70 expression. After the infection of the retroviral vectors, T cells were grown for 2 weeks and then were stimulated with each L cell transfectant. The inhibitory effect on TCR down-modulation was shown by histogram (A) and its MFI level (B). TCR was stained with anti-TCR-αβ mAb after 6 h stimulation. GFP-positive cells were gated and analyzed. The results are representative of three independent and reproducible experiments.

endogenous ZAP-70 from binding to tyrosine-phosphorylated TCR-ζ [29]. To investigate whether the DN ZAP-70 binds to the phosphorylated TCR-ζ chain in the stimulated T cells, DN ZAP-70 and R190K were immunoprecipitated with anti-FLAG mAb after 72 h of infection and the immunocomplexes were then subjected to Western blot analyses. The anti-FLAG mAb co-precipitated two forms (p21 and p23) of phosphorylated TCR-ζ with DN ZAP-70 from the lysate of T cells stimulated with M12DR4. On the other hand, similar to the result shown in Fig. 1, the anti-FLAG mAb co-precipitated only the p21 form of phosphorylated TCR-ζ from the lysate of Q59GDR4 stimulated T cells (Fig. 6). No TCR-ζ was detected in the immunocomplex from the T cells expressing R190K. These results indicate that DN ZAP-70, but not R190K, bound to the phosphorylated TCR-ζ chain and hence DN ZAP-70 prevents the recruitment of endogenous ZAP-70 to the TCR complexes. Importantly, these data suggest that the DN ZAP-70/TCR-ζ association suppresses the T cell responses stimulated not only with M12DR4 but also with OPALs.

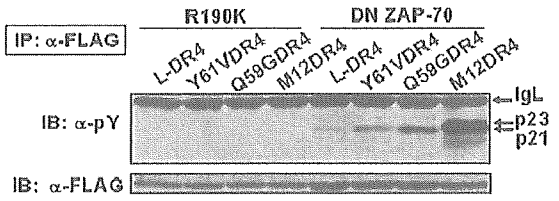

Fig. 6. The association of phosphorylated TCR-ζ with DN ZAP-70. T cells expressing R190K or DN ZAP70 were stimulated for 5 min with each L cell transfectant. R190K or DN ZAP-70 was immunoprecipitated by anti-FLAG mAb (α-FLAG) and phosphorylated forms of TCR-ζ associated with DN ZAP-70 were immunoblotted with the anti-phosphotyrosine mAb (α-pY). The blots were reprobed with anti-FLAG mAb.

The inhibitory effects of DN ZAP-70 on PLC-γ1 phosphorylation and PLC-γ1/ZAP-70 association in stimulated T cells

A possible explanation for the significance of the binding of kinase-inactive ZAP-70 to TCR-ζ is the recruitment of other signaling molecules to the TCR complexes. As one such candidate molecule, we investigated tyrosine-phosphorylation of PLC-γ1 and its association to ZAP-70 in the stimulated T cells. As shown in Fig. 7, M12DR4-stimulation induced an intense tyrosine-783 phosphorylation of PLC-γ1. Q59GDR4-stimulation also induced an increased tyrosine-phosphorylation of PLC-γ1 in comparison to that of the T cells co-cultured with L-DR4. The tyrosine-783 phosphorylation of PLC-γ1 was suppressed in DN ZAP-70 expressing T cells stimulated with Q59GDR4 and M12DR4. These observations suggest that DN ZAP-70 associated with tyrosine-phosphorylated TCR-ζ inhibits the tyrosine-phosphorylation of PLC-γ1 in T cells stimulated with M12DR4 and Q59GDR4.

To check the possibility that ZAP-70 could help PLC-γ1 phosphorylation by recruiting it to TCR complexes, we investigated the phosphorylation of PLC-γ1 and its association with ZAP-70. PLC-γ1 phosphorylation in M12DR4

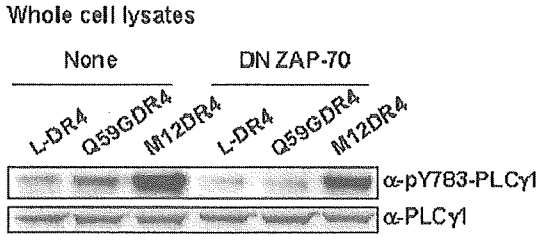

Fig. 7. The inhibitory effects of DN ZAP-70 on PLC-γ1 phosphorylation. After 72 h of infection, 3×10^6 T cells (about 50% cells were positive for GFP) were stimulated with each L cell transfectant for 10 min at 37 °C. Whole cell lysates were immunoblotted with anti-phospho-PLC-γ1 (Tyr783) Ab (α-pY783-PLC-γ1). The same blot was reprobed with anti-PLC-γ1 mAb (α-PLC-γ1) to confirm the equal loading. The data shown are representative results from three independent and reproducible experiments.

stimulated T cells was stronger than that in OPAL-stimulated T cells. Notably, ZAP-70 was co-immunoprecipitated with PLC-γ1 using anti-PLC-γ1 mAb in T cells stimulated with M12DR4, Q59GDR4, or Y61VDR4 (Fig. 8A). Although no phosphorylation of ZAP-70 was observed in the T cells stimulated with OPALs, the level of ZAP-70/PLC-γ1 association increased in comparison to that of the L-DR4 stimulated T cells. These results suggest that OPAL stimulation induces recruitment of unphosphorylated and kinase-inactive ZAP-70 and its association with incompletely phosphorylated TCR-ζ, and that ZAP-70/PLC-γ1 association results in the phosphorylation of PLC-γ1 leading to the T cell responses.

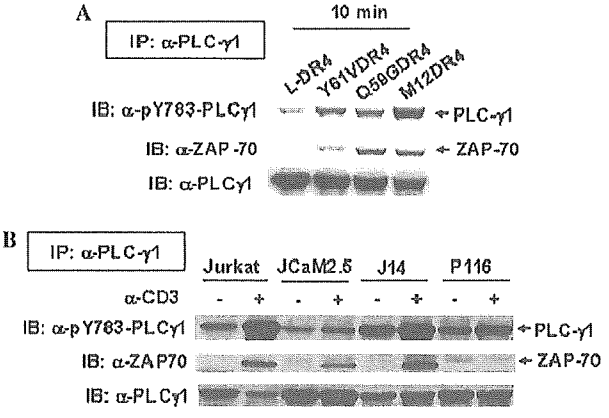

Fig. 8. The physical association of PLC-γ1 with ZAP-70 in TCR stimulated T cells. (A) The association of PLC-γ1 with unphosphorylated ZAP-70 in OPAL-stimulated T cells. T cells (5×10^6) were stimulated with each L cell transfectant for the indicated time, lysed, and subjected to immunoprecipitation using anti-PLC-γ1 mAb. As a control, T cells stimulated with L-DR4 were used. The anti-PLC-γ1 immunocomplexes were resolved by 10% SDS–PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-phospho-PLC-γ1 (Tyr783) antibody (α-pY783-PLC-γ1). The blots were reprobed with anti-PLC-γ1 mAb. (B) The association of PLC-γ1 with ZAP-70 in Jurkat cells. Cells were stimulated with anti-CD3ε mAb or left untreated and goat anti-mouse IgG on ice for 30 min and incubated at 37 °C for 1 min. Cells were lysed and immunoprecipitated with anti-PLC-γ1 mAb. The immunoprecipitates were subjected to Western blot analysis using anti-phosphoTyr783-PLC-γ1 (top) and anti-ZAP-70 mAb (middle). The membrane was reprobed with anti-PLC-γ1 mAb (bottom).

The association of PLC-γ1 with ZAP-70 is not specific to the T5-32 cells. The association of PLC-γ1 with ZAP-70 could also be detected in Jurkat T cells stimulated with anti-CD3 mAb, but not in unstimulated cells (Fig. 8B). ZAP-70 was co-precipitated with PLC-γ1 in wild-type Jurkat cells, the LAT deficient (JCaM2.5), and the SLP-76 deficient (J14) Jurkat cell lines, but not in ZAP-70-deficient (P116) cell line using anti-PLC-γ1 mAb immunoprecipitation. These results indicate that PLC-γ1 associates with ZAP-70 in TCR stimulated T cells even in the absence of LAT or SLP-76.

Discussion

Many models of T cell activation have been proposed to explain the differential effects of T cell stimulation between the full agonist and the APLs. Our previous studies have shown that single amino acid substitutions in the antigenic peptide can affect a range of responses such as the magnitude or level of proliferation, cytokine production, and the expression of various cell surface molecules in $CD4^+$ T cell clone, YN5-32, which recognizes the streptococcal M12p54-68 peptide and its APLs in the context of HLA-DR4 [16]. To prepare large amounts of cells in order to investigate the function of signaling molecules in YN5-32 after APL stimulation, the YN5-32 T cell clone was transformed with *H. saimiri* to establish T5-32 (manuscript in preparation, H. Tsukamoto et al. and [26]). T5-32 proliferates in response to exogenous IL-2 even in the absence of feeding with irradiated PBMC pulsed with the cognate peptide. T5-32 could be maintained and expanded with an IL-2-supplemented medium. T5-32 exhibited a magnitude of reactivity to the antigenic peptides similar to that of YN5-32.

The stable introduction of DN ZAP-70 into T5-32 has heretofore not been achieved, due to the very low transfection efficiency of T5-32 and significant cytotoxicity induced by the transfection method such as electroporation. Importantly, T5-32 stably expressing DN ZAP-70 or R190K could be successfully established using our retroviral system. In M12DR4 stimulated T5-32 cells, the expression of DN ZAP-70 inhibited the tyrosine-phosphorylation of ZAP-70 and significantly suppressed the TCR down-modulation while the expression of R190K had no such effects (Fig. 3). Since DN ZAP-70 could associate with both p21 and p23 forms of phosphorylated TCR-ζ, but R190K could not, the inhibitory effects of DN ZAP-70 seemed to be due to the prevention of endogenous ZAP-70 to bind to phosphorylated TCR-ζ (Fig. 6).

Contrary to its inhibitory effect on ZAP-70 phosphorylation, no significant difference in the magnitude of IFN-γ production was observed in M12DR4-stimulated T cells expressing DN ZAP-70, R190K or the mock vector. The lack of inhibitory effects on IFN-γ production in DN ZAP-70 expressing T cells stimulated with M12DR4 (Fig. 4A) suggests the following possibility; The T cell

activation stimulated with M12DR4 highly over-expressing HLA-DR4/M12p54-68 complexes is too strong to be inhibited by DN ZAP-70. Further supporting this possibility, the expression of DN ZAP-70 in T cells markedly inhibited the response to L-DR4 cells prepulsed with the fully agonistic M12p54-68 peptide (Fig. 4B).

We also checked the IL-2 production of the stimulated T cells at a single cell level using different methods, such as intracellular staining of IL-2 and cell surface detection of secreted IL-2. Compared with IFN-γ production, the IL-2 production was quite small even in the M12DR4 stimulated T cells. Because IL-2 production was hardly detected in the stimulated T cells, we could not evaluate the inhibitory effects of DN ZAP-70 on IL-2 production. It remains to be analyzed using other sophisticated methods in the future.

ZAP-70 was also involved in the TCR down-modulation stimulated with OPALs. The extent of TCR down-modulation correlated to the capacity of each APL to induce TCR signaling [19–22]. While full agonists induce the maximal degree of TCR down-modulation, partial agonists induce a lower level of TCR down-modulation [19,20] and this phenomenon correlates with the activity of ZAP-70 [30]. The ZAP-70-deficient Jurkat cell P116 and the kinase-dead ZAP-70 containing DK33 T cell showed a reduced TCR internalization by anti-CD3 antibody stimulation. P116 reconstituted with ZAP-70 restored TCR internalization to the level achieved in wild-type Jurkat cells [30]. As expected, an intense stimulator M12DR4 can induce a strong TCR down-modulation and OPALs can induce a weak but definite TCR down-modulation (Fig. 5).

It must be noted that, although the tyrosine-phosphorylation of ZAP-70 was not detected in T5-32 T cells stimulated with OPAL, the expression of DN ZAP-70 resulted in decrease of IFN-γ production (Fig. 4A) and an inhibition of the TCR down-modulation (Fig. 5). In addition, unphosphorylated ZAP-70 associated with partially phosphorylated TCR-ζ in OPAL-stimulated T cells. These observations suggested that the association of ZAP-70 with TCR-ζ might medicate activation of other signaling molecules regardless of its kinase activity. As one such candidate molecule, we chose PLC-γ1 and examined the tyrosine-phosphorylation of PLC-γ1 in T cells expressing DN ZAP-70 stimulated with the OPAL, because (1) we provided evidence that activation of protein kinase Cμ was involved in the T cell activation stimulated with Q59GDR4, thus suggesting the production of diacylglycerol by PLC-γ1 activity, and (2) a specific inhibitor for PLC (U-73122) inhibited the Q59GDR4 stimulated T cell responses, such as IFN-γ production and T cell proliferation [26]. As a result, we found that PLC-γ1 phosphorylation induced in TCR-stimulated T cells was inhibited in the presence of DN ZAP-70 (Fig. 7).

PLC-γ1 directly binds to the phosphorylated linker for activation of T cells (LAT) in TCR stimulated T cells. However, OPAL stimulation does not induce the tyrosine-phosphorylation of ZAP-70 and LAT [26], and we observed the association of PLC-γ1 with ZAP-70 even in LAT or SLP-76 deficient Jurkat T cells stimulated with anti-CD3ε antibody (Fig. 8B). Therefore, LAT and SLP-76 seemed to be dispensable for the PLC-γ1/ZAP-70 association. Williams et al. showed that a tyrosine (corresponding to Tyr-319) phosphorylated peptide derived from interdomain B of ZAP-70 binds to a GST fusion protein with the C-terminal-side SH2 domain of PLC-γ1 [32]. In addition, even the unphosphorylated ZAP-70 peptide was also shown to be weakly associated with the PLC-γ1 C-terminus SH2 protein. Therefore, it seems feasible that unphosphorylated ZAP-70 and PLC-γ1 may be directly associated through the interdomain B of ZAP-70 and C-terminus SH2 of PLC-γ1. In our experimental system, how unphosphorylated ZAP-70 and PLC-γ1 associate remains to be elucidated in OPAL-stimulated T cells. Although the tyrosine-phosphorylation of ZAP-70 was undetectable, an increased PLC-γ1/ZAP-70 association was detected in OPAL-stimulated T cells compared with the findings in unstimulated T cells. Therefore, we presume that the PLC-γ1/ZAP-70 association correlates with the phosphorylation of PLC-γ1.

In summary, we investigated whether unphosphorylated ZAP-70 is required for the activation of T cells in response to OPAL by expression of DN ZAP-70. Our findings suggest that the association of unphosphorylated ZAP-70 with the incompletely phosphorylated TCR-ζ chain is necessary for T cell activation induced by OPAL. These observations provide a new insight into the unidentified role of ZAP-70. The further characterization of those steps in the TCR signaling pathway after APL stimulation is important to understand the activation, homeostatic proliferation, and differentiation of T cells, and the introduction of dominant negative forms of signaling molecules into T5-32 is expected to help elucidate these phenomena.

Acknowledgments

We thank Drs. T. Kitamura, N.R. Landau, and A. Wiss for providing retroviral vectors (pMX-IRES-GFP and MLV-gagpol-IRES-bsr), vector encoding for the amphotropic envelope glycoprotein (SV-A-MLV-Env), and mutant Jurkat cells (JCam2.5 and J14), respectively. We also thank Dr. T. Ejima for providing human recombinant IL-2 and Ms. M. Ohara and Dr. B. Quinn for helpful comments on the manuscript. This work was supported in part by Grants-in-Aid 12051203, 14370115, and 15510165 from the Ministry of Education, Science, Technology, Sports and Culture, Japan.

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

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Received 31 May 2005
Available online 28 June 2005

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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doi:10.1016/j.bbrc.2005.06.096

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

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

Results

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

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

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

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

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

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

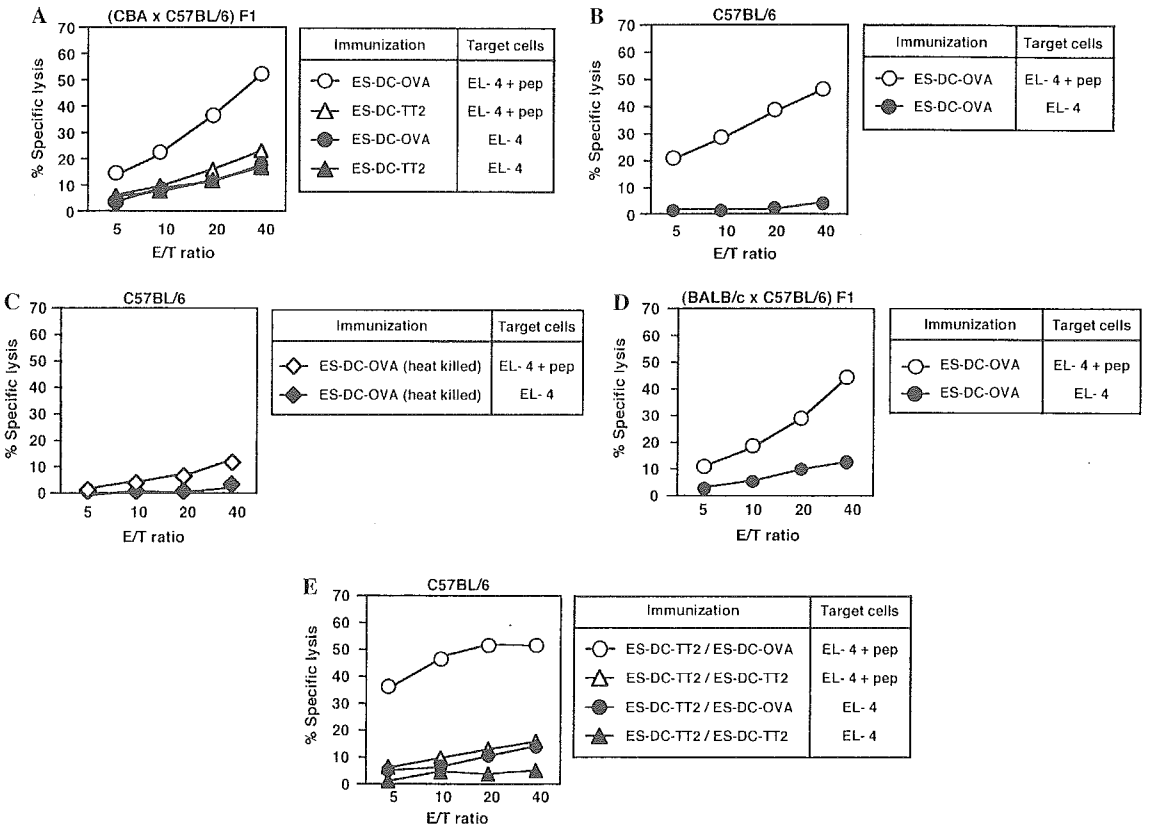

Fig. 1. Stimulation of OVA-specific CTL by ES-DC expressing OVA in syngeneic and semi-allogeneic mice. (CBA × C57BL/6) F1 (A), C57BL/6 (B, C), or (BALB/c × C57BL/6) F1 (D) mice were injected i.p. twice with ES-DC-OVA or ES-DC-TT2 (1×10^5/injection/mouse) on days −14 and −7. In (C), ES-DC-OVA were heat-killed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

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

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

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

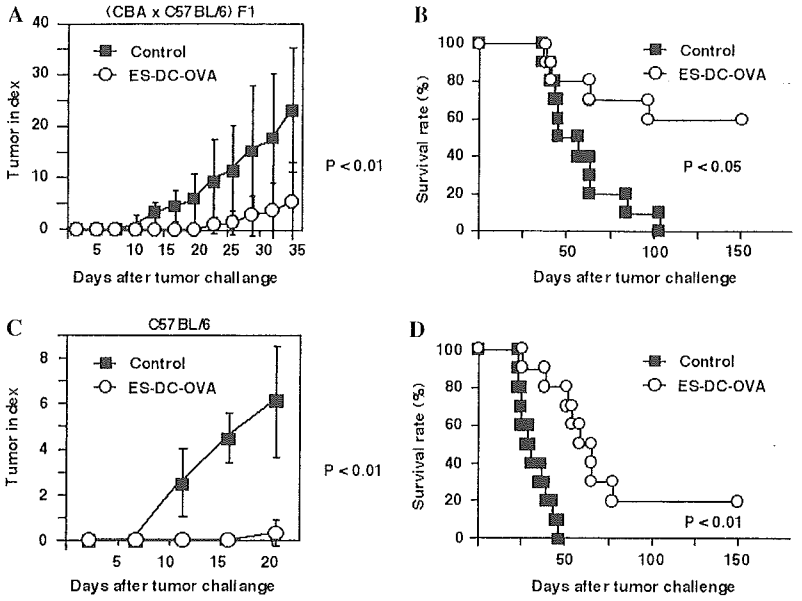

Fig. 2. Induction of protective immunity by ES-DC expressing OVA against OVA-expressing tumor cells in syngeneic and semi-allogeneic mice. (CBA × C57BL/6) F1 mice were injected i.p. twice on days −14 and −7 with ES-DC-OVA (2×10^4/injection/mouse) or medium (RPMI-1640) only as control and were challenged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

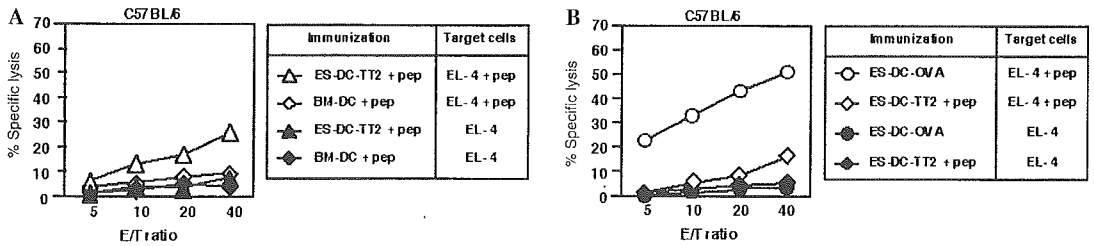

Fig. 3. Priming of OVA-specific CTL by OVA peptide-loaded DC or OVA-expressing ES-DC in semi-allogeneic mice. BM-DC or ES-DC-TT2 (non-transfectant ES-DC) were pulsed with $OVA_{257-264}$ synthetic peptide (10 μM) for 2 h and injected i.p. into C57BL/6 mice (1×10^5/injection/mouse) (A). ES-DC-OVA or OVA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

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

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

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

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

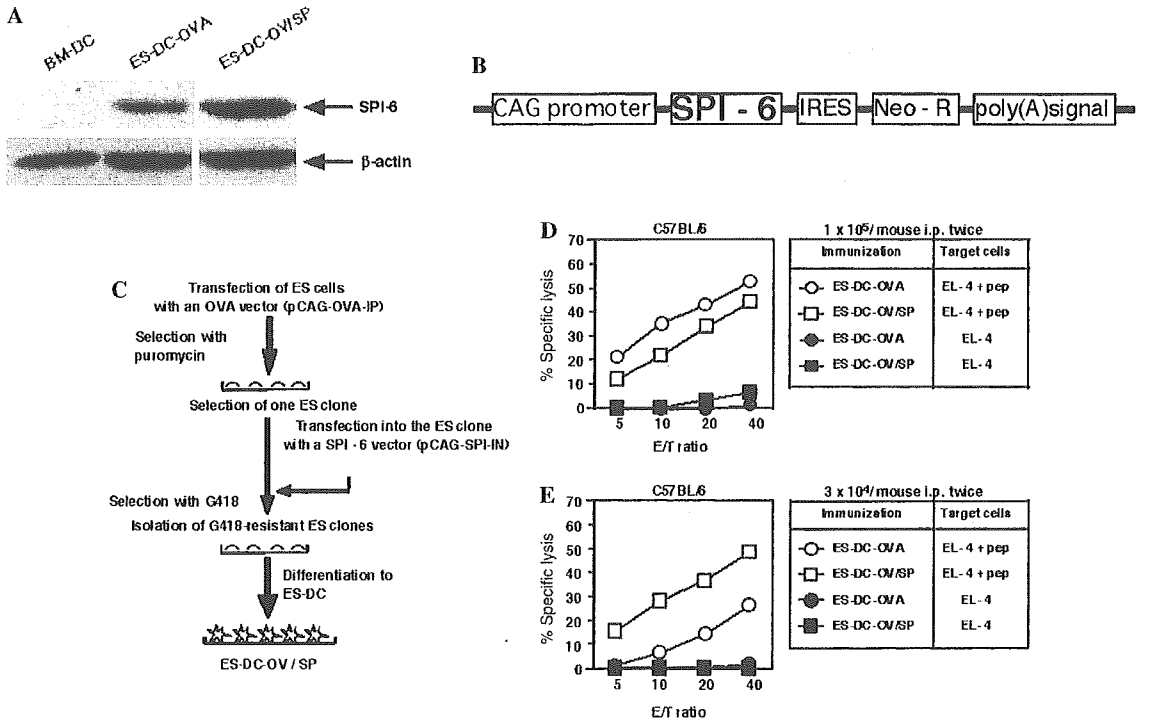

Fig. 4. Expression of SPI-6 in DC and priming of antigen-specific CTL by ES-DC expressing OVA and SPI-6 in semi-allogeneic mice. (A) The levels of expression of SPI-6 in BM-DC, ES-DC-OVA, and ES-DC-OV/SP were analyzed by a Western blotting analysis. The same samples were analyzed also for β-actin expression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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

Acknowledgments

Grant support: Grants-in-Aid 12213111, 14370115, 14570421, and 14657082 from the Ministry of Education, Science, Technology, Sports, and Culture, Japan and a Research Grant for Intractable Diseases from Ministry of Health, Labour and Welfare, Japan, and grants from the Tokyo Biochemical Research Foundation and the Uehara Memorial Foundation are acknowledged.

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ELSEVIER

Coculture of Th Cells With Interleukin (IL)-7 in the Absence of Antigenic Stimuli Induced T-Cell Anergy Reversed by IL-15

Yu-Zhen Chen, Zhong-Fang Lai, and Yasuharu Nishimura

ABSTRACT: Interleukin-7 (IL-7) is an important survival factor for T cells. We report here for the first time that it has another important role, facilitating T-cell clonal unresponsiveness, or anergy. The anergy was induced by a 20-day coculture of activated-human $CD4^+$ T-cell clones with IL-7 and irradiated peripheral blood mononuclear cells without antigenic stimuli. T-cell survival, but not T-cell anergy induction, was dependent on direct cell contacts between T cells and irradiated peripheral blood mononuclear cells. The anergic T cells exhibited no or very low expression of IL-7 receptor α chain (IL-7Rα), IL-2 receptor α chain (IL-2Rα), and common γ chain (γc), and did not express cytotoxic T-lymphocyte–associated protein 4, but expressed IL-15Rα. Coculture for 3 to 9 days of anergic T cells with a T-cell–activating cytokine IL-15, but not IL-2, restored the responsiveness of IL-7–induced anergic T cells together with reexpressions of IL-7Rα, IL-2Rα, and γc. The anergy induction by IL-7 and restoration of responsiveness by IL-15 suggest novel mechanisms for regulation of helper T-cell responses, induction of peripheral tolerance, and breakdown of T-cell self-tolerance. *Human Immunology 66, 677–687 (2005).* © American Society for Histocompatibility and Immunogenetics, 2005. Published by Elsevier Inc.

KEYWORDS: human; $CD4^+$ T cells; anergy; IL-7; IL-15

ABBREVIATIONS

APC	antigen-presenting cell
CTLA4	cytotoxic T-lymphocyte–associated protein 4
GAD65	glutamic acid decarboxylase 65
HLA-DR	human leukocyte antigen–DR
ICOS	inducible costimulator
IL	interleukin
IL-2Rα	IL-2 receptor α chain
IL-7Rα	IL-7 receptor α chain
mAb	monoclonal antibody
MHC	major histocompatibility complex
NK	natural killer
PBMC	peripheral blood mononuclear cell
PD-1	programmed death-1
PPD	purified protein derivative of tuberculin
rh	recombinant human
TCRαβ	T-cell receptor αβ chains
γc	common γ chain

INTRODUCTION

Interleukin (IL)-2 is an important growth and survival factor for T lymphocytes and sensitizes these cells to Fas ligand-mediated activation-induced cell death [1]. After stimulation by antigen, IL-2 promotes T-cell proliferation and promotes T-cell survival, probably by inducing the expression of Bcl-2 and related proteins [2–4]. However, the mice that lack expression of IL-2 or IL-2 receptor-β (IL-2Rβ) chain gene accumulate activated T cells and develop autoimmunity [5, 6], which suggests the important role of IL-2 in terminating T-cell response and maintaining tolerance *in vivo*. Thus, IL-2 plays a critical role in the regulation of immune responses of T cells. IL-7 is produced predominantly by stromal tissues [7] as well as by dendritic cells [8]. Mice with targeted disruption of IL-7 or the IL-7 receptor (IL-7R) gene exhibited greatly dimin-

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Received June 1, 2004; revised February 14, 2005; accepted February 16, 2005.

Human Immunology 66, 677–687 (2005)

0198-8859/05/$–see front matter
doi:10.1016/j.humimm.2005.02.006

ished lymphoid development, with a 10–20-fold decrease in the total number of T cells and the absence of B cells [9, 10]. Humans with mutations in the IL-7R gene exhibit a greatly diminished T-cell development, but in contrast with the mice, humans have a normal B-cell development, resulting in T-B$^+$ natural killer cell (NK)$^+$ severe combined immunodeficiency [11]. In the periphery, IL-7 is critical for survival and homeostatic proliferation of naive T cells as a means of maintaining a stable number of T cells [12, 13].

IL-15, a 14–18-kDa cytokine originally discovered as a T-cell stimulatory activity present in culture supernatants of a simian kidney epithelial cell line, has biologic activities similar to those of IL-2 [14–17]. IL-15 can support the growth of activated T cells and can activate cytolytic effector T cells and NKT cells [18]. IL-15 can also serve as a chemoattractant for T lymphocytes [19] and can exert B4 cell costimulatory activity for proliferation and antibody production [20]. Members of the IL-2 subfamily of type I cytokines, such as IL-2, IL-4, and IL-7, potentially compete with each other for the recruitment of common γ-chain (γc), because receptors for these cytokines share γc in addition to other receptor subunit(s) unique to each cytokine. And IL-2 markedly reduced the effect of IL-15 on T-cell proliferation [21, 22].

Recent reports have demonstrated that dendritic cells produced IL-15 in response to type 1 interferon and that dendritic cell–derived IL-15 controls the induction of CD8$^+$ T-cell immune responses [23, 24]. These findings suggest important roles of these cytokines on regulation of physiologic and pathologic functions of T cells. In the present study, we investigated the effects of IL-7 on survival of human CD4$^+$ T-cell clones in the absence of antigenic stimuli, and we observed that IL-7 can induce T-cell anergy that can be reversed by IL-15.

MATERIALS AND METHODS

Generation of CD4$^+$ T-Cell Clones Specific to Purified Protein Derivative of Tuberculin

Purified protein derivatives of tuberculin (PPD)-specific CD4$^+$ T-cell lines were established from a donor as previously described [25]. Cloning of T cells was performed with Terasaki plates by limiting dilution at 0.3 T cells/well in the presence of 3×10^4/well irradiated (3000 cGy) autologous peripheral blood mononuclear cells (PBMCs) pulsed with PPD in RPMI 1640 medium supplemented with 10% heat-inactivated male plasma, antibiotics, L-glutamine, and recombinant human IL-2 (rhIL-2). Growing microcultures were then expanded at weekly intervals by feeding with irradiated PBMCs pulsed with PPD and complete medium supplemented with rhIL-2.

Persistent Culture of T-Cell Clones With rhIL-7 in the Absence of Antigenic Stimulus

TM1–6 T-cell clones specific to PPD and a T-cell clone specific to self-peptide from human glutamic acid decarboxylase 65 (GAD65) [26, 27] were used. After the T cells were cultured with irradiated (4500 cGy) PBMCs, antigen, and rhIL-2 for 11 to 15 days (the first culture), the T cells were washed in RPMI 1640 medium and cultured in complete medium supplemented with or without rhIL-7 or rhIL-2 in the absence of antigen for 20 to 30 days (the second culture). Major processes were as follows. On day 12 from the initiation of the first culture, T cells were washed three times in RPMI 1640 medium, and T cells (2×10^6/well in 48-well plates) were cultured with irradiated PBMCs (2×10^5/well in 48 well/plate) in the presence of rhIL-7 (100 ng/ml or 50 ng/ml), rhIL-2 (100 ng/ml), or medium alone. This second culture was continued for 20~30 days in which medium was exchanged every 3~4 days for fresh medium supplemented with or without the same cytokine as described above.

Measurement of Surviving T Cells

For monitoring the survival of T cells, T cells harvested on day 20 of the second culture were doubly stained with PI (5 μg/ml) (Sigma) and FITC-conjugated annexin V (Pharmingen) [28, 29]. Cells were analyzed by FACScan (Becton Dickinson) flow cytometry. In some experiments, we used 0.4 μm PET trancewell (Becton-Dickinson, 24 wells), which can allow for vectorial transport but does not allow for passage of mammalian cells. In the insert well, 2×10^5/well irradiated PBMCs were plated; 2×10^6 T cells were plated in the lower well, so that the T cells could not make contact with the PBMCs. After 20 days of culture, the T cells were analyzed. In another well, anti–human leukocyte antigen (HLA) class II monoclonal antibodies (mAbs) (a mixture of anti-DR, anti-DP, and anti-DQ mAbs) was added to investigate effects of HLA class II on the survival of T cells.

Cell Surface Phenotypes of T Cells

To observe changes in levels of cell surface molecules expressed on the T-cell clone, the T cells were harvested from the culture under different conditions. T cells were then incubated with mAbs directed against human CD28 (Becton Dickinson), CD3ε, CD4, CD8 (ATCC), CD40L, HLA-DR, T-cell receptor αβ chains (TCRαβ), CD3, CD25, CD44, cytotoxic T-lymphocyte–associated protein 4 (CTLA4), inducible costimulator (ICOS) (PharMingen), IL-7 receptor α chain (IL-7Rα; R&D Systems), CD132 (γc), or programmed death-1 (PD-1) (Bioscience), respectively, followed by staining with FITC-conjugated goat antimouse immunoglobulin G antibody when antibodies

were not directly labeled with fluorescence. Subsequently, cells were analyzed by FACScan.

Proliferative Response of T-Cell Clones

The GAD65p115-127 was synthesized and purified as described [25, 30–32]. Antigen-specific proliferation of the T-cell clones was investigated as previously described [25]. In brief, T-cell clones (3×10^4) were cultured with various doses of antigens PPD (1–20 μg/ml) or GAD65p115-127 (1–25 μg/ml) in the absence or presence of irradiated (3000 cGy) PBMCs (1.5×10^5) for 48 hours and pulsed with 1.0 μCi/well of ^{3}H-thymidine for the last 16 hours. Then the cells were harvested, and the incorporated radioactivity was measured with a micro-β scintillation counter [25, 27].

RESULTS

Influence of IL-7 on Survival of Th-Cell Clones in the Absence of Antigenic Stimuli

The survival of mature T cells is controlled in mice by a number of factors [33]. To further investigate the relationship between survival of human $CD4^+$ T cells *in vitro* and these factors, we established several $CD4^+$, TCRαβ+ human T-cell clones specific to PPD and selected TM5-1 and TM2-2 clones. To exclude the possibility that experimental data merely represent characteristics of T-cell clones specific to PPD, we selected another $CD4^+$, $TCR\alpha\beta^+$ T-cell clone autoreactive to GAD65p115-127, SA32.5.

After the T cells had been cultured with complete medium supplemented with rhIL-2, irradiated PBMCs as antigen-presenting cells (APCs) and PPD for 12 days (the first culture), these T cells were cultured with rhIL-7, medium only (Figure 1A), or rhIL-2 (Figure 1B) in the presence of irradiated PBMCs without antigenic stimuli (the second culture). T cells cultured with rhIL-7 exhibited a slight proliferation during the first 3 to 5 days; then the cell numbers decreased continuously in a time-dependent manner. On days 25 to 30 of the second culture, the numbers of surviving T cells were one fifth or one sixth of the starting cell numbers (2×10^6) in all three clones, whereas all T cells cultured with medium alone (Figure 1A) or rhIL-2 (Figure 1B) resulted in cell death by day 12 or day 20, respectively, of the second culture.

Loss of Antigen-Induced Proliferation in T Cells Cultured With IL-7

To determine whether these surviving T cells cultured with IL-7 can respond to antigen, we measured proliferative responses of surviving T cells to antigens on days 5, 10, 15, and 20 of the second culture. The results revealed that proliferative responses of TM5-1, TM2-2, and

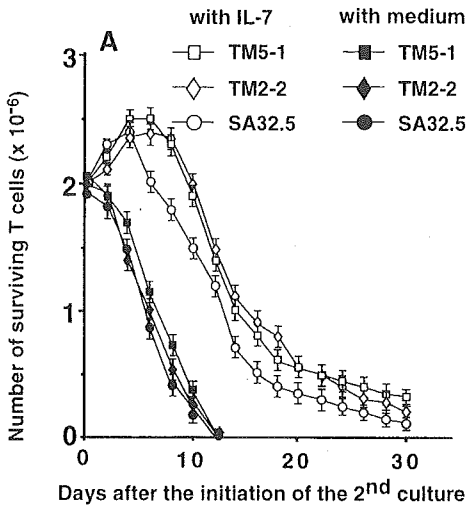

FIGURE 1 Survival of three T helper cell clones cultured with interleukin (IL)-7 and irradiated peripheral blood mononuclear cells (PBMCs) in absence of antigenic stimuli. After first culture of T cells with antigen-pulsed irradiated PBMCs in the presence of recombinant human (rh)IL-2 (100 U/ml) for 12 days, T cells were continually cultured with rhIL-7 (100 ng/ml) (A, open symbols), rhIL-2 (50 U/ml) (B), or medium alone (A, solid symbols) and irradiated PBMCs (2×10^5/well) for 30 days as the second culture. A total of 2×10^6/well of T cells were cultured at start of the second culture in a 48-well plate. The number of surviving and viable T cells, TM5-1 (square), TM2-2 (diamond), and SA32.5 (circle) clones were counted by the Trypan blue dye exclusion method, each with 2 days of culture. Mean values of one set of representative data from three experiments with similar results are shown. Standard errors of mean values did not exceed 10%.

SA32.5 T-cell clones decreased on days 10 and 15. On day 20, these three T-cell clones did not respond to stimuli of relevant antigens, PPD or GAD65p115-127 at all (Figure 2A) not only in 48-hour cultures but also

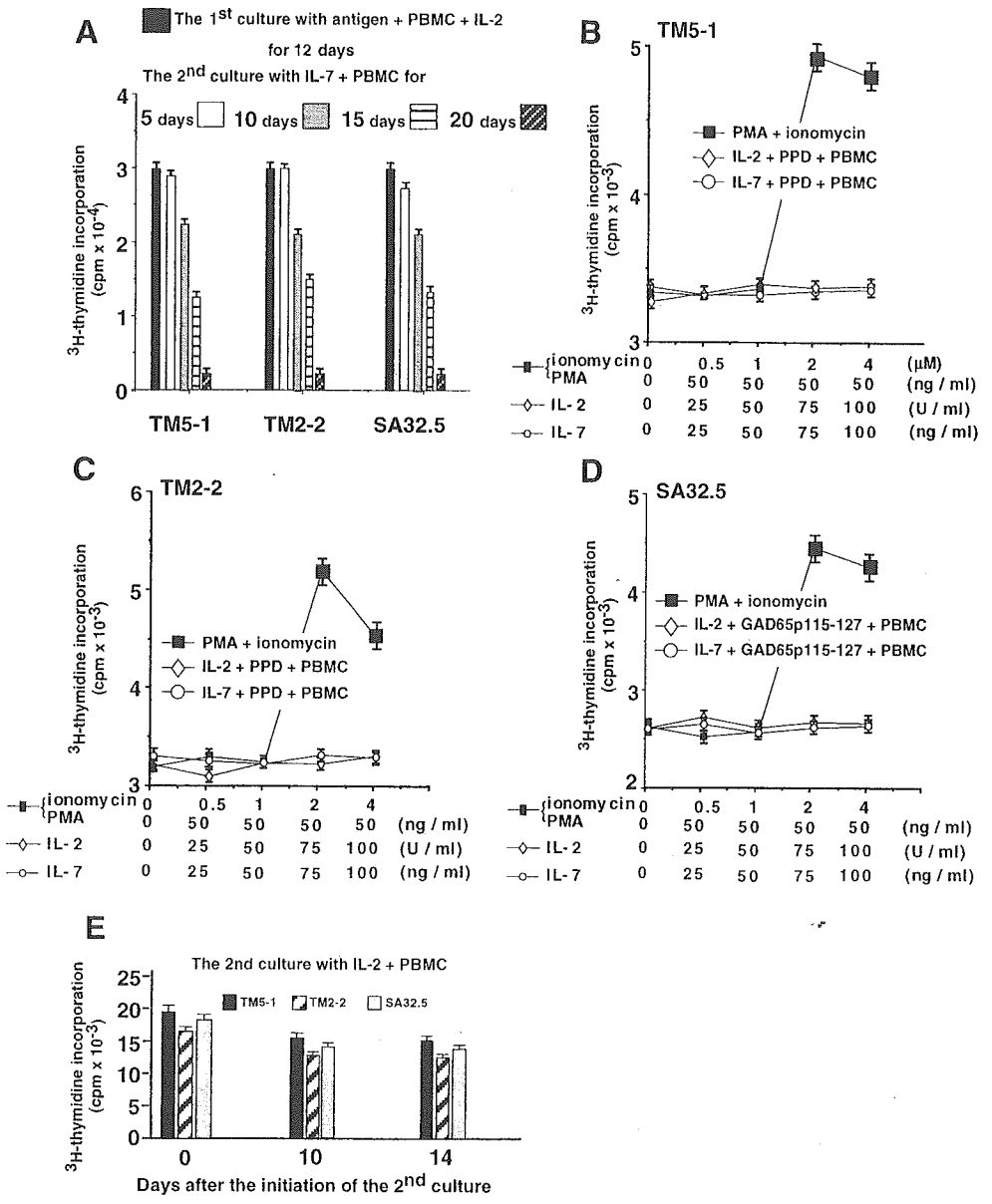

FIGURE 2 Surviving T cells cultured with interleukin (IL)-7 but not IL-2 exhibited T-cell anergy. (A) Induction of T-cell anergy by culture of T cells with IL-7 and irradiated peripheral blood mononuclear cells (PBMCs) without antigenic stimuli. After the first culture of T cells for 12 days. T cells were harvested, washed, and assayed for proliferative response to purified protein derivative of tuberculin (PPD; 10 μg/ml) or glutamic acid decarboxylase (GAD)65p115-127 (5 μg/ml) (solid bar) in presence of irradiated PBMCs. T cells from the second recombinant human (rh)IL-7 cultures (50 ng/ml) for 5 days (open bar), 10 days (shadow bar), 15 days (horizontally lined bar), and 20 days (shaded bar), respectively, were washed and assayed for proliferative response to PPD or GAD65p115-127. (B–D) Responses of anergic T cells recovered from the second culture, TM5-1 (B), TM2-2 (C), and SA32.5 (D) to ionomycin + PMA (solid square), or to antigen in presence of irradiated PBMCs and IL-2 (open diamond) or IL-7 (open circle). Concentrations of IL-2, IL-7, ionomycin, and PMA were as indicated. Numbers of T cells or PBMCs and concentrations of antigens were as described in (A). (E) Prolonged second culture of T cells with IL-2 and PBMCs did not induce T-cell anergy. On days 12~14 of the second culture with IL-2 + irradiated PBMCs, T cells were harvested, washed, and assayed for proliferative response to PPD (10 μg/ml) or GAD65p115-127 (5 μg/ml) (solid bar) in the presence of irradiated PBMCs. Largest mean value of the triplicate assay observed for T cells cultured with medium alone was 1085 cpm in all proliferative assays shown. One set of representative data obtained from three experiments with similar results is shown. Each assay was performed in duplicate culture, and mean values are indicated. Standard errors of mean values did not exceed 10%.

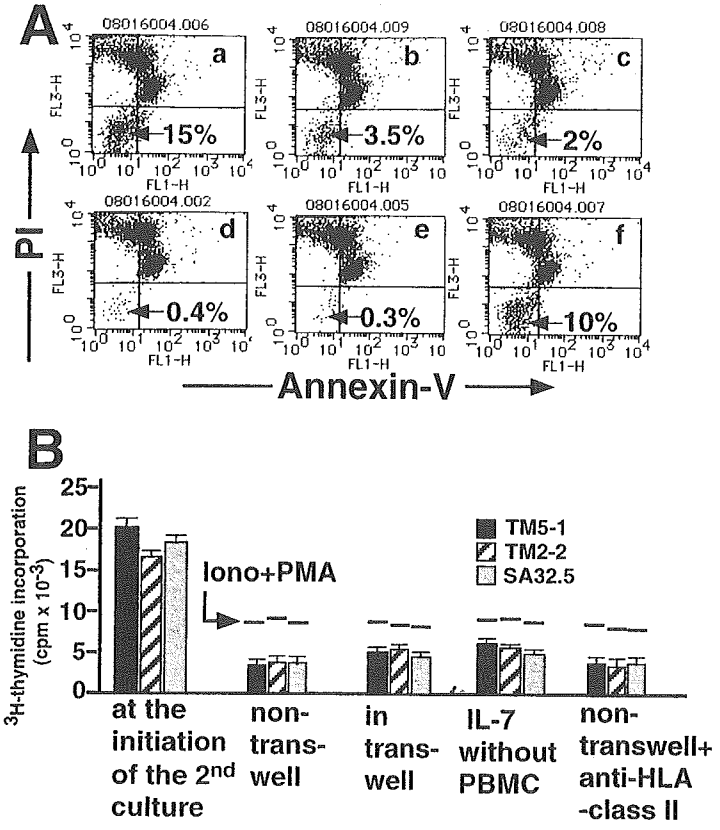

FIGURE 3 Importance of direct cell contacts between T cells and peripheral blood mononuclear cells (PBMCs) for survival of T cells in second culture. (A) Flow cytometric analysis of survived cells by PI–annexin V staining method. Experimental conditions are the same as those in Figure 1. Insert wells were used in some experiments. On day 20 in the second culture, cells were harvested and doubly stained with FITC-conjugated annexin V and 0.5% PI, and assayed immediately; lower right numbers indicate percentages of double negative and live cells. (A-a) T cells + recombinant human interleukin-7 (rhIL-7) + irradiated PBMCs. (A-b) T cells + rhIL-7 + irradiated PBMCs in insert wells. (A-c) T cells + rhIL-7. (A-d) IL-7 + irradiated PBMCs. (A-e) T cells + irradiated PBMCs. (A-f) anti–human leukocyte antigen (HLA) class II (a mixture of α-DR, α-DQ, and α-DP) 100 μg/ml + T cells + rhIL-7 + irradiated PBMCs. (B) Analysis of antigen-specific proliferative responses of surviving T cells. T cells with different culture conditions were washed and assayed for proliferative responses to cognate antigen, respectively, or to ionomycin + PMA (indicated by short lines above bars) in presence of irradiated PBMCs. Largest mean value of triplicate assay observed for T cells cultured with medium alone was 1018 cpm in all proliferative assays. One set of representative data from three reproducible experiments is shown.

72-hour, 96-hour, or 7-day cultures in the presence of antigen, irradiated PBMCs, and exogenous IL-2 or IL-7. On the other hand, PMA + ionomycin stimulated a weak but a definite proliferation of these three T-cell clones (Figure 2B–D).

Then we asked whether T-cell response could be restored in the presence of rhIL-2 or rhIL-7 together with antigenic stimuli. We found that both IL-2 and IL-7, in the presence of antigen and irradiated PBMCs, could not restore any responses of the surviving T cells in all three T-cell clones (Figure 2B–D) at all four concentrations of cytokines indicated in Figure 2. Therefore, coculture of T-cell clones with IL-7 and irradiated PBMCs alone for 20 days can induce T-cell anergy, and the anergy, once induced, cannot be rescued by either IL-2 or IL-7 even in the presence of antigen and APCs. On the other hand, T cells cultured with rhIL-2 and irradiated PBMCs for 15 to 18 days as the second culture exhibited complete cell death (Figure 1B), whereas on days 10 to 14 of the culture, proliferative responses of the living T cells to cognate antigen were conserved (Figure 2E).

Importance of Direct Cell Contact Between T Cells and PBMCs for Survival of T Cells But Not T-Cell Anergy Induced by IL-7

To investigate the requirement of PBMCs for supporting T-cell survival and T-cell anergy induction in the presence of IL-7, we used a transwell system to separate T cells from direct contact with PBMCs (Figure 3). Results of annexin V and PI staining revealed that numbers of

double-negative surviving T cells (15%) in the IL-7+PBMC group (Figure 3A-a) were markedly larger than the numbers of double-negative cells in other groups, including coculture of T cells with rhIL-7 alone. When an insert well was used, surviving T cells decreased to 3.5% (Figure 3 A-b). Under the culture condition with T cells + IL-7, the surviving T cells decreased to 2% (Figure 3 A-c). Yet under the culture condition with irradiated PBMCs+ IL-7 (Figure 3A-d) or T cell + irradiated PBMCs (Figure 3A-e), there were almost no surviving T cells (0.4% or 0.3%, respectively). The result further indicated that cell-cell contact between T cells and PBMCs in the presence of IL-7 played an important role for T-cell survival. When anti–HLA class II was added in the culture (Figure 3A-f), surviving T cells decreased to 10%, indicating that T-cell receptor-major histocompatibility class (TCR-MHC) interaction plays a partial role in the T-cell survival.

To determine whether cell-cell contact also plays a role during anergy induction of T cells by IL-7 and PBMCs, we compared specific responses to cognate antigens of these surviving cells harvested from transwell, nontranswell, and other cultures, even though purification of surviving T cells by exclusion of dead cells was very difficult in the transwell group and the IL-7–only group (Figure 3B). When response to antigens of T cells harvested from the initiation of the second culture was taken as 100%, the response of T cells harvested from nontranswell cultures was less than 10%, whereas the response of T cells harvested from transwell cultures was 13%, and in T cells from the IL-7–only group the response was 15%. T cells harvested from the coculture of T cells + IL-7 + irradiated PBMCs + anti-HLA class II (a mixture of anti-DR, -DQ, -DP) mAbs also did not respond to antigen (10% response). On the other hand, α-CD3 + PMA stimulated a weak but a definite proliferation of these anergic T cells (Figure 3B). These results indicated that cell-cell contact between T cells and PBMCs played only a small role in anergy induction. In contrast, survival of the anergic cells was very poor when T cells were separately cultured with PBMCs in a transwell culture, indicating the cell-cell contact between T cells and PBMCs plays an important role in maintenance of survival of anergic T cells.

Cell Surface Phenotype of the Anergic T Cells

The cell surface phenotype of the anergic T cells (TM5-1) was investigated (Figure 4), because characteristic cell surface phenotypes, such as no expression of CTLA4 or ICOS [34, 35], loss of CD40L or CD28 expression [36, 37], and reduction of CD4 molecule expression [36, 37], were reported to be related to the anergy induction in T cells.

The results demonstrated that the anergic T cells expressed significant levels of TCR, CD4, and IL-15Rα, but expression levels of CTLA4, ICOS, PD-1, IL-7Rα, IL-2 receptor α chain (IL-2Rα), and γc were markedly decreased or could not be detected (Figure 4A). The anergic T cells expressed CD28 and CD40L, although the expression levels were reduced. The kinetic changes of expression levels of these molecules during the second culture were also investigated (Figure 4B). The results suggest that loss of three cytokine receptors, including IL-7Rα, IL-2Rα, and γc, correlates with induction of T-cell anergy. The same cell surface phenotypes were also observed in anergic TM2-2 and SA32.5 clones induced by IL-7 (data not shown).

IL-15 Restored Immune Responsiveness of Anergic T Cells Induced by IL-7

Cytokine IL-15, which is produced by dendritic cells, activated monocyte/macrophages, placenta, skeletal muscle, and kidney [14–17], plays an essential role in T-cell homeostasis [38]. Because the anergic T cells expressed IL-15Rα, we investigated effects of IL-15 on anergic T cells induced by IL-7. At first, these anergic T cells (TM5-1, TM2-2, and SA32.5) were cultured with IL-15 without both APCs and antigen (the third culture). IL-15 induced a small increase in cell number during days 1~3 (Figure 5A). The T-cell number reached a peak, with about a 1.5 to 1.8-fold increase from the starting cell numbers on days 6 to 7 of the third culture, and maintained a plateau level of cell number during days 6 to 10. Then the live T cells decreased to a complete cell death by day 20. The IL-2 or IL-2 + IL-7 did not induce any increase in cell number of the anergic T cells; instead, almost all cells died within 4 days (Figure 5B).

Before being cultured with IL-15, the anergic T cells were directly assayed in the presence of IL-15, antigen, and irradiated PBMCs to determine the incorporation of ^{3}H-thymidine and T cells did not exhibit proliferative responses (data not shown). After the third culture with IL-15 for 3 days, T cells began to restore responsiveness to antigens in the presence of irradiated PBMCs without IL-15 (Figure 5C). This T-cell response to antigen increased on days 6 and 9 of the third culture with IL-15. Until days 15 to 18 of the culture with IL-15, the T-cell responsiveness to antigens were conserved. To determine whether other cytokines would restore responsiveness of the anergic T cells to antigen, we first selected IL-2, because IL-2 was found to restore response to antigens in some anergic T cells induced by other mechanisms [39–41]. The results indicate that IL-15, but not IL-2, plays an important role in restoration of T-cell responsiveness in the IL-7–induced anergic T cells. Furthermore, IL-15 also partially restored expressions of cytokine receptors IL-7Rα, IL-2Rα, and γc in T cells, when IL-15 reversed

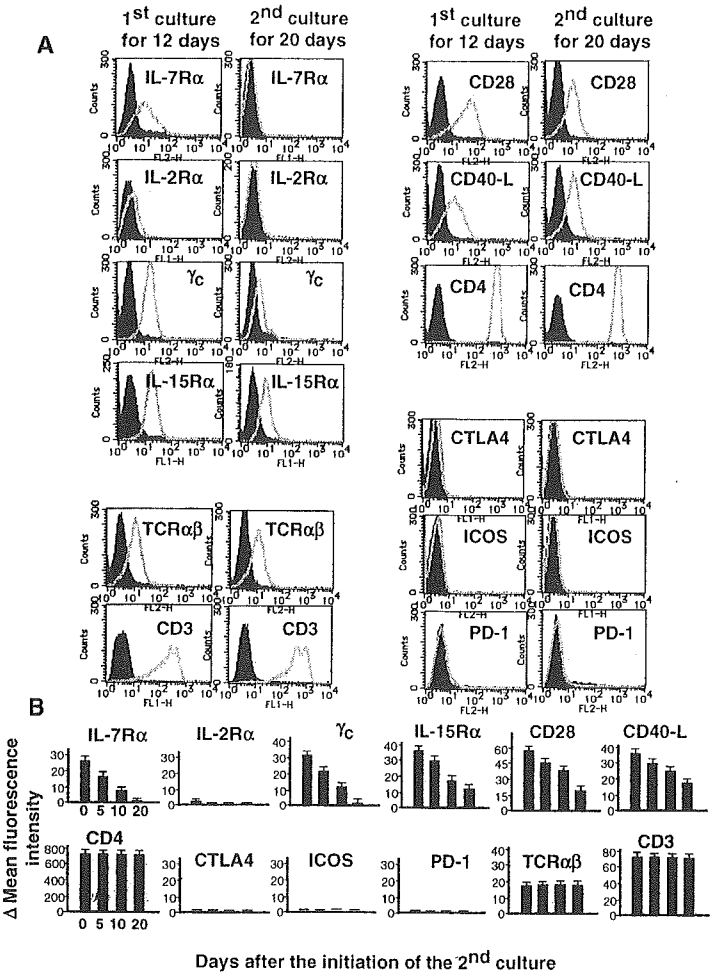

FIGURE 4 Cell surface phenotypes of anergic T cells. (A) Characteristic cell surface phenotype of anergic T cells on day 20 of the second culture. (B) Summary for data obtained from cell surface phenotype of T cells recovered on days 0, 5, 10, 15, and 20 during the second culture. Representative data using TM5-1 clone are shown. Conditions of the first or second culture were as described for Figure 2. Same-cell surface phenotypes were also observed in TM2-2 and SA32.5 clones. ΔMFI indicates that MFI of T cells stained with each monoclonal antibody subtracted with MFI of T cells stained with isotype-matched control antibodies.

T-cell anergy on day 6 of the third culture (Figure 5D), suggesting that these reexpressions of cytokine receptors on T cells may correlate with restoration from the T-cell anergy. The T cells did not express PD-1 and ICOS after the culture with IL-15 (data not shown). Similar phenomena were also observed in all three T-cell clones tested.

DISCUSSION

In this report, we demonstrated that prolonged culture of human $CD4^+$ T-cell clones with rhIL-7 in the presence of irradiated PBMCs without antigenic stimuli induced T-cell anergy. The anergic T cells had lost expression of cytokine receptors, IL-7Rα, IL-2Rα, and γc. Once anergy was induced, antigen-specific proliferative responses of T cells cannot be rescued even by addition of exogenous IL-2. However, culture of anergic T cells with rhIL-15 for 6 days did restore responsiveness of T cells to antigen.

The anergy induction by IL-7 and restoration of responsiveness by IL-15 may provide a good experimental model *in vitro* for further investigation of maintenance of peripheral tolerance and its breakdown. IL-7 is an important survival factor for resting T cells [33]. Because the anergy induction described in this study required a relatively long culture time with IL-7, the process of the anergy induction seems to be complex. We postulated that MHC molecules might be involved in the process, because in this study, T-cell survival depended on the presence of irradiated PBMCs (Figure 2). It was reported that T-cell survival induced by IL-7 required recognition