一つで癌細胞の増殖，悪性化に関与しており，乳癌の約25％に過剰発現している．Her2陽性の転移性再発性乳癌患者に対する臨床試験において，Herceptin単剤で10％，抗がん剤paclitaxel単剤で15％，Herceptinとpaclitaxelの併用により40％の奏効率を示した[22]．

(3) cetuximab(EGFレセプターに対する抗体)：EGFレセプターもErbBチロシンキナーゼ受容体ファミリーであり，頭頸部癌，肺癌，前立腺癌，大腸癌などの多くの癌で高発現している分子である．IMC-C225は多くの適応で臨床試験が実施されているが，最も高い奏効率が得られているのは頭頸部癌である．IMC-C225と放射線療法の併用により，頭頸部癌患者15例全例に奏効し，特に13例では腫瘍が完全に退縮した[23]．

癌特異的分子を標的とした抗体医薬の開発が進み，腫瘍拒絶効果のメカニズムも明らかになってくるに従い，抗体療法の難しさも明らかとなってきた．抗体医薬は造血系の腫瘍に対しては，ある程度の効果が認められるものの，抗体単独での固形腫瘍に対する腫瘍拒絶効果は，それほど認められていないのが現状である．今後，腫瘍拒絶効果を高めるために，より発現頻度の高い抗原を標的とした抗体療法を行ったり，抗体に抗がん剤やラジオアイソトープなどを結合させ抗体1分子当たりの腫瘍拒絶効果を高めたり，遺伝子操作によりADCC活性を増強させた抗体を作製するなどの工夫をしていくことが必要であろう．

7. ペプチドワクチン療法

WT1(Wilms' tumor gene)は白血病および，様々な固形癌において高発現する遺伝子で，それらの癌に対する免疫療法の標的分子として有望であると考えられていた．杉山らは，乳癌，肺癌および白血病の患者19人にWT1ペプチドワクチン療法を行い，11人において腫瘍サイズの縮小や腫瘍マーカーの低下など何らかの効果があったことを報告している[24]．その中に，乳癌が原発で肺転移した患者において，2回目のWT1ペプチドワクチン施行後から肺の腫瘍の縮小と血清中のCEA値の低下がみられるようになり，最終的にはCTによる所見において劇的な腫瘍の縮小が確認された症例が観察されている．実際の治療は9個のアミノ酸からなるWT1ペプチドを，CpGオリゴデオキシヌクレオチドとともに皮内へ2週間ごとに3回ワクチンすることにより行われ，これによりWT1ペプチド特異的CTLの誘導が19人中10人に観察されている．この結果より，ペプチドワクチンが，その標的とする腫瘍と免疫方法を選ぶことにより有効となることが示された．

8. 免疫療法の効果を増強するための様々な工夫

獲得免疫を効率よく誘導するためには，自然免疫系の活性化が重要である．従来知られていたアジュバント効果の多くは，自然免疫系の活性化で説明できる．マクロファージ，DC，NKおよびNKT細胞を有効に活性化できるアジュバントの開発が望まれるが，その一つとしてToll様受容体(Toll-like receptor: TLR)を活性化できるTLRリガンドが重要である．例えば，リポ多糖(LPS)はTLR4を介して，また非メチル化CpGを含むDNA(CpG DNA)はTLR9を介してDCなどを活性化することができる．Meliefらは，ヒトパピローマウイルス由来のCTLエピトープとThエピトープの両方を含んだ35アミノ酸からなる長いペプチドを，DCを活性化できるアジュバントであるオリゴデオキシヌクレオチド(ODN)-CpGとともにマウスの皮下に免疫することにより，従来の9アミノ酸からなる短いCTLエピトープペプチドのみによる免疫よりも効率よくCTLを誘導することができ，抗腫瘍効果も著しく増強することを報告した[25]．Clynesらは，腫瘍抗原だけを負荷したDCワクチンより，腫瘍抗原と抗体の免疫複合体を負荷したDCワクチンの方が，Fcレセプターを介したDCへの抗原の取り込みが促進されるために，Th細胞およびCTLの両方を活性化することができ，圧倒的に強い抗腫瘍効果を示すことを報告した[26]．

著者らは，*in vitro*で，マウスES細胞(embry-

onic stem cell)からDC(ES-DC)を分化誘導する方法を開発した[27]. ES-DCは骨髄細胞系DCに相当する細胞表面マーカーを発現しており，強力なリンパ球混合培養反応(MLR)刺激活性と効率よい抗原提示能をもつ．更に，ES細胞の段階で複数の遺伝子を効率よく導入できた．これを利用して，卵白アルブミン(OVA)とともにT細胞遊走を誘導するケモカインであるSLCあるいはMigを発現したES-DCをマウスに移入することにより，OVA特異的キラーT細胞ならびに移植したOVA発現腫瘍細胞(MO4)の拒絶を*in vivo*において非常に効率よく誘導できた[28]．現在，ES-DCの臨床応用への可能性について検討中である．

9. Homeostatic proliferationを利用した新しい免疫細胞療法

前述のように癌細胞は，様々な機序により免疫機構から逃避して腫瘍を増大させるが，癌細胞に特異的で免疫原性のある抗原を標的として強力な免疫操作を行うことにより腫瘍を排除できる可能性がある．実際メラノーマにおいて，Rosenbergらは長年取り組んできた養子免疫療法に，最近注目されているT細胞のhomeostatic proliferationという考え方を組み合わせたユニークな免疫療法を行った[29]．homeostatic proliferationとは，体内のリンパ球の数は一定に保たれており，その数を減らす操作を加えると，新たに移入されたリンパ球が生き延びて一定数に達するまで増殖するという現象である．TheofilopoulosらとMuleらは，その考え方を抗腫瘍免疫療法に応用して，マウスにおいて抗腫瘍効果を増強させることに成功した[30,31]．Rosenbergらは，まさにその発想をヒトに応用した．つまり，あらかじめ，化学療法により患者のリンパ球数を減らしておいて，そこへ大量に増やしておいた，癌細胞を傷害するCTLを移入した．その結果CTLが体内で長期にわたって生存し，ついには転移性メラノーマ13例中6例に50％以上の腫瘍縮小をもたらすなど，今までの癌の免疫療法では考えられないほどの抗腫瘍効果が観察された[29]．

この結果は，癌の免疫療法が，3大治療に匹敵するほどの潜在能力をもっていることを期待させるものである．転移性の進行癌にも有効であったことから，比較的早期の癌治療あるいは癌の予防や再発予防といったところにも，十分免疫療法の活躍できる余地はあると考えられる．更に従来の免疫療法に加え，ペプチドワクチン[32]やDNAワクチン[33]，あるいはDCワクチン[34]も十分利用できると考えられる．今後は，手に負えない進行癌だけでなく，対象を早期癌から前癌状態(癌予備群)にまで広げた臨床試験が行われる可能性も考えられる．

一方，この治療で用いられたCTLは，MART-1，gp100といったメラノサイト分化抗原由来のペプチドを用いて誘導したもので，この治療により，正常メラノサイトへの攻撃による白斑やブドウ膜炎などの自己免疫現象も観察された[29]．このことは，癌を拒絶できるほどの免疫療法が行われた場合，そのCTLを誘導するのに使われた抗原が自己の正常臓器にも発現するものであれば，その臓器を傷害してしまう可能性があることを示している．すなわち我々は，癌特異的に発現するが，重要な正常臓器には発現しない腫瘍拒絶抗原を同定して免疫療法に利用しなければならない．

10. NKT細胞を利用した腫瘍免疫療法の可能性

NKT細胞は，TCRとNK細胞マーカーをともに発現する細胞で，ヒトNKT細胞はTCR遺伝子として選択的にVα24Vβ11を発現する．NKT細胞はTh1型サイトカイン(IFN-γなど)とTh2型サイトカイン(IL-4など)の両方を産生することができ，更に，活性化されたNKT細胞はパーフォリンやグランザイムを産生し，癌細胞に対して傷害作用を示す．

NKT細胞は，DCなどの抗原提示細胞の表面に発現するCD1d/α-galactosylceramideを認識し，活性化されることが知られている．マウスモデルでは，NKT細胞が多く分布している肝臓や肺の腫瘍に対して抗腫瘍効果が認められた．腫瘍免疫療法として，α-galactosylceramide

(α-Galcer)の直接投与，α-Galcerを負荷したDCの投与が考えられる．また，NKT細胞にはTh1様のNKT細胞とTh2様のNKT細胞の2種類が存在しており，大量のIFN-γを産生するTh1様NKT細胞の養子免疫療法などが考えられている．

おわりに

一度は影をひそめた抗体療法が，ヒト化抗体の導入により著明な効果をもたらしている．また，癌ペプチド療法のトランスレーショナル・リサーチが花盛りで，癌の抗体療法とともに，今再び，癌の免疫療法のブームが到来している．多くの臨床応用の結果は，一見，我々に失望を与えたかにみえていたが，実は，多くの論文や報告は，生体の中で確かに抗腫瘍免疫応答は起きており，工夫すれば十分癌の治療法として有効であることを示している．今こそ我々は，第4の治療法としての免疫療法でなく，手術・抗がん剤(化学療法)・放射線療法に並ぶ免疫療法を確立するために，様々な観点から研究を突き進めていかなければならない．数多くの癌患者が恩恵を受けられるように．

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STEM CELLS

EMBRYONIC STEM CELLS

Genetically Manipulated Human Embryonic Stem Cell-Derived Dendritic Cells with Immune Regulatory Function

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Key Words. Dendritic cells • Embryonic stem cells • Cell differentiation • Cell therapy

ABSTRACT

Genetically manipulated dendritic cells (DC) are considered to be a promising means for antigen-specific immune therapy. This study reports the generation, characterization, and genetic modification of DC derived from human embryonic stem (ES) cells. The human ES cell-derived DC (ES-DC) expressed surface molecules typically expressed by DC and had the capacities to stimulate allogeneic T lymphocytes and to process and present protein antigen in the context of histocompatibility leukocyte antigen (HLA) class II molecule. Genetic modification of human ES-DC can be accomplished without the use of viral vectors, by the introduction of expression vector plasmids into undifferentiated ES cells by electroporation and subsequent induction of differentiation of the transfectant ES cell clones to ES-DC. ES-DC introduced with invariant chain-based antigen-presenting vectors by this procedure stimulated HLA-DR-restricted antigen-specific T cells in the absence of exogenous antigen. Forced expression of programmed death-1-ligand-1 in ES-DC resulted in the reduction of the proliferative response of allogeneic T cells cocultured with the ES-DC. Generation and genetic modification of ES-DC from nonhuman primate (cynomolgus monkey) ES cells was also achieved by the currently established method. ES-DC technology is therefore considered to be a novel means for immune therapy. STEM CELLS *2007;25:2720–2729*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Embryonic stem (ES) cells are characterized by pluripotency and infinite propagation capacity, and the methods for genetic modification of ES cells, including targeted gene modification, have been well-established. This laboratory and others have devised methods to generate dendritic cells (DC) in vitro from mouse ES cells [1, 2]. The functions of mouse ES cell-derived DC (ES-DC), including stimulation of allogeneic T cells, processing and presentation of antigenic proteins, and migration upon in vivo transfer, are comparable to those of DC generated in vitro from bone marrow cells [3]. This laboratory has also established a strategy for the genetic modification of mouse ES-DC [1]. Expression vectors were introduced into ES cells by electroporation, and subsequently the transfectant ES cell clones were induced to differentiate to ES-DC. Studies using mice have demonstrated that in vivo transfer of genetically engineered mouse ES-DC is very useful for modulating immune responses both positively and negatively. It is possible to induce anticancer immunity [3–6] and prevent autoimmune disease [7, 8] in mouse models with genetically engineered ES-DC.

In the present study, looking toward future clinical application of ES-DC technology, a method was developed to generate ES-DC from human ES cells. The morphology and the results of functional and flow cytometric analyses indicate that human ES-DC possess the characteristic features of DC. cDNA microarray analysis revealed that the change of gene expression profile during generation and maturation of human ES-DC partially mimics that of monocyte-derived DC (Mo-DC). The currently established method was also applicable to cynomolgus monkey (*Macaca fascicularis*) ES cells.

MATERIALS AND METHODS

Cell Lines, Cytokines, and Reagents

The use of human ES cells was done in accordance with the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells (2001) of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, after approval by the Institutional Review Board. The human ES cell lines KhES-1 and KhES-3 have recently been established and maintained on mouse

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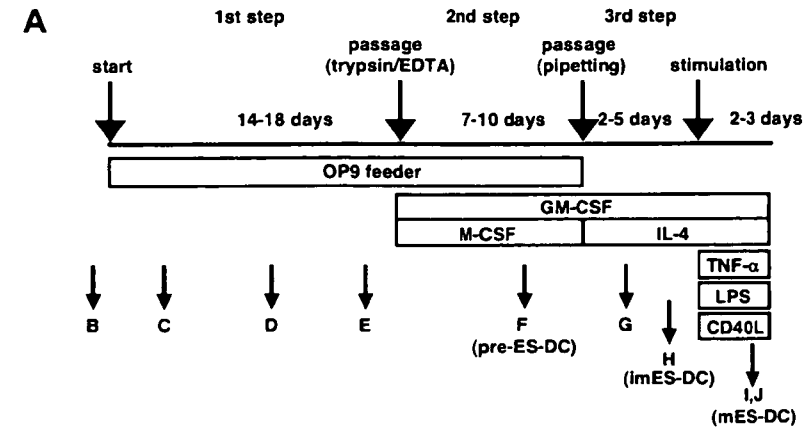

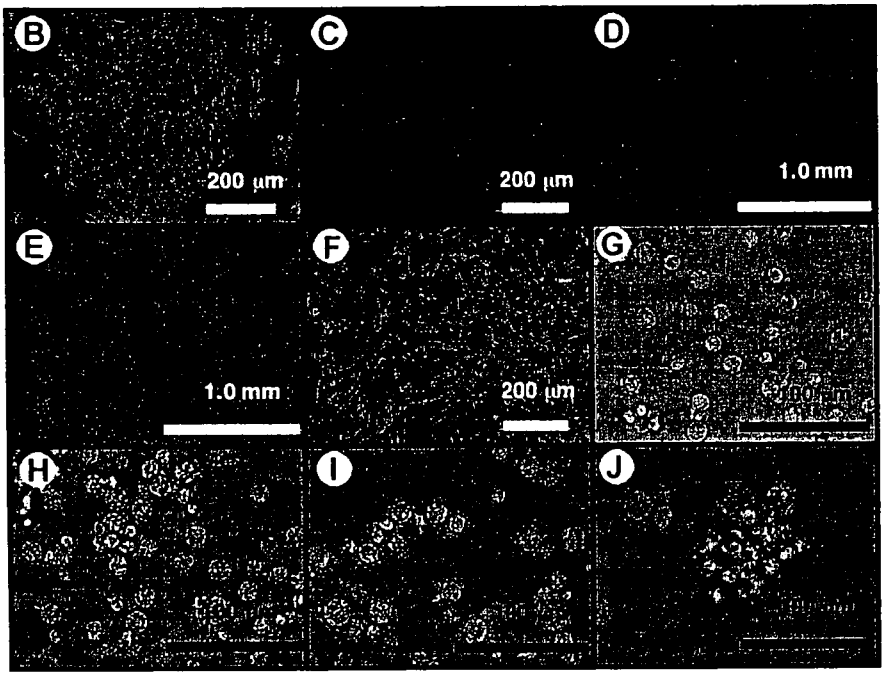

Figure 1. Culture protocol and morphological changes of human embryonic stem (ES) cell-derived cells during differentiation culture. **(A):** The schedule for the culture to induce differentiation of human ES cells into ES-DC is schematically depicted. **(B):** Undifferentiated human ES cells on primary embryonic fibroblast feeder layer. **(C–E):** ES cell-derived cells on day 3 **(C)**, day 11 **(D)**, and day 15 **(E)** in the first step. **(F):** Cells on day 6 in the second step. **(G–J):** Cells on day 1 **(G)**, day 3 **(H)**, and day 6 **(I, J)** in the third step. Cells shown in **(I, J)** had been stimulated with TNF-α plus LPS for 2 days. Abbreviations: ES-DC, embryonic stem cell-derived dendritic cells; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; imES-DC, immature embryonic stem cell-derived dendritic cells; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; mES-DC, mature embryonic stem cell-derived dendritic cells; TNF-α, tumor necrosis factor α.

primary embryonic fibroblast (PEF) feeder layers as previously described [9, 10]. Mouse-derived hematopoietic stromal cell line OP9 was treated with mitomycin C (10 μg/ml) for 1 hour before plating onto gelatin-coated tissue culture dishes to make feeder cell layers. The establishment and maintenance of cynomolgus monkey ES cell line CMK6 was also reported [11, 12]. Recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), interleukin-4 (IL-4), tumor necrosis factor α (TNF-α), and soluble CD40-ligand were purchased from Peprotech (London, http://www.peprotech.com). Lipopolysaccharide (LPS) from *Escherichia coli* and OK-432 were purchased from Sigma-Aldrich (St. Louis, http://www.sigmaaldrich.com) and Chugai Pharmaceutical (Tokyo, http://www.chugai-pharm.co.jp/hc/chugai_top_en.jsp), respectively.

Induction of Differentiation of ES Cells into ES-DC

The procedure for differentiation culture was composed of three steps (Fig. 1A). Step 1 was as follows: undifferentiated ES cells maintained on PEF were rinsed with phosphate-buffered saline (PBS) and treated with dissociation solution containing 1 mg/ml collagenase, 0.25% trypsin, and 20% knockout serum replacement (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) in PBS [10] and cultured on OP9 feeder cell layers in minimum essential medium-α supplemented with 20% fetal calf serum (FCS) and 2-mercaptoethanol (50 μM). Culture of cells was continued for 14–18 days with human ES cells and for 11–13 days with cynomolgus monkey ES cells, and the medium was changed once every 3 days. At the end of this step, the cells were rinsed with PBS, treated with trypsin-EDTA (PBS containing 0.25% trypsin and 1 mM EDTA) for 30–40 minutes, and recovered. After resuspension in culture medium, the cells were plated onto culture dishes and incubated for 2–4 hours. Thereafter, floating or weakly adherent cells were recovered by pipetting, and any firmly adherent cells were discarded. Step 2 was as follows: after being passaged through nylon mesh (Cell Strainer 100 μm; BD Biosciences, Bedford, MA, http://www.bdbiosciences.com), cells recovered from one 90-mm dish were plated in two dishes with freshly prepared OP9 feeder layers. On the following day, the culture medium was exchanged with a medium containing GM-CSF (100 ng/ml) and M-CSF (50 ng/ml). The culture was continued for 7–10 days, depending on the propagation of floating cells on the feeder layers. Step 3 was as follows: ES cell-derived floating cells were recovered by pipetting; resuspended in RPMI 1640 medium containing 10% FCS, GM-CSF (100 ng/ml), and IL-4 (10 ng/ml); and cultured in Petri dishes ($3–5 \times 10^5$ cells per dish) without a feeder layer (Locus, Tokyo). To induce maturation, IL-4 (10 ng/ml), TNF-α (10 ng/ml), LPS (3 μg/ml), and, in some experiments, soluble CD40-ligand (20 ng/ml) or OK-432 (10 μg/ml) were simultaneously added on day 3 or 5 of this step, and the culture was continued for an additional 2–3 days. Differentiating cells were microscopically analyzed on an inverted microscope (IX70; Olympus, Tokyo, http://www.olympus-global.com).

Flow Cytometric Analysis

The following monoclonal antibodies (Ab) conjugated with fluorescein isothiocyanate or phycoerythrin were purchased from BD Pharmingen (San Diego, http://www.bdbiosciences.com/index_us.shtml) or eBioscience Inc. (San Diego, http://www.ebioscience.com): anti-human histocompatibility leukocyte antigen (HLA)-DR (clone L243, mouse IgG2a); anti-HLA-A, B, and C (clone G46–2.6, mouse IgG1); anti-human CD80 (clone L307.4, mouse IgG1); anti-human CD83 (clone HB15e, mouse IgG1); anti-human CD86 (clone FUN-1, mouse IgG1); anti-human CD40 (clone 5C3, mouse IgG1); anti-human B7-H1/programmed death-1-ligand-1 (PD-L1) (clone MIH1, mouse IgG1); and anti-human CD74 (clone M-B741, mouse IgG2a). As isotype-matched controls, mouse IgG2a (clone G155–178) and mouse IgG1 (clone MOPC-21) were used. The cell samples were treated with FcR-blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com) for 10 minutes, stained with the fluorochrome-conjugated Ab for 30 minutes, and washed three times with PBS/2% FCS. Intracellular staining with anti-CD74 monoclonal Ab was done by using IntraPrep (Beckman Coulter, Marseille, France, http://www.beckmancoulter.com). Stained cell samples were analyzed on a FACScan flow cytometer, and, in some experiments, the DC fraction was gated by forward and side scatters.

Enzyme-Linked Immunosorbent Assay to Detect Cytokine Production by ES-DC

Cells were cultured in 96-well flat-bottomed culture plates (1.2×10^5 cells in 150 μl of medium per well) in the presence or absence of soluble CD40-ligand, LPS, or OK432. After 60 hours of culture, supernatant was collected, and the concentration of TNF-α and IL-12 p70 was measured by using enzyme-linked immunosorbent assay (ELISA) kits (Pierce, Rockford, IL, http://www.piercenet.com).

Allogeneic T-Cell-Stimulation Assay

Mononuclear cells were isolated from heparinized peripheral blood of a human or a cynomolgus monkey housed in the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan), using Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden, http://www.amersham.com). T cells were purified using the Pan T cell isolation kit for humans or the kit for nonhuman primates (Miltenyi Biotec). The T cells (4×10^4/well) were cocultured with graded numbers of x-ray-irradiated (40 Gy) stimulator cells in RPMI 1640 medium supplemented with 10% human plasma in 96-well round-bottomed culture plates for 5 days. [^{3}H]-Methyl-thymidine (247.9 GBq/mmol) was added to the culture (0.037 MBq/well) for the last 16 hours. At the end this time, the cells were harvested onto glass fiber filters (PerkinElmer Life and Analytical Sciences, Boston, http://www.perkinelmer.com), and the incorporation of [^{3}H]-thymidine was measured by scintillation counting. In the experiment using PD-L1-transfectant ES-DC, anti-PD-L1 blocking Ab (clone MIH1; eBioscience) or control mouse IgG1 Ab (eBioscience) was added to the culture (10 μg/ml).

Recombinant Antigenic Protein

A DNA fragment encoding human glutamic acid decarboxylase (GAD65) p96–174 protein fragment was cloned into the prokaryotic expression vector pGEX-4T-3 (Amersham Biosciences), to generate a vector for glutathione *S*-transferase-fused GAD65 protein fragment (GST-GAD). The induction of the production of recombinant protein in *E. coli* (DH5α) and the extraction of the recombinant protein from bacterial inclusion bodies was done according to Frangioni and Neel [13]. The purification of the recombinant protein with glutathione-agarose (Sigma-Aldrich) was done as described in our previous report [14, 15]. The purity and integrity of the recombinant protein was confirmed by SDS-polyacrylamide gel electrophoresis. The protein was concentrated and separated from small peptide fragments, if any, with Centricon-10 (Millipore, Bedford, MA, http://www.millipore.com), and the solvent was changed from the elution buffer to the culture medium by dialysis.

Antigen Presentation Assay

A human CD4$^+$ T-cell clone, SA32.5, recognizing GAD65p111–131 in the context of HLA-DR53 molecule (DRA*0101+DRB4*0103) was established and maintained as previously described [16]. In the assay with the synthetic peptide, ES-DC stimulated with TNF-α (10 ng/ml) plus LPS (3 μg/ml) were harvested, incubated in the presence of peptide (6 μM) for 3 hours, washed four times with culture medium, and x-ray-irradiated (35 Gy). A T-cell proliferation assay was set up in a 96-well flat-bottomed culture plate with SA32.5 T cells (3×10^4 cells per well) and graded numbers of the peptide-loaded ES-DC in RPMI 1640 medium supplemented with 10% human plasma. In the assay with recombinant protein, the indicated amount of GST or GST-GAD protein was added to the coculture of SA32.5 T cells (3×10^4 cells per well) and irradiated ES-DC (1×10^4 cells per well). After 48 hours of culture, [^{3}H]-thymidine was added, and then after an additional 16 hours of culture, the cells were harvested and the incorporated radioactivity was counted.

Plasmid Construction

cDNA for human PD-L1 was isolated by polymerase chain reaction (PCR) with Pyrobest DNA polymerase (Takara, Osaka, Japan, http://www.takara.co.jp) using cDNA clone CS0DI011, purchased from Invitrogen (Carlsbad, CA, http://www.invitrogen.com), as a template. Double-stranded oligo DNA (5′-atgaacattttacttcagtatgtg-gtgaaaagtttcgat-3′) coding for GAD65p115–127 (the core epitope for SA32.5 T-cell clone) was ligated to human invariant chain (Ii)-based epitope presentation vector pCI [17] to generate GAD65-epitope-fused Ii. A cDNA fragment for HLA-DRB4*0103 was generated by reverse transcriptase (RT)-PCR from RNA isolated from peripheral blood mononuclear cells positive for *HLA-DRB4*0103*. The coding DNA fragments were cloned into a mammalian expression vector, pCAG-IRES-Neo, which is driven by the CAG promoter and includes an internal ribosomal entry site (IRES)-neomycin-resistance gene cassette [3].

Transfection of ES Cells

Human ES cells were harvested using CTK solution, dissociated into clusters of 50–100 cells by pipetting, and washed twice with Dulbecco's modified Eagle's medium (DMEM). The cells harvested from two 90-mm culture dishes with subconfluently growing ES cells were suspended in 0.1 ml of DMEM and mixed with 50 μg of linearized plasmid DNA dissolved in 0.1 ml of PBS in a 4-mm-gap cuvette. The electroporation of human ES cells was performed at 150 V and 200 μF on a Gene Pulser (Bio-Rad, Hercules, CA, http://www.bio-rad.com). The transfection of cynomolgus monkey ES cells was done as previously described [18], with some modifications. Cynomolgus monkey ES cells were harvested after treatment with trypsin-EDTA. ES cells ($1–1.5 \times 10^7$) suspended in 0.7 ml of DMEM were mixed with 50 μg of plasmid DNA in 0.1 ml of PBS in a 4-mm-gap cuvette. Electroporation was done at 250 V and 500 μF. After electroporation, the ES cells were cultured on G418-resistant PEF feeder layers in 90-mm culture dishes or six-well plates. Selection with G418 (150 μg/ml) was done from 2 to 4 days after the transfection, and G418-resistant ES cell colonies were picked up using a micropipette under microscopic observation on days 15–18 for human ES cells and on day 11 for monkey ES cells. The transfectant clones were transferred to 24-well culture plates with PEF and expanded in the presence of G418. ES cell transfectant clones with relatively high levels of expression of the transgene were selected on the basis of the resistance to a high dose (1–3 mg/ml) of G418 and the results of the RT-PCR analysis. Thereafter, the clones were subjected to the differentiation procedures. At the proper stages of differentiation, the cells were screened to select ES cell clones that highly expressed the transgene after differentiation, based on a flow cytometric analysis for PD-L1 and Ii transfectant human ES cells and on the antigen-presenting capacity for HLA-DRB4 transfectant cynomolgus monkey ES cells.

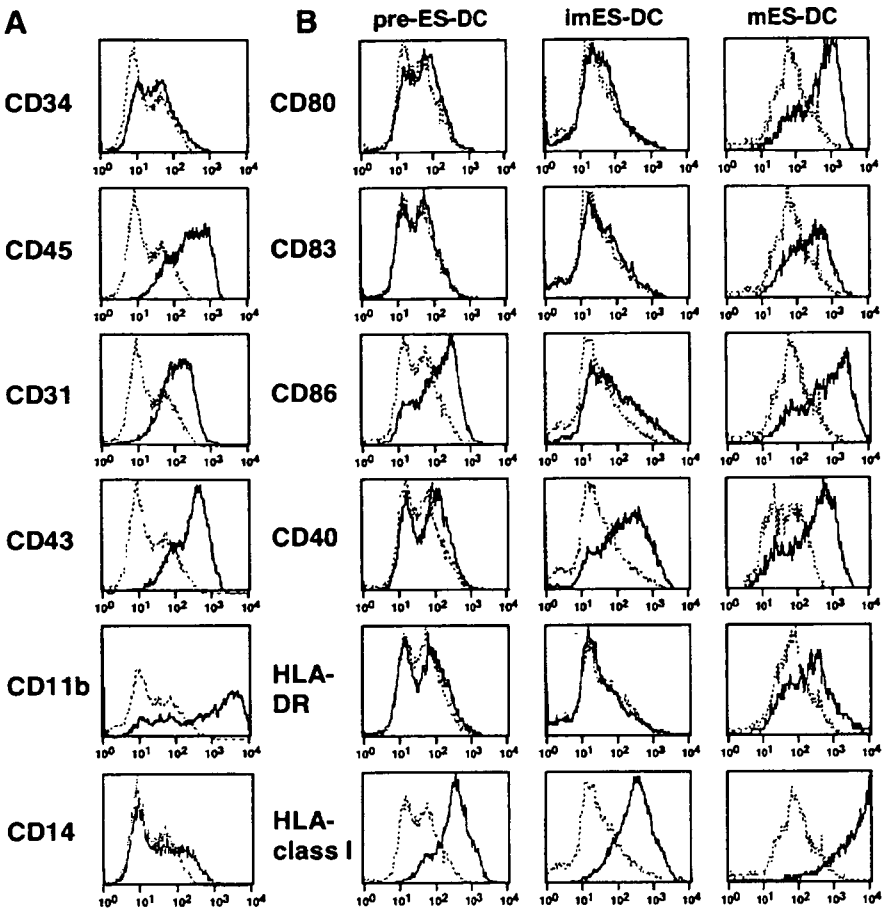

Figure 2. Cell surface phenotypes of human ES-DC. (**A**): ES cell-derived floating cells harvested on day 6 in the second step were analyzed for the cell surface expression of CD34, CD45, CD31, CD43, CD11b, and CD14. (**B**): ES cell-derived cells harvested on day 8 in the second step (pre-ES-DC) and from the third step before (imES-DC) and after (mES-DC) addition of maturation stimuli were analyzed for the cell surface expression of CD80, CD83, CD86, CD40, HLA-DR, and HLA class I. Staining profiles with specific antibody (Ab) (thick lines) and isotype-matched control Ab (thin, broken lines) are shown. Abbreviations: ES-DC, embryonic stem cell-derived dendritic cells; HLA, histocompatibility leukocyte antigen; imES-DC, immature embryonic stem cell-derived dendritic cells; mES-DC, mature embryonic stem cell-derived dendritic cells.

RT-PCR for Detection of the Transgene-Derived Transcripts

cDNA was synthesized from total cellular RNA with random hexamer primers and SuperScript II reverse transcriptase (Invitrogen). The following PCR primer sets were used: 5′-gctggattacatcaaagcactgaa-3′ and 5′-caacaaagtctggcttatatccaa-3′ for hypoxanthine-guanine phosphoribosyl transferase and 5′-ctgactgaccgcgttactcccaca-3′ and 5′-ttggttatagatgtatctgatcaggt-3′ for transgene-derived DRB4 transcript.

RESULTS

Differentiation of Human ES Cells to ES-DC

Based on previous experience in the generation of dendritic cells from mouse ES cells [1] and also based on the findings in a preliminary study using cynomolgus monkey ES cells, the feeder cell-coculture method was adopted for the generation of dendritic cells from human ES cells, instead of the embryoid body (EB)-based method. The human ES cell line selected was KhES-1; this line exhibited the highest growth rate among the three lines of human ES cell lines established in a recent study [9, 19]. For feeder cells, three lines of mouse stromal cell lines (ST2, OP9, and PA6) were evaluated for their capacity to induce hematopoietic differentiation of KhES-1 ES cells, and OP9 had the best yield among them (data not shown).

The protocol for the differentiation culture to generate ES-DC from human ES cells developed in the current study is composed of three steps, as shown in Figure 1A. At the beginning of the differentiation culture, undifferentiated ES cells maintained on mouse PEF feeders (Fig. 1B) were harvested using dissociation solution CTK [9] and plated on OP9 feeder cell layers (step 1). Next, the ES cells grew and formed clusters composed mostly of epithelial cell-like large flat cells (Fig. 1C, 1D). Clusters of round, cobblestone-like cells also appeared at approximately day 8, and those resembled the mesodermally differentiated cell clusters observed in hematopoietic differentiation culture of mouse ES cells [1, 20]. The size and number of round cell clusters gradually increased, and by around day 15, they covered 20%–30% of the surface area (Fig. 1E).

On days 15–18 of the first step, cells were recovered from the dishes using trypsin/EDTA and isolated nonadherent cells, and then they were seeded onto freshly prepared OP9 cell layers, to begin the second step. On the next day, the culture medium was exchanged for medium containing GM-CSF and M-CSF. Thereafter, small round cells, floating or loosely adhering to the feeder layer, appeared and gradually increased in number (Fig. 1F). The growth of the round cells depended primarily upon GM-CSF, thus suggesting that they grew in response to that factor. The cells were recovered and analyzed for their expression of hematopoietic cell lineage markers by flow cytometry (Fig. 2A). The cells expressed CD34 and CD45, thus indicating that they followed a hematopoietic cell lineage. They also expressed CD31, CD43, and CD11b, thus collectively indicating a commitment to a myeloid cell lineage. The double peaks seen in the histograms in Figure 2 reflect the heterogeneity of the analyzed cells in size and intensity of autofluorescence.

On days 7–10 of the second step, the floating or loosely adherent cells were harvested by pipetting and transferred to Petri dishes without feeder cells. We then cultured the cells in the presence of GM-CSF and IL-4 to start the third step. Following this passage, the cells changed their morphology from round to irregular shapes, and some had protrusions (Fig. 1G).

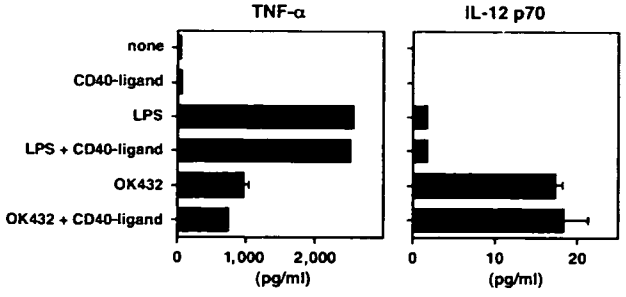

Figure 3. Production of TNF-α and IL-12 by embryonic stem cell-derived dendritic cells (ES-DC). ES-DC were recovered from the culture (third step, day 4) and replated (1.2×10^5 cells per 150 μl in 96-well culture plates) in the presence or absence of soluble CD40-ligand (20 ng/ml), LPS (3 μg/ml), or OK432 (10 μg/ml) as indicated. After 60 hours, supernatant was collected, and concentration of TNF-α and IL-12 p70 was measured by enzyme-linked immunosorbent assay. Data are indicated by mean value + SD of duplicate cultures. Abbreviations: IL, interleukin; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α.

Cells with protrusions gradually increased, and more than 50% of the cells exhibited DC-like irregular shapes after 2–3 days (Fig. 1H). The floating cells expressed CD86 and CD40 but scarcely expressed CD80 or CD83 (Fig. 2B). Expression of HLA-DR at this stage differed between experiments.

Figure 1I and 1J shows the cells after the simultaneous addition of TNF-α, LPS, soluble CD40-ligand, and IL-4. Generally, they exhibited longer protrusions than before the stimulation, and some of the protrusions were veil-like. Many of the cells formed aggregates. Flow cytometric analysis showed the increased expression of CD86 and the expression of CD80, CD83, and HLA-DR (Fig. 2B). Collectively, the cells exhibited the characteristics of DC in their morphology and expression of surface molecules, and thus they were designated human ES-DC.

Production of IL-12 and TNF-α by ES-DC was measured by ELISA (Fig. 3). Production of TNF-α was profoundly induced by either LPS or OK432. OK432, but not LPS, induced the production of IL-12, consistent with the reports that OK432 is an efficient inducer of IL-12 [21, 22]. Addition of CD40-ligand showed little effect on the production of these cytokines by human ES-DC.

ES cell-derived floating cells first appeared during the second step of the culture for differentiation (pre-ES-DC) and could readily be isolated by the pipetting procedure. Their morphology, pattern of expression of surface molecules, and T-cell-stimulation capacity (described below) continuously changed until the final maturation. To determine the change in gene expression associated with such changes in the phenotypes, the gene expression profiles of pre-ES-DC, immature ES-DC, and mature ES-DC were analyzed using cDNA microarrays. For reference purposes, human peripheral blood monocytes and immature and mature Mo-DC were also analyzed. The data for genes with relevance to immune functions were selected from the total microarray data and are shown in supplemental online Table 1. Consistent with the results of flow cytometric analysis (Fig. 2B), upregulation of the expression of genes encoding cell surface molecules such as HLA class I, HLA class II, CD86, and CD40, along with differentiation of ES-DC, was observed. In addition, expression of the genes related to DC function, including CD74/invariant chain, CCR7, and CCL17/TARC, was increased during the differentiation. Clustering analysis indicates similarity between change of the gene expression pattern from monocytes to immature Mo-DC and that from pre-ES-DC to immature ES-DC, as well as that from immature Mo-DC to mature Mo-DC and that from immature ES-DC to mature ES-DC (supplemental online Fig. 1).

The protocol of differentiation culture described thus far was originally developed using the KhES-1 line of human ES cells. This differentiation procedure was also applied to KhES-3, another human ES cell line. KhES-3 differentiation was similar to KhES-1 except that KhES-3 differentiated slightly more quickly than KhES-1, and a first-step culture of 14–15 days was sufficient for the differentiation of KhES-3.

Function of Human ES-DC

The capacity of the human ES-DC to stimulate T cells was examined based on the proliferative response of allogeneic T cells cocultured with ES-DC (Fig. 4A). ES cell-derived floating cells recovered from the second step (pre-ES-DC) showed little capacity to induce a response of T cells. In contrast, ES-DC following the third step before the addition of maturation stimuli (immature ES-DC) showed a weak but definite stimulation, and following exposure to the maturation stimuli (mature ES-DC) they showed a strong capacity to stimulate allogeneic T cells to proliferate.

Next, the antigen-presenting capacity of ES-DC was examined. KhES-1 is positive for the *HLA-DRB4*0103* gene encoding the β chain of HLA-DR53 molecule. Presumably, ES-DC derived from KhES-1 should express the DR53 molecule, and their ability to present antigen to DR53-restricted $CD4^+$ T cells was determined. As shown in Figure 4B, KhES-1-derived ES-DC preloaded with GAD65-derived synthetic peptide stimulated GAD65-specific DR53-restricted human T-cell clone SA32.5 to proliferate. To examine the capacity to process antigenic protein and present epitope, recombinant protein was used as the antigen (Fig. 4C). The SA32.5 T-cell clone cocultured with the ES-DC in the presence of recombinant GAD65 protein also showed a proliferative response, thus indicating that ES-DC processed the antigenic protein and presented the epitope derived from the protein in the context of HLA class II molecules.

Genetic Modification of Human ES-DC

Previous research established a strategy for the genetic modification of mouse ES-DC [1]. Briefly, the expression vectors were introduced into ES cells by electroporation, and subsequently the transfectant ES cell clones were induced to differentiate to ES-DC. The following experiments were performed to determine whether or not this strategy could be applicable to human ES cells.

PD-L1/B7-H1 is known to downmodulate responses of T cells upon interaction with the ligand, PD-1 on T cells [23]. An expression vector for human PD-L1 was introduced to KhES-1 by electroporation. The expression vector used was pCAG-INeo, driven by the CAG promoter and containing an IRES-neomycin-resistance gene cassette (Fig. 5A). Among the transfectant clones, 23 ES cell clones showing resistance to high does of G418 (2 mg/ml) were selected and subjected to the ES-DC-differentiation culture.

The expression of PD-L1 of the transfectant clones was examined by a flow cytometric analysis at the stage of immature ES-DC, harvested on day 2 of the third step of the differentiation culture. Although even nontransfectant ES-DC evidently expressed PD-L1 after maturation (data not shown), only a small population of them expressed PD-L1 at this stage (Fig. 5B, K1ES-DC). On the basis of the results of the analysis, one transfectant clone, KhES1-PD28, expressing the highest level of PD-L1 after the differentiation into immature ES-DC, was selected (Fig. 5B). Allogeneic T cells cocultured with immature ES-DC-PD28 showed a significantly lower response than those cocultured with nontransfectant immature ES-DC ($p < .05$; Fig.

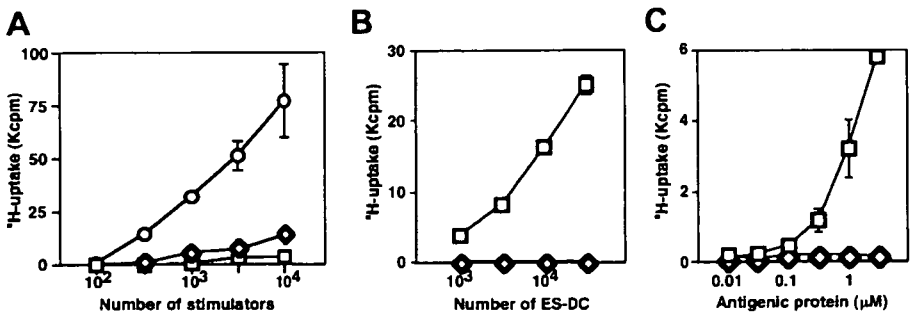

Figure 4. Stimulation of allogeneic T cells and antigen presentation by human ES-DC. **(A):** The indicated numbers of mature ES-DC (circles), immature ES-DC (diamonds), and pre-ES-DC (squares) were x-ray-irradiated (40 Gy) and cocultured with allogeneic human peripheral blood T cells (4×10^4 cells per well) in a 96-well round-bottomed culture plate for 5 days. Proliferation of T cells in the last 16 hours of the culture was measured based on [^{3}H]-thymidine uptake. The data are indicated as the mean value ± SD of duplicate cultures. **(B):** The indicated numbers of KhES-1-derived mature ES-DC prepulsed with glutamic acid decarboxylase (GAD) $65_{111-131}$ peptide (squares) and those left unpulsed (diamonds) were cocultured with a GAD65-specific, HLA-DR53-restricted human CD4$^+$ T-cell clone, SA32.5 (3×10^4 T cells per well) for 3 days. Proliferation of the T cells in the last 16 hours of the culture was measured by [^{3}H]-thymidine uptake. **(C):** Mature KhES-1-derived ES-DC (1×10^4 cells per well) were cocultured with SA32.5 T cells (3×10^4 cells per well) in the presence of the indicated concentrations of glutathione *S*-transferase (GST)-GAD65 recombinant protein (squares) or GST protein (diamonds) for 3 days. Proliferation of the T cells in the last 16 hours of the culture was measured by [^{3}H]-thymidine uptake. Abbreviation: ES-DC, embryonic stem cell-derived dendritic cells.

5C). The proliferation-reducing effect of the transgene-derived PD-L1 was abrogated by the addition of anti-PD-L1 blocking Ab ($p < .01$), ruling out the possibility that the introduction of the PD-L1 expression vector impaired the differentiation of ES-DC. Collectively, these results suggest that forced expression of PD-L1 on ES-DC downmodulated the proliferative response of cocultured allogeneic T cells via the interaction of PD-L1 with PD-1 on the T cells.

ES-DC carrying an epitope-presenting vector and expressing recombinant human invariant chain (Ii/CD74), which included GAD65p115–127 in the class II-associated invariant chain peptide region, were also generated (Fig. 5D). It was expected that the epitope could be efficiently targeted to the major histocompatibility complex (MHC) class II pathway [17]. Using a protocol similar to that used for the generation of PD-L1 transfectants, the vector was introduced into KhES-1 ES cells, and a transfectant clone, KhES-1-Ii23, highly expressing transgene-derived recombinant CD74, was selected by a flow cytometric analysis at the pre-ES-DC stage. The expression of CD74 was detected even in the nontransfectant pre-ES-DC, reflecting intrinsic expression of CD74 (Fig. 5E). The transfectant exhibited an increased expression of CD74 in comparison to the nontransfectants, thus indicating additional expression of the molecule derived from the transgene. The ability of the transfectant ES-DC, ES-DC-Ii23, to stimulate the GAD epitope-specific T-cell clone SA32.5 in the absence of antigenic peptide or protein was next examined. As a result, ES-DC-Ii23 stimulated SA32.5 T cells and induced their proliferation, thus demonstrating functional expression of the epitope-presentation vector in the transfectant ES-DC (Fig. 5F). The in vivo transfer of ES-DC transfected with this antigen-presenting vector is therefore expected to be useful for controlling the immune response in an antigen-specific manner [7].

Generation and Genetic Modification of Cynomolgus Monkey ES-DC

The differentiation protocol established using human ES cells was then applied to nonhuman primate ES cells. An ES cell line derived from cynomolgus monkey, CMK6 [11], was subjected to the ES-DC differentiation culture. Following the transfer to OP9 feeder layers, CMK6 cells grew and differentiated more rapidly than did human ES cells KhES-1 and KhES-3. The optimal duration of the first step of the differentiation culture for CMK6 was 11–13 days, whereas the duration ranged from 14 to 18 days for human ES cells. Figure 6A–6C illustrates the morphological changes of CMK6-derived cells following the second step of the differentiation culture. The surface phenotypes of the CMK6-derived pre-ES-DC, immature ES-DC, and mature ES-DC were then analyzed by flow cytometry (Fig. 6D). The double peaks seen in the histograms in Figure 6D reflect the heterogeneity of the analyzed cells in size and intensity of autofluorescence. Cynomolgus monkey ES-DC had the capacity to stimulate allogeneic cynomolgus monkey T cells (Fig. 6E), as human ES-DC did.

The expression vector for *HLA-DRB4*0103* (Fig. 7A) was introduced to CMK6. An analysis of the partial nucleotide sequence of *DRA* (*CyLA-DRA*) gene of CMK6 showed that the predicted amino acid sequence of the CyLA-DRα chain is very similar to that of HLA-DRα, with only one amino acid difference in α1 domain (GenBank accession no. AY591919). This suggested that the transgene-derived HLA-DRβ chain could associate with the intrinsic CyLA-DRα chain expressed in cynomolgus ES-DC and present an antigen to human T cells. The expression of the transgene before and after the ES-DC differentiation was confirmed by an RT-PCR analysis (Fig. 7B). ES-DC derived from a transfectant ES cell clone, cES-53-23, were prepulsed with synthetic GAD65 peptide and cocultured with the HLA-DR53-restricted, GAD65-specific T-cell clone SA32.5. Figure 7C shows that the GAD65 peptide-pulsed transfectant ES-DC stimulated the T cells to proliferate. In contrast, ES-DC originating from parental ES cells prepulsed with the peptide could not stimulate the T-cell clone. In addition, DR53-transfectant cynomolgus ES-DC had the capacity to process and present a protein antigen to the T cells (Fig. 7D). These results demonstrate the antigen-processing and presenting capacity of cynomolgus ES-DC and also the functional expression of the transgene that had been introduced into the ES cells before the differentiation. Thus, the effect and safety of the immune therapy by the in vivo transfer of ES-DC can be examined by preclinical studies using cynomolgus monkeys.

DISCUSSION

To establish the current culture protocol, various culture conditions were tested. As feeder cell lines, three lines of mouse stromal cells, OP9, PA6, and ST2, were comparatively evaluated. As a result, the use of OP9 was thus observed to produce

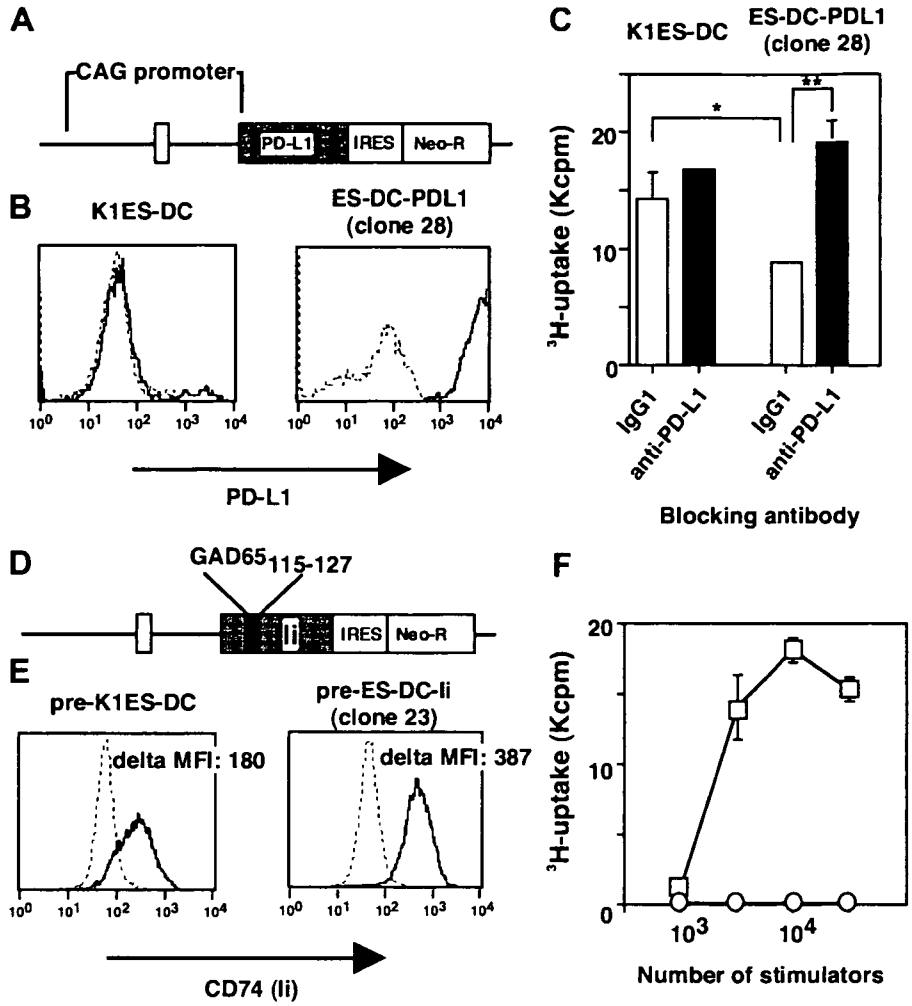

Figure 5. Genetic modification of human ES-DC. **(A):** Structure of the expression vector for human PD-L1. The expression of PD-L1 was driven by the CAG promoter, and the PD-L1-coding sequence was followed by IRES-neomycin-resistance gene (Neo-R), a selection marker. The open box in the CAG promoter indicates exon 1 of the rabbit β-actin gene contained in CAG promoter. **(B):** Transgene-derived PD-L1 expressed in immature ES-DC originated from transfectant embryonic stem (ES) cells was detected by flow cytometric analysis (ES-DC-PD-L1, clone 28). As a control, the staining profile of ES-DC derived from parental ES cell line (K1ES-DC) is shown. Specific stainings with anti-human-PD-L1 monoclonal antibody (Ab) (thick line) and isotype-matched control staining (thin, broken line) are shown. **(C):** The alloreactive response of T cells (4×10^4 cells per well) cocultured with immature ES-DC (1×10^4 cells per well) derived from the PD-L1-transfectant ES cells (ES-DC-PD-L1, clone 28) or those derived from parental ES cell line (K1ES-DC) is shown. The culture was done under the same conditions as those shown in Figure 3A except that anti-PD-L1 blocking Ab or isotype-matched mouse IgG1 was added to the culture. The statistical significance of the differences between the T-cell responses is indicated by asterisks (*, $p < .05$; **, $p < .01$). **(D):** Structure of expression vector for human Ii (Ii/CD74) including GAD65-derived epitope. The class II-associated invariant chain peptide region of the Ii-coding sequence was replaced with an oligo DNA-encoding $GAD65_{115-127}$. **(E):** Intracellular CD74 expressed in pre-ES-DC originated from transfectant ES cell clone (pre-ES-DC-human Ii [hIi], clone 23) and parental ES cell line (pre-K1ES-DC) was detected by a flow cytometric analysis. Specific staining with anti-human-CD74 monoclonal Ab (thick lines) and isotype-matched control staining (thin, broken lines) are shown. The values in the figure indicate the delta MFI between staining with the anti-CD74 and the isotype-matched control Ab. **(F):** SA32.5 T cells (3×10^4 cells per well) were cocultured with the indicated numbers of mature ES-DC-hIi clone 23 (squares) or nontransfectant ES-DC (circles) in the absence of exogenous antigen for 3 days. The proliferation of the T cells in the last 16 hours of the culture was measured by the [^{3}H]-thymidine uptake. Abbreviations: delta MFI, difference of mean fluorescence intensity; ES-DC, embryonic stem cell-derived dendritic cells; GAD, glutamic acid decarboxylase; Ii, invariant chain; IRES, internal ribosomal entry site.

the highest yield of ES-DC. Although ST2 also worked as feeder cells in the second step, the yield of ES-DC was approximately half of that obtained using OP9. It was also essential to remove any firmly adherent cells, when transferring the cells from the first to second step, by the procedure described in the Materials and Methods. At the end of the first step, many flat, adherent ES cell-derived cells were observed to form monolayers in the dishes. They probably differentiated into cell lineages other than mesoderm, and unless removed, they grew rapidly in the second step and inhibited the growth of hematopoietic cells.

Previously, two other groups reported the generation of functional antigen-presenting cells or DC from human ES cells. Zhan et al. adapted embryoid body-based induction of hematopoietic differentiation [24]. Slukvin et al. recently reported a method using OP9 [25]. Although there are some similarities between the method of Slukvin et al. [25] and the one reported here, the two methods differ in the following points.

In both methods, human ES cells were cocultured with OP9 feeder cells at the initial differentiation step (the first step). However, the duration of this culture step in our method (14–18 days) is significantly longer than the 10 days in the method of Slukvin et al. [25]. In our system, cells with morphology indicating mesodermal differentiation first appeared on day 8 or 9, and the extension of the first step of culture to days 14–18 significantly improved the yield of hematopoietically differentiated cells (Fig. 1D, 1E). In addition, we pretreated OP9 cells

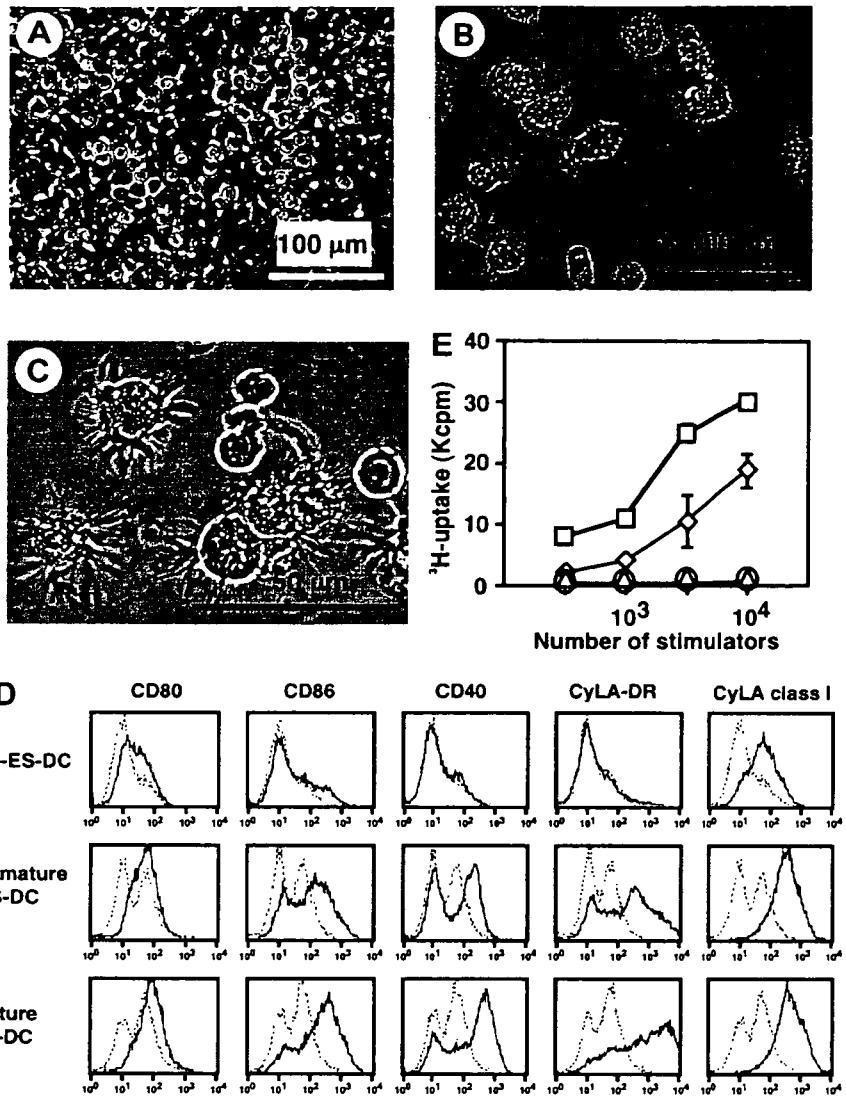

Figure 6. Generation of ES-DC from cynomolgus monkey embryonic stem (ES) cells. **(A–C):** The morphologies of cynomolgus monkey ES cell-derived differentiating cells (pre-ES-DC) at day 7 in the second step **(A)** and those in the third step before **(B)** and after **(C)** the addition of maturation stimuli are shown. **(D):** Cynomolgus monkey ES cell-derived cells harvested on day 8 in the second step (pre ES-DC) and from the third step before (immature ES-DC) and after (mature ES-DC) addition of maturation stimuli were analyzed for the cell surface expression of CD80, CD86, CD40, CyLA-DR, and CyLA class I. Staining patterns with specific monoclonal antibody (thick lines) and isotype-matched controls (thin, broken lines) are shown. **(E):** The indicated numbers of mature ES-DC (squares), immature ES-DC (diamonds), pre-ES-DC (circles), and undifferentiated cynomolgus ES cells (triangles) were x-ray-irradiated (40 Gy) and cocultured with allogeneic cynomolgus monkey peripheral blood T cells (4×10^4 cells per well) in a 96-well round-bottomed culture plate for 5 days. The proliferative responses of T cells in the last 16 hours of the culture were measured based on the [^{3}H]-thymidine uptake. Abbreviation: ES-DC, embryonic stem cell-derived dendritic cells.

with mitomycin C before use as feeder cells, and this was essential for efficient generation of hematopoietic cells. Treatment with mitomycin C may not only inactivate the mitosis of OP9 but also enhance the capacity of OP9 to support hematopoietic differentiation [26].

In the method of Slukvin et al., cells harvested from the first step of culture were directly transferred to 2-hydroxyethyl methacrylate-coated culture containers for the second step of culture [25]. In our method, cells harvested from the first step of culture were incubated in tissue culture-coated dishes for 2–5 hours to remove adherent cells. Removal of cells committed to nonmesodermal lineages by this procedure is essential. In addition, the second step of culture was also done with OP9 feeder in our method.

After the second step of culture, removal of dead cells and aggregated cells may be necessary in the method of Slukvin et al., as described in their report [25]. Indeed, we observed many dead cells, as well as DC-like cells, when we tried that method. In our method, most of recovered cells after the second step were viable, and removal of dead cells was not necessary.

The issues of safety and efficacy are critical for the establishment of ES-DC therapy. It is presumed that preclinical in vivo studies with the nonhuman primates will be required. Therefore, the ability to generate ES-DC from cynomolgus monkey ES cells is also considered to be important. It is probable that ES-DC can be generated from the ES cells of other nonhuman primates used in medical research, such as the rhesus monkey (*Macaca mulatta*) [27] and the common marmoset (*Callithrix jacchus*) [28]. For clinical application of the ES-DC technology, development of a feeder-free differentiation method may be required. Embryoid body-mediated differentiation methods may be one way to resolve this issue. In the mouse system, induction of mesodermal differentiation of ES cells using type IV collagen-coated culture plates has been reported [29, 30]. Several molecules have been reported to be involved in support of hematopoietic cell growth or differentiation by stromal cells [31–33]. Information on the molecular basis of the interaction between differentiating ES cells and feeder cells is valuable for the development of a feeder-free differentiation system.

Considering clinical applications, manipulation of function of ES-DC by genetic modification without use of viral vectors, demonstrated in the present study, has a significant advantage. However, random integration of multiple copies of transgenes into various genomic loci of ES cells is accompanied by risks such as activation of cellular oncogenes. Thus, a method to integrate transgenes into intended loci of the genome of human ES cells needs to be established.

Previously, we demonstrated a method for efficient targeted integration of expression vectors into specific genomic sites of mouse ES cells, using exchangeable gene-trap vector with Cre-

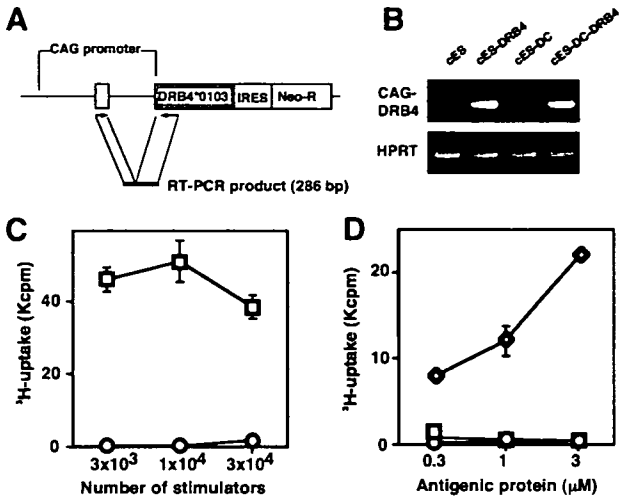

Figure 7. Antigen presentation to human T cells by genetically modified cES-DC. **(A):** The structure of HLA-DRB4*0103 expression vector is shown. The open box indicates the noncoding first exon of rabbit β-actin gene included in the CAG promoter. RT-PCR with PCR primers indicated by arrowheads generated PCR products of 286 base pairs from the transgene-derived mRNA. **(B):** Results of an RT-PCR analysis of parental cES and a transfectant embryonic stem cell clone (cES-DRB4) and derivative embryonic stem cell-derived dendritic cells (ES-DC) on the expression of transgene-derived mRNA (CAG-DRB4). The PCR products for HPRT transcript amplified from the same cDNA samples are also shown as control. **(C):** The indicated numbers of DRB4-transfectant ES-DC (squares) or nontransfectant ES-DC (circles) were preloaded with $GAD65_{111-131}$ peptide, x-ray-irradiated (40 Gy), and cocultured with SA32.5 T cells (3×10^4 cells per well) for 3 days. The proliferation of the T cells in the last 16 hours of the culture was measured by the [^{3}H]-thymidine uptake. **(D):** DRB4-transfectant ES-DC (diamonds) (1×10^4 cells per well) or nontransfectant ES-DC (squares) were cocultured with SA32.5 T cells (3×10^4 cells per well) in the presence of the indicated concentration of glutathione *S*-transferase (GST)-GAD recombinant protein for 3 days. DRB4-transfectant ES-DC and SA32.5 T cells were cocultured also in the presence of GST protein (circles). The proliferation of the T cells in the last 16 hours of the culture was measured by the [^{3}H]-thymidine uptake. Abbreviations: cES, cynomolgus embryonic stem cells; cES-DC, cynomolgus embryonic stem cell-derived dendritic cells; HPRT, hypoxanthine-guanine phosphoribosyl transferase; IRES, internal ribosomal entry site; RT-PCR, reverse transcriptase polymerase chain reaction.

Lox-mediated recombination system [1]. We are now trying to develop a system for targeted integration of transgenes into human ES cell genome. In this system, at first, gene-targeting vector conveying a drug resistance marker gene flanked by lox sequences is introduced, and then ES cell clones carrying the vector properly integrated by homologous recombination are selected. Subsequently, expression vectors with lox sequences are introduced with the aid of the Cre-Lox recombination system. Integration of a single copy of the transgene into the intended locus can be verified by Southern blot analysis. By this strategy, we can obtain ES cell clones with defined genetic modification, thus avoiding the risks accompanying the random integration of exogenous genes.

Allogenicity caused by differences in the genetic background between human ES cell lines and the recipients is considered to be a critical problem in medical application of ES-DC. We previously reported that mouse ES-DC administered into semiallogeneic recipients, sharing one MHC haplotype with the ES-DC, effectively primed antigen-specific cytotoxic T lymphocytes (CTL), suggesting that ES-DC can survive for a period long enough to stimulate antigen-specific CTL restricted by the shared MHC class I [4]. However, in the same semiallogeneic setting, we also observed five times that injection of no antigen-loaded ES-DC significantly reduced the efficiency of priming of antigen-specific CTL induced by the subsequent injection of antigen-loaded ES-DC (unpublished observations). Thus, repetitive stimulation with ES-DC expressing allogeneic MHC may result in activation and expansion of allogeneic MHC class I-reactive CTL, and in such recipients, subsequently transferred ES-DC may be rapidly eliminated. Repeated immunization may be required in clinical applications, for example, to induce antitumor immunity. Thus, we should resolve the problem of the histoincompatibilty between ES cell lines and recipients.

Methods for targeted gene modification of human ES cells and for targeted chromosome elimination of mouse ES cells have been developed [34–36]. To overcome the problem of histoincompatibility, genetic modification to inhibit expression of endogenous HLA class I in ES-DC may be effective. Deletion of more than 1,000 kilobases of entire HLA class I region of human ES cell genome by gene targeting is infeasible by currently available technology. However, disruption of the genes of molecules necessary for the cell surface expression of HLA class I molecules, such as transporter associated with antigen processing (TAP) or β2-microglobulin (β2M), is presumably feasible. In our plan, we will introduce expression vector encoding the β2M-linked form of recipient-matched HLA class I heavy chain into TAP1- or β2M-deficient human ES cells. We are now testing this strategy by using a mouse system.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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特集　動物MHC

MHCとは何か

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はじめに

MHC（主要組織適合遺伝子複合体：major histocompatibility complex）は，マウスを用いた皮膚移植の実験において，移植片生着の成否，すなわち組織適合性を決定する主要な遺伝的因子として同定された。その後の研究により，*MHC*領域に存在する遺伝子によってコードされるMHC分子が，T細胞に対して抗原ペプチドを提示する分子であり，その遺伝子多型が免疫応答の個体差を規定していることが明らかにされた。

免疫系は，感染性微生物の侵入から生体を防御するために不可欠なシステムである。体内に侵入した微生物に由来するタンパク質は，獲得免疫系により，抗原としてアミノ酸配列のレベルで識別される。抗原認識は，個々のリンパ球が発現する抗原特異的なレセプターにより担われている。Bリンパ球（B細胞）の抗原レセプターは，免疫グロブリン（抗体）であり，Tリンパ球（T細胞）の抗原レセプターは，T細胞レセプター（T cell receptor：TCR）である。免疫グロブリンが抗原分子に直接結合するのに対し，T細胞レセプターは，抗原タンパクの限定分解の結果生じたペプチドがMHC分子に結合し，MHC-抗原ペプチドの複合体を形成したものを認識するという特徴がある。

MHC分子は，クラスⅠとクラスⅡの2種類に大別される。MHCクラスⅠとクラスⅡでは，その構造，組織分布，結合する抗原ペプチドの由来，さらに，それを認識するT細胞の種類が異なる。T細胞が，T細胞レセプターを介してペプチドとMHC分子の複合体を認識すると，活性化状態となり，細胞増殖，サイトカインの産生，細胞傷害活性の発現などの反応を示す。そして，MHCクラスⅠとクラスⅡいずれもが，免疫系による抗原の認識と，その後の感染性微生物の排除による生体防御機構において必須の役割を担っている。本稿では，ヒトのMHC（*HLA*：human histocompatibility leukocyte antigen）とマウスの*MHC*（*H-2*）についての知見から，哺乳動物のMHC分子の構造と機能について概説する。なお，本稿ではMHCクラスⅠ分子として，著明な遺伝的多型性を有し，ペプチド抗原の提示を行なう，古典的MHCクラスⅠについてのみ言及する。

MHCクラスⅠの組織分布と構造

MHCクラスⅠは，マウスではH2-K，D，Lの3種類の分子があり，ヒトではHLA-A，B，Cの3種類の分子がある。MHCクラスⅠ分子は，約340個のアミノ酸より成る45kDaのα（重）鎖と，99個のアミノ酸からなる12kDaのβ2ミクログロブリン（β2M）とが非共有結合により結合して細胞膜表面に発現する（図1）。MHCクラスⅠ分子は，すべての有核細胞に発現しており，核の無い細胞のうち，血小板には発現しているが，赤血球には発現していない。MHCクラスⅠ分子のα鎖は，

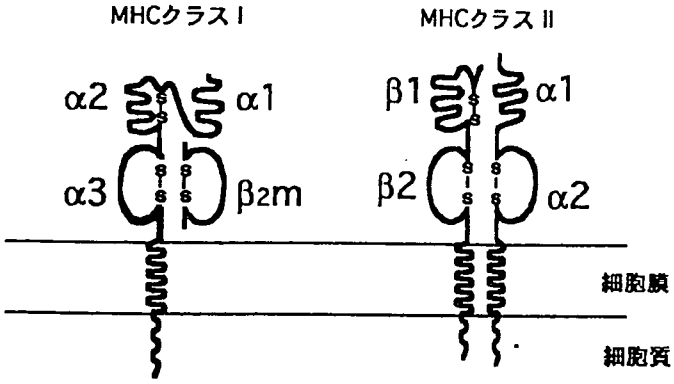

図1　MHC分子の構造

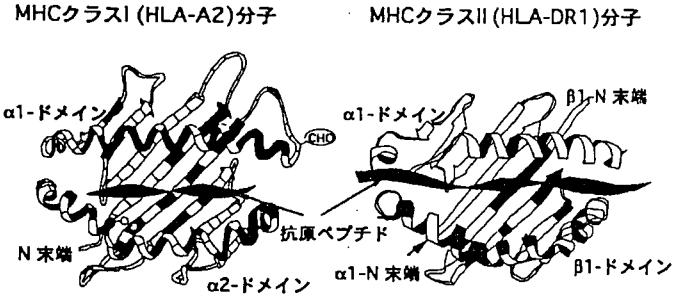

図2　MHCクラスI (HLA-A2) およびクラスII (HLA-DR1) 分子の立体構造。この図は，上面（T細胞レセプター側）より見たものであり，黒く塗りつぶしたアミノ酸残基は多型を示す。これらはHLA分子のペプチド収容溝の底面や側壁に多く認められる。

それぞれ約90個のアミノ酸からなるα1，α2およびα3の3つの細胞外ドメインと，膜通過部分（39あるいは40アミノ酸）および短い細胞質内部分（25あるいは28アミノ酸）を有する。α2およびα3ドメインには各々2個ずつのシステイン残基があり，その間に各ドメイン内でジスルフィド（S-S）結合が形成されている。X線結晶解析により数種類のMHCクラスI分子の立体構造が解明され，MHCクラスI分子の先端の部分を構成するα1とα2ドメインには，αヘリックス構造が側壁を，またβシート構造が底面を構成する溝状の構造（ペプチド収容溝）が存在し，この溝にペプチドが結合していることが明らかになった（図2）。

MHCクラスI分子の機能

MHCクラスI分子のペプチド収容溝には，細胞内で合成されたタンパク質に由来するペプチドが結合する。この中には，細胞に感染したウイルスなどのタンパク質に由来するペプチドも含まれる。まず，これらのタンパク質にユビキチンが結合したものが，プロテアソームと呼ばれるタンパク質分解装置に取り込まれ分解される。その結果，タンパク質は9個前後のアミノ酸からなるペプチド断片となり，ペプチドトランスポーター（TAP：transporter associated with antigen processing）により小胞体内腔へと輸送される（図3）。なお，プロテアソームを構成するサブユニットの一部，ならびにTAPの遺伝子は共に，*MHC*遺伝子領域内に存在する。小胞体の内腔で，MHCクラスIの重鎖-β2M-ペプチドの複合体が形成され細胞表面へ輸送され，CD8陽性キラーT細胞へ提示される。

MHCクラスIに結合するペプチドは8～12個の

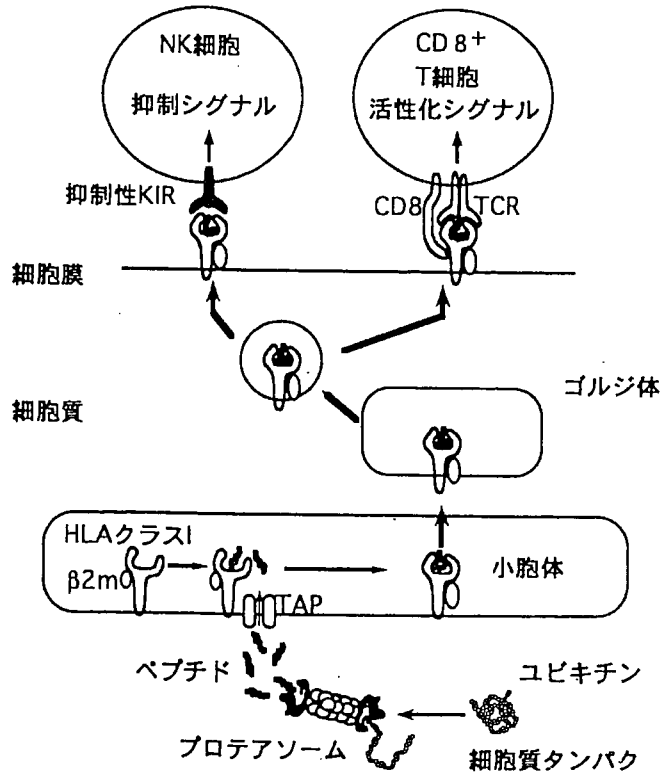

図3　MHCクラスI分子による抗原提示の経路

アミノ酸からなるペプチドで，両端（NおよびC末端側）のアミノ酸残基（アンカー残基）がクラスⅠ分子の先端のペプチド収容溝に存在する窪み（ポケット）にうまく収容されると，ペプチドはMHCクラスⅠ分子に結合する。MHCの型によってポケットの構造が異なっているので，そこに結合できるアンカー残基の種類は，MHCの型によって異なる。

感染等のない正常な細胞では，細胞表面に存在するMHCクラスⅠ分子はその細胞が本来産生するタンパク質に由来するペプチドを結合している。このような自己のMHCクラスⅠ分子と自己由来のペプチドの複合体を認識するT細胞は，胸腺における分化の過程で除かれているか，あるいは機能的に不活性化され反応しない状態になっている（自己に対する免疫寛容）。

ウイルスや細胞内寄生性の細菌，原虫などが感染した細胞では,細胞表面のMHCクラスⅠ分子のごく一部にこれらの感染性微生物に由来する抗原ペプチドが結合している。CD8陽性細胞傷害性（キラー）T細胞は，MHCクラスⅠ分子にこのような非自己抗原ペプチドが結合した複合体を認識して活性化する。活性化キラーT細胞は，パーフォリンを分泌して標的細胞の細胞膜に穴をあけ，さらにグランザイムなどのタンパク質分解酵素を細胞質内に送り込み，アポトーシスを誘導することにより標的細胞を破壊する。この際にキラーT細胞の表面に発現する糖タンパク質であるCD8分子は，標的細胞上に発現するMHCクラスⅠ分子の$\alpha 3$ドメインに結合して，CD8陽性T細胞と標的細胞との間の接着を高めると同時に，T細胞内に活性化シグナルを伝達する。キラーT細胞がウイルスが感染した細胞を破壊することにより，細胞内に存在しているウイルスの増殖が阻止される。このメカニズムは，ウイルス感染に対する重要な防御機構である。また，がん細胞上のMHCクラスⅠに提示されているペプチドの中には，正常細胞では発現していないか，あるいは，極めてわずかしか発現していないタンパク質に由来するものがある。このようながん関連抗原に由来するペプチドを認識するキラーT細胞が体内に存在すれば，そのがん細胞を傷害することが可能である。すなわち，免疫系は，がん細胞特異的ペプチドを目印にして，正常細胞とがん細胞を識別し，がん細胞を攻撃することが可能である。

NK（ナチュラルキラー）細胞は血球系の細胞であるが，代表的な免疫担当細胞であるマクロファージ，樹状細胞，T細胞あるいはB細胞のいずれにも属さない細胞である。NK細胞は特定のウイルスあるいは細菌に感染した細胞，あるいは腫瘍細胞を破壊する。NK細胞の表面にはKIR（killer-cell immunoglobulin-like receptors）と総称される一群のレセプターが発現している。KIRの中にはリガンドを認識した結果としてNK細胞の細胞傷害活性を活性化するものと抑制するものがあるが，抑制性KIRの中には標的細胞上のMHCクラスⅠ分子に結合するものがある。NK細胞が，体内の正常な細胞に発現しているMHCクラスⅠ分子を認識すると，このような抑制性KIRを介してNK細胞内に細胞傷害活性の発現を抑制するシグナルが伝達されるため，このような正常な自己細胞は傷害しない（図3）。一方，ウイルス感染やがん化などにより，MHCクラスⅠ分子を発現しなくなった細胞に対しては，MHCクラスⅠ分子を認識する抑制性KIRが働かないので，NK細胞による攻撃の対象となる。

MHCクラスⅡ分子の組織分布と構造

MHCクラスII分子は，マウスではI-AとI-Eの2種類があり，ヒトではHLA-DR，DQ，DPの3種類の分子がある。MHCクラスII分子は，樹状細胞，マクロファージやB細胞などの，いわゆるプ

ロフェッショナル抗原提示細胞に発現する。また，ヒトでは抗原刺激などにより活性化されたT細胞にも発現する。さらに，インターフェロン-γの作用により，一部の非血球細胞にも発現する。

MHCクラスII分子はα鎖とβ鎖が結合して出来ており，細胞膜に結合してその大部分を細胞外に発現する膜結合型タンパク質である。MHCクラスII分子は，約230個のアミノ酸からなる33-35kDaのα鎖と，約230個のアミノ酸からなる約27-29kDaのβ鎖が非共有結合により結合して細胞膜表面に発現する（図1)。MHCクラスII分子のαおよびβ鎖の細胞外部分は，それぞれ約90個のアミノ酸からなる2つのドメイン に分かれており，α1, α2, β1および β2 ドメイン により構成されている。MHCクラス I 分子と同様に，数種類のMHCクラスII分子についても，X線結晶解析によってその立体構造が明らかにされた。その結果，α1および β1 ドメインが組み合わさって，MHCクラス I 分子の α1 および α2 ドメインが形成するのと同様のペプチド収容溝を作ることが明らかとなった。さらに，この部分にペプチドが線状に結合していることが示された。

MHCクラス II 分子の機能

MHCクラスII分子による抗原提示の経路の概要は，図4に示す通りである。MHCクラスII分子には，細胞外から取り込まれたタンパク質，あるいは細胞内で合成されたタンパク質のうち分泌タンパクあるいは膜タンパク質に由来するオリゴペプチドが結合する。

MHCクラスII分子は，前述したように主にプロフェッショナル抗原提示細胞に発現しており，微生物感染等に際して，CD4陽性ヘルパーT細胞に対して抗原ペプチドを提示し，ヘルパーT細胞を活性化することにより免疫応答を開始する，という役割を有している。

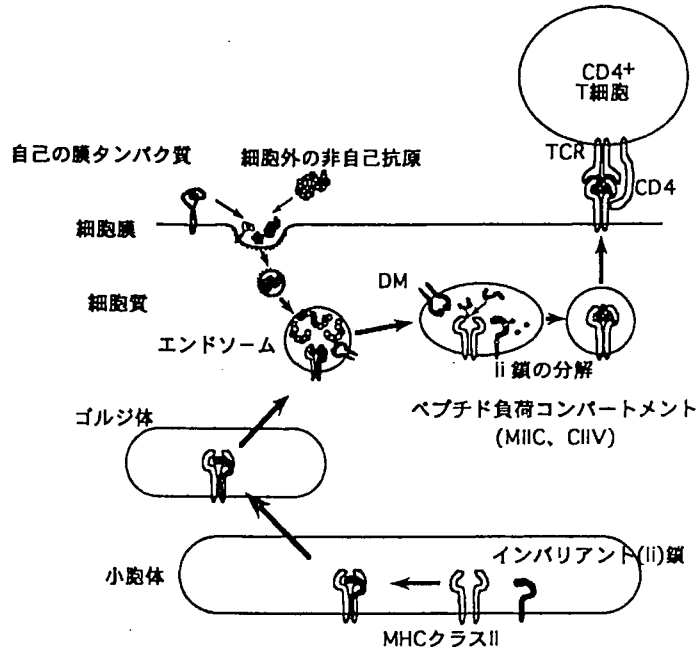

図4 MHCクラスII分子による抗原提示

樹状細胞などの抗原提示細胞は，細胞外にある物質をピノサイトシス，エンドサイトシス，あるいはファゴサイトシスにより細胞内へ取り込む。取り込まれたもののうちタンパク質は，細胞内のエンドソーム内においてタンパク分解酵素により限定分解され，その結果オリゴペプチドが産生される。

一方，MHCクラスII分子は，小胞体で合成された後，ゴルジ体を経てエンドソームへ移行するが，この間，インバリアント鎖（Ii鎖）と称される分子と会合している。Ii鎖は，MHCクラスII分子をエンドソームに導くシャペロン分子としての機能と，MHCクラスII分子のペプチド収容溝を塞いでエンドソーム以外の場所でペプチドが結合するのを防ぐ役割を有している。Ii鎖のこの二つの働きにより，MHCクラスII分子は，細胞内で合成されたタンパク質でなく，細胞外から取り込まれたタンパク質に由来する抗原ペプチドと選択的に結合する。

Ii鎖とMHCクラスII分子の複合体がエンドソームに到達すると，まず，Ii鎖がタンパク分解酵素

により分解され，MHCクラスII分子から解離する。空になったMHCクラスII分子のペプチド収容溝に，前述の外来性のタンパク質等に由来するペプチドが結合する。この過程は，遺伝子がMHC領域内に存在し，MHCクラスII分子と類似した構造を有するがペプチド結合能を持たないDM分子により促進される（図4)。このようにして形成されたMHCクラスII分子・ペプチド複合体が抗原提示細胞の細胞膜表面へ移動し，CD4陽性T細胞に対して提示される。

クラスIIに結合する抗原ペプチドはクラスI結合性ペプチドより長く，通常13～23個のアミノ酸からなる。ペプチド上で数個のアミノ酸を隔てて位置する2～5個のアミノ酸残基（アンカー残基）の側鎖が，MHCクラスII分子のペプチド収容溝にある大小数個のポケットに収容され，MHCクラスII分子との結合に重要な役割を担っている。

感染性微生物などが生体内に侵入してきた場合，生体内各組織に分布する樹状細胞がこれを捕捉し，リンパ節などの所属リンパ組織へ遊走し，その微生物由来のペプチドをMHCクラスII分子に結合させ複合体として提示する。CD4陽性T細胞は，T細胞レセプターを介してMHCクラスII分子・ペプチド複合体を認識し，活性化シグナルをT細胞内に伝えることにより，種々のサイトカイン およびサイトカインレセプター 遺伝子などを発現した後に増殖する。この際にCD4分子は，MHCクラスII分子のβ2ドメインに結合して，T細胞による抗原認識を補助すると共に細胞内に活性化シグナルを送る。

活性化されたCD4陽性ヘルパーT細胞は，IL (interleukin) -4の作用および細胞同士の直接の接触を介してB細胞を増殖させ，形質細胞への分化を誘導して抗体産生を促す。また，IL-2，IL-4，GM-CSF，IFN (interferon) -γなどの作用により，CD8陽性キラーT細胞の増殖と活性化，および抗原提示細胞の分化と活性化を誘導する。非自己抗原が存在しない場合には，自己の細胞膜タンパクあるいは分泌タンパクに由来するペプチドがMHCと結合するが，CD8陽性キラーT細胞の場合と同様に，この自己MHC-自己ペプチド複合体を認識するCD4陽性T細胞は通常存在しないか，存在しても免疫応答を示さない（自己に対する免疫寛容)。

MHCの遺伝的多型性の生物学的意味

*MHC*遺伝子には，著しい遺伝的多型性が認められる。ヒトの*MHC*である*HLA*の場合，その多くの遺伝子座について数十から数百もの対立遺伝子（アリル）が報告されている。*MHC*の遺伝子多型性は，MHC分子の中でもペプチド収容溝を形成し，T細胞レセプターによって認識されるドメインにおいてとくに顕著である（図2)。異なるアリルのMHC分子では，そのペプチド収容溝の形状が違うため，そこに結合できるペプチドの構造も異なる。このため，個体が発現する*MHC*のアリルによって，体内でT細胞へ提示可能な抗原ペプチドが規定される。すなわち，*MHC*の遺伝的多型により，免疫応答性の個体差が生じる。

*MHC*遺伝子が複数存在し，それぞれが著明な多型性を示すことは，個体が所有するMHC分子の種類を増大させ，個体がT細胞を介して免疫応答できる抗原ペプチドの種類を増やし，免疫力を高めるという利点をもたらしている。さらにMHC分子の高度な多型性は，個体ではなく集団のレベルで多数の抗原に対処することを可能にしている。つまり伝染病の流行などに対して，個体によってはT細胞が免疫応答することができずに死に至ることがあっても，人類という種全体の中には，病原微生物に対してうまくT細胞の免疫応答を誘導することのできる*HLA*を持つ個体が存在

し，これが免疫応答を示して生き残ることにより，感染症による種の絶滅を回避してきたと考えられる。

また，特定の*HLA*アリルを有する個体が，特定の疾患への感受性が高い（疾患発症と特定の*HLA*アリルとの正の相関）ことが知られている。このような疾患の多くは，自己免疫疾患など免疫応答の異常に起因するものであり，このような相関は*HLA*によって規定される免疫応答の個体差の違いにより説明される。また，疾患の真の原因遺伝子は*HLA*ではないが，この遺伝子と*HLA*遺伝子との連鎖不平衡により，疾患感受性と特定の*HLA*のアリルとの間に相関が見られる場合もある。

T細胞によるアロMHC分子の認識と移植医療における意義

MHC分子の多型性は，T細胞によるアロ（同種異系）細胞認識の最大の原因となっている。前述したように，T細胞レセプターは，MHC分子の中でも多型がとくに顕著なペプチド結合部位にペプチドが結合した複合体の構造を認識する。ある個体の体内に存在するT細胞の集団（T細胞レパトア）は，某大な認識多様性を有するものであるが，その中の1～7％がその個体が発現しないMHC分子，すなわちアロMHCに何らかの抗原ペプチドが結合したものを認識すると言われている。この頻度は，例えば特定のMHCと特定の外来抗原ペプチドが結合した複合体を認識するT細胞の頻度と比較して極端に高いものである。このため，アロMHCを発現する細胞に対しては，微生物などの外来抗原などに対して通常惹起される応答等よりも非常に強力なT細胞応答が起こる。臓器移植における拒絶反応は，レシピエント体内のT細胞が移植臓器を異物として認識し，攻撃することを大きな一因として発生する。このことが，移植医療において，ドナーとレシピエントの間での*HLA*の一致度が，臓器移植の成否を決定する重要な因子となる理由である。

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