

# 学位論文

## Doctor's Thesis

Activation of antigen-specific cytotoxic T lymphocytes by utilizing allogeneic ES cell-derived dendritic cells with genetic modification of MHC class I expression

(MHC クラス I を自己化したアロジェニック ES 細胞由来の樹状細胞を用いた抗原特異的細胞傷害性 T 細胞の誘導)

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## 2. Summary

A method for the genetic modification of dendritic cells (DC) was previously established based on the *in vitro* differentiation of embryonic stem (ES) cells to DC (ES-DC). The unavailability of human ES cells genetically identical to the patients will be a problem in the future clinical application of this technology. This study attempted to establish a strategy to overcome this issue. The TAP1 or  $\beta$ 2-microglobulin ( $\beta$ 2m) gene was disrupted in 129 (H-2<sup>b</sup>)-derived ES cells and then expression vectors for the H-2K<sup>d</sup> or  $\beta$ 2m-linked form of K<sup>d</sup> ( $\beta$ 2m-K<sup>d</sup>) were introduced, thus resulting in two types of genetically engineered ES-DC, TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC and  $\beta$ 2m<sup>-/-</sup>/ $\beta$ 2m-K<sup>d</sup> ES-DC. As intended, both of the transfectant ES-DC expressed K<sup>d</sup> but not the intrinsic H-2<sup>b</sup> haplotype-derived MHC class I.  $\beta$ 2m<sup>-/-</sup>/ $\beta$ 2m-K<sup>d</sup> and TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC were not recognized by pre-activated H-2<sup>b</sup>-reactive CTL and did not prime H-2<sup>b</sup> reactive CTL *in vitro* or *in vivo*.  $\beta$ 2m<sup>-/-</sup>/ $\beta$ 2m-K<sup>d</sup> ES-DC and TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC had a survival advantage in comparison to  $\beta$ 2m<sup>+/-</sup>/ $\beta$ 2m-K<sup>d</sup> ES-DC and TAP1<sup>+/-</sup>/K<sup>d</sup> ES-DC, when transferred into BALB/c mice. K<sup>d</sup>-restricted RSV-M2-derived peptide-loaded ES-DC could prime the epitope-specific CTL upon injection into the BALB/c mice, irrespective of the cell surface expression of intrinsic H-2<sup>b</sup> haplotype-encoded MHC class I.  $\beta$ 2m<sup>-/-</sup>/ $\beta$ 2m-K<sup>d</sup> ES-DC were significantly more efficient in eliciting immunity against RSV M2 protein-expressing tumor cells than  $\beta$ 2m<sup>+/-</sup>/ $\beta$ 2m-K<sup>d</sup> ES-DC. The modification of the  $\beta$ 2m or TAP gene may therefore be an effective strategy to resolve the problem of HLA class I allele mismatch between human ES or induced pluripotent stem cells and the recipients to be treated.

### 3. Publication list

- Matsunaga, Y; Fukuma, D; Hirata, S; Fukushima, S; Haruta, M; Ikeda, T; Negishi, I; Nishimura, Y; Senju, S. Activation of antigen-specific cytotoxic T lymphocytes by beta2-microglobulin or TAP1 gene disruption and the introduction of recipient-matched MHC class I gene in allogeneic embryonic stem cell-derived dendritic cells. *J Immunol* 181: 6635-6643, 2008
- Senju, S; Suemori, H; Zembutsu, H; Uemura, Y; Hirata, S; Fukuma, D; Matsuyoshi, H; Shimomura, M; Haruta, M; Fukushima, S; Matsunaga, Y; Katagiri, T; Nakamura, Y; Furuya, M; Nakatsuji, N; Nishimura, Y. Genetically manipulated human embryonic stem cell-derived dendritic cells with immune regulatory function. *Stem Cells* 25: 2720-2729, 2007
- Fukushima, S; Hirata, S; Motomura, Y; Fukuma, D; Matsunaga, Y; Ikuta, Y; Ikeda, T; Kageshita, T; Ihn, H; Nishimura, Y; Senju, S. Multiple antigen-targeted immunotherapy with alpha-galactosylceramide-loaded and genetically engineered dendritic cells derived from embryonic stem cells. *J Immunother* in press
- Senju, S; Haruta, M; Matsunaga Y; Fukushima, S; Ikeda, T; Takahashi, K; Okita, K; Yamanaka, S; Nishimura, Y. Characterization of dendritic cells and macrophages generated by directed differentiation from mouse induced pluripotent stem cells. *Stem Cells* in division

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## 5. Abbreviations

APC,	Antigen presenting cell;
$\beta$ 2m, $\square$	$\beta$ 2-microglobulin;
BM-DC,	Bone marrow-derived dendritic cell;
CMTMR,	Chloromethyl-benzoyl-amino- tetramethyl-rhodamine;
CMFDA,	Chloromethylfluorescein diacetate;
CTL,	Cytotoxic T lymphocyte;
ELISA,	Enzyme-linked immunosorbent assay;
ELISPOT,	Enzyme-linked immunospot;
ER,	Endoplasmic reticulum;
ES cell,	Embryonic stem cell;
ES-DC,	Embryonic stem cell-derived dendritic cell;
FITC,	Fluorescein isothiocyanate;
GM-CSF,	Granulocyte macrophage colony stimulating factor;
HA,	Hemagglutinin;
HIV,	Human immunodeficiency virus;
HLA,	Human leukocyte antigen;
IFN,	Interferon;
IL,	Interleukin;
IRES,	Internal ribosomal entry site;
i.p.,	Intraperitoneally;
iPS cell,	Induced pluripotent stem cell;
Luc,	Luciferase;
MHC,	Major histocompatibility complex;
PBS,	Phosphate-buffered saline;
PCR,	Polymerase chain reaction;
RSV,	Respiratory syncytial virus;
s.c.,	Subcutaneously;
TAP,	Transporter associated with antigen processing;
TNF,	Tumor necrosis factor;

## 6. Introduction

### 6.1. DC vaccine against cancer

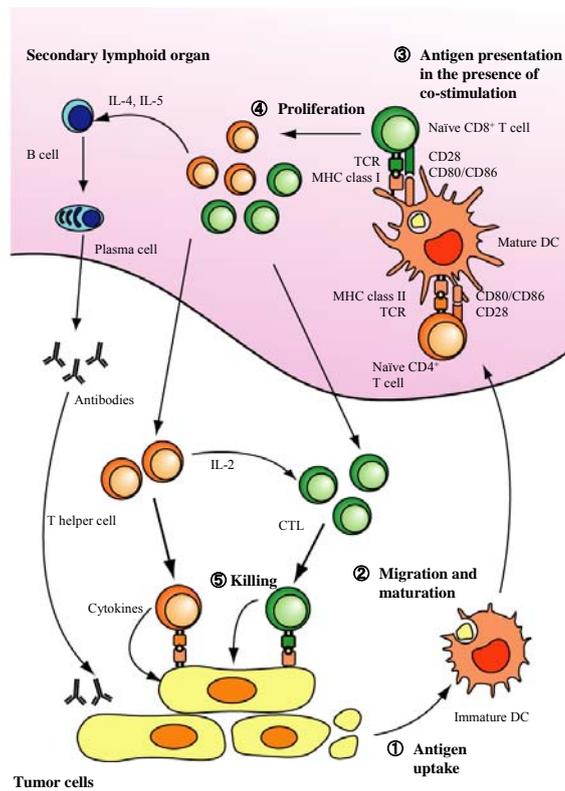
Dendritic cells (DC) are potent stimulators of B and T lymphocytes (1). B cells, which are important in humoral immunity, can directly recognize native antigen through their B cell receptors. However, T lymphocytes, which are important in cellular immunity, require the antigen to be processed and presented to them by antigen presenting cells (APC), including DC. The T cell receptors (TCR) recognize antigenic peptides in the context of the major histocompatibility complex (MHC)-encoded molecules expressed on the cell surface of APC. The mature DC express peptide-MHC complex, together with appropriate co-stimulate molecules on the cell surface. This allows the priming of naive CD4<sup>+</sup> T helper and CD8<sup>+</sup> cytotoxic T lymphocyte (CTL). DC also can activate natural killer cells (2) and natural killer T cells (3). So, DC can conduct all of the elements of the immune orchestra, and they are therefore an attractive target and tool for cancer immunotherapy (Fig.1).

Ex vivo-generated, antigen-loaded DC have now been used as vaccines to induce strong T cell immunity (4). Mice studies have shown that DC loaded with tumor antigen can induce therapeutic and protective antitumor immunity (5). Numerous reports have shown that DC loaded with dead tumor cells, tumor cell lysates, tumor antigenic proteins or peptides can induce immunity and clinical responses (6-9). Banchemereau. et al. compared clinical outcomes in several Phase I and Phase II vaccine trials in which DC and other types of vaccine were used for the treatment of patients with melanoma (Table 1). Although the rate of objective tumor regression is still limited, these outcomes warrant further exploration to establish the therapeutic value of vaccination with DC.

**Table 1. Clinical outcomes of cancer vaccines in patients with melanoma.**

Vaccine	Total Patients	Responding Patients	Response Rate (%)	References
Peptide vaccine	410	11	2.7	10, 11, 12
Viral vector	160	3	1.9	10
Tumor cells	43	2	4.6	13, 14
Dendritic cells	116	11	9.6	10, 15, 16, 17,18, 19

Table referred from REF.10



**FIGURE 1. Tumor immunity.**

Tumor regression *in vivo* is mediated by a complex interplay between two main mechanisms: innate and adaptive immune response. Innate mechanism may trigger inflammatory events in the tumor microenvironment and, in the presence of a local adequate cytokine combination (IL-2, IL-12, IL-18, IL-23), stimulate DC to react against tumor specific surface antigens. Immature DC incorporate tumor antigens and are activated to migrate to peripheral lymphoid tissues. Here they mature into cells that express high levels of co-stimulatory molecules (CD80/CD86) and the adhesion molecules that mediate interactions with the naïve T cells. Naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells recognize antigens presented by MHC class II and MHC class I molecules, respectively. Activated naïve CD4<sup>+</sup> T cells differentiate into helper T cells in at least two subpopulations (TH1 and TH2 cells). TH1 cells produce IL-2, IFN- $\gamma$ , TNF- $\alpha$  and GM-CSF that increase the activity of macrophages, and the expression of MHC class I molecules on the surfaces of CD8<sup>+</sup> cells. TH1 cells also stimulate proliferation and activation of CD8<sup>+</sup> T cells. TH2 cells secrete another group of cytokines, IL-4, IL-5 and IL-10, that induce naïve B cells to differentiate into plasma cells secreting immunoglobulin. Tumor-specific antibodies of the correct isotypes might be able to direct the lysis of the tumor cells by NK cells and macrophages through activation of Fc receptors bound by immune complexes. Naïve CD8<sup>+</sup> T cells differentiate into CTL that kill tumor cell by granule- and Fas-mediated mechanisms.

Genetic modification of DC with genes encoding for antigen or immunomodulatory molecules offers the potential of activating immune responses. For loading tumor antigens to DC for anticancer immunotherapy, the gene-based antigen expression by DC has several advantages over using DC simply pulsed with tumor antigenic proteins or peptides (20). The expression of tumor antigens by DC circumvents the need for identifying specific CTL epitopes within the protein, and by that the antigens are continuously supplied for presentation. Another advantage of using DC engineered to express tumor antigens is their potential for generating CTL responses against multiple class I-restricted epitopes derived from the antigen, thereby eliciting a broad antitumor effector response (21). Various antigens have been used to transfect murine and/or human DC and induced tumor antigen-specific immune responses, including MAGE-1, gp100, MART1, hTRP2, MUC-1 and NY-ESO-1 (22-27). An alternative method to enhance the ability of DC to present tumor antigens is the introduction of immunomodulatory molecules such as cytokines, chemokines and co-stimulatory molecules into the DC. DC genetically engineered to constitutively express immunomodulatory gene (GM-CSF, TNF- $\alpha$ , IL-12, lymhotactin and CD40L etc.) possess adjuvant-like properties and thereby augment antitumor immune responses (28-32).

Although DC-based cancer vaccine is a promising approach for anticancer immunotherapy, the medical application of DC vaccine strategy has several drawbacks (33). A problem is the source of DC for vaccination with DC. DC are generated from monocytes obtained from peripheral blood of the patients. Apheresis is necessary to obtain a sufficient number of monocytes as a source for DC. However, this procedure is sometimes invasive for patients with cancer and has the risk of contamination with residual malignant patient cells. Furthermore, 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. The way of introducing immunomodulatory genes into DC is another problem. Adenovirus vector is used to introduce genes to human monocyte-derived DC. However, random integration of multicopies of transgenes into various genomic loci of the DC is accompanied with risks such as activation of cellular oncogenes. In addition, use of virus-based vectors outside of isolated laboratories is prohibited by law in many

countries.

These considerations help illustrated the pressing need for quality control of preparations of DC intended for clinical use, so as to ensure both safety and efficacy of the procedure.

## 6.2. Embryonic stem cell-derived DC

Embryonic stem (ES) cells exhibit the remarkable properties of self-renewal, pluripotency and tractability for genetic modification. By exploiting ES cells as a novel source of DC, these features may be harnessed in addition to those of the DC themselves, to enhance their suitability as a candidate of cellular vaccine for cancer immunotherapy. This laboratory and others have established methods to generate DC from mouse and human ES cells (34-38). The capacity of ES cell-derived DC (ES-DC or esDC) to stimulate alloreactive T cells and to prime antigen-specific and syngeneic CTL is comparable to that of conventional bone marrow derived-DC (BM-DC). Genetically modified ES-DC can be readily generated by introducing expression vectors into ES cells and the subsequent induction of their differentiation into ES-DC (39). Genetically modified DC generated in this laboratory are summarized in Table 2.

**Table 2. Genetically modified ES-DC generated in our laboratory.**

Genes introduced into ES-DC	Expected effects	References
<b>Activation of immune response</b>		
OVA along with CCL21	Antigen presentation and Attraction of T cells	40, 41
OVA along with CXCL9	Antigen presentation and Attraction of T cells	40
OVA along with XCL1	Antigen presentation and Attraction of T cells	40
Glypican-3	Presentation of natural tumor antigen	42
TRP2, hgp100 or SPARC	Presentation of natural tumor antigen	43
<b>Suppression of immune response</b>		
MOG peptide along with TRAIL	Antigen specific immunosuppression	39, 44
MOG peptide along with PD-L1	Antigen specific immunosuppression	39
<b>Others</b>		
SPI-6	Avoidance of apoptosis of DC	45

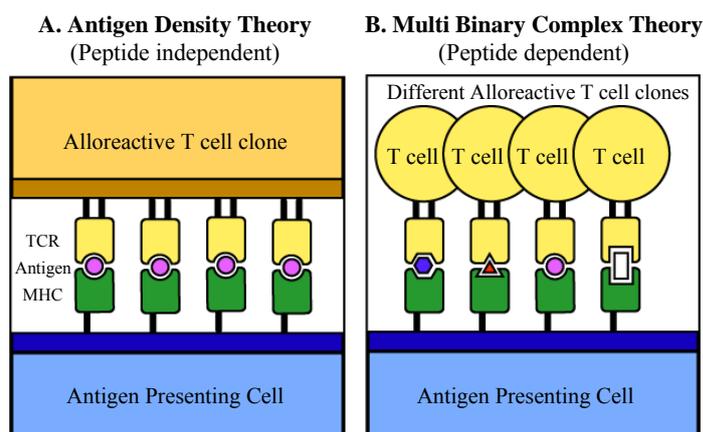
The transfection of ES cells can be done by electroporation with plasmid vectors and the use of virus-based vectors is not necessary. Once an ES cell clone with proper genetic modification is established, it then serves as an infinite source for genetically modified DC. Mouse models have demonstrated that vaccination with genetically engineered ES-DC expressing tumor antigens (42) and T cell-attracting chemokines (41) is very effective for the induction of anti-tumor immunity.

### **6.3. Immunological problems of ES-DC therapy**

In general, ES cells genetically identical to patients will not be available. In the future, the clinical application of ES-DC technology will therefore require a solution to the problem of histoincompatibility between patients to be treated and the ES-DC (46). Alloreactivity is the immune response caused by the histoincompatibility among other members of the same species. MHC antigens are exceptionally important in stimulating alloresponses. Alloreactivities of T cells to MHC class I and class II occur at a similar frequency, magnitude and clinical importance in organ and bone marrow transplantation (47). Normally, the frequency of alloreactive T cells is about 1000 times higher than the frequency of T cells specific for foreign antigens ( $1:10^3$  versus  $1:10^6$ ), which results in a strong primary response (48). Two different theories have been proposed to explain the high frequency of alloreactive T cells (49): (i) the “antigen density” theory and (ii) the “multiple binary complexes” theory as described below.

The first theory proposes an affinity-based response, termed the “antigen density model” (Fig. 2, left). It proposes that engrafted cells will contain a high concentration of allo-MHC molecules, such that an alloreactive T cell will recognize the differences occurring within the MHC molecule (independent of the bound peptide) as antigenic, thus initiating a cellular immune response against the graft. This peptide independent theory would account for the higher numbers of alloreactive T cells compared to the lower frequencies of most antigen-specific T cells that focus their recognition upon the peptide. The theory suggests that all of the allogeneic MHC expressing cells simultaneously present thousands of antigenic determinants per cell in the form of the polymorphisms within the MHC molecule itself, thus activating lower affinity T cells. The second theory outlines alloreactivity

as being peptide-dependent, termed the “multiple binary complexes theory” (Fig. 2, right). It assumes the allogeneic MHC molecule is similar enough to the self-MHC molecule for the alloreactive TCR to bind to it and recognize the presented peptide as foreign. Under this theory, also referred to as “determinant frequency”, the peptide is the key element controlling the specificity of the response. This could result from the alloreactive TCR recognizing the deceptively similar allogeneic MHC molecule bound to one or more endogenous peptides, that appear foreign because they have not undergone the negative selection process. Alternatively, recognition could be due to a peptide that is common to the graft and host, but looks foreign to the alloreactive TCR due to adoption of a distinct conformation induced by the polymorphic residues within the allogeneic MHC molecule.



**FIGURE 2. Two different hypotheses to explain of the high frequency of alloreactive T cells.** The antigen density theory suggested the alloreactive T cells recognize the differences in the allogeneic MHC molecules and the high density of these molecules on the antigen presenting cell would induce a strong alloresponse. The multiple binary complexes theory proposes that allorecognition is the result of recognition by T cells of many different peptides presented by allogeneic MHC molecules. Figure is cited from REF 49 with minor modifications.

Both these mechanisms may contribute to the high frequency of T cells that can respond to allogeneic MHC molecules expressed on allogeneic APC. Therefore, one

approach to overcome the histoincompatibility between the patients and ES-DC is to inhibit cell surface expression of the allogeneic MHC molecules on ES-DC.

According to a previous study by another group (50), BM-DC inoculated into allogeneic recipient mice are eliminated within a few days and the number of DC detected in the draining lymph node is lower than that of DC syngeneic to the recipient mice. In such circumstances, the rapid elimination of APC is mainly mediated by CD8<sup>+</sup> T cells reactive to allogeneic MHC class I (51). Thus, this study targeted to allogeneic MHC class I on ES-DC to minimize the alloreactivity.

#### **6.4. Target molecules for minimizing the allogeneic barriers**

However, the genomic region including the MHC class I genes spans more than 1,000 kb in both the mouse and human genome and complete elimination of such a large genomic region by gene-targeting technique is currently infeasible. The obvious candidates for modification are the genes encoding and controlling the cell-surface expression of MHC class I molecules.

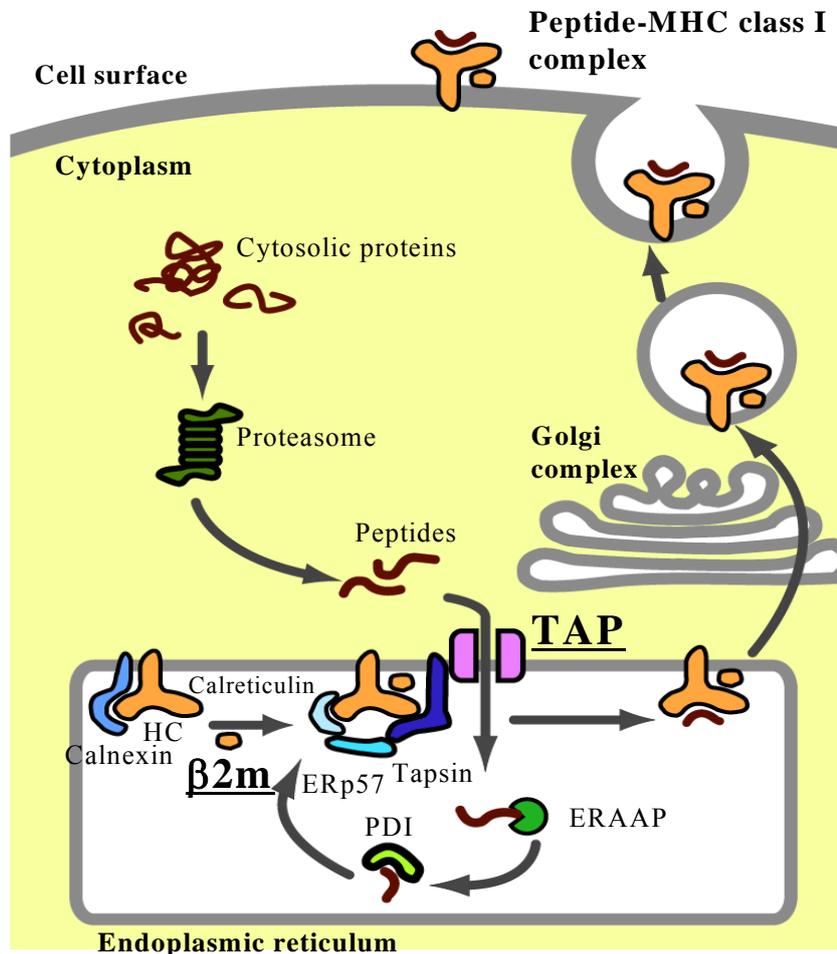
Overview of the classical pathway for MHC class I-mediated antigen presentation is shown in Figure 3. Stable cell surface expression of MHC class I requires the assembly of peptide-MHC class I and  $\beta$ 2m. This assembly occurs in the ER. Peptides generated in the cytoplasm must be translocated across the ER membrane in order to be bound by MHC class I molecule (52). This is achieved by the transporter associated with antigen processing (TAP) localized in the ER. TAP is a heterodimer composed of two units, TAP1 and TAP2, both of which are essential and sufficient for peptide translocation (53). On the other hand, the MHC class I heavy chain (HC) is cotranslationally translocated into the ER; it is glycosylated and associates successively with chaperones BIP and calnexin. Toward the end of the folding process, HC binds to  $\beta$ 2-microglobulin ( $\beta$ 2m) and dissociates from calnexin; the newly formed HC- $\beta$ 2m complex is incorporated into an ensemble of accessory molecules composed of TAP, tapasin, calreticulin, Erp57 and the protein disulfide isomerase (54-56). Most HC- $\beta$ 2m complexes are held in association with the loading complexes until they stably assemble with peptide. Once formed, the peptide-MHC class I complexes then leave ER and are transported to the cell surface via the Golgi apparatus to present peptides to CD8<sup>+</sup> T cells. Without the peptide transporter, empty

dimers of HC with  $\beta 2m$  are formed, but these are unstable (57). Excess free peptide would rescue the cell surface expression of MHC class I by stabilizing the new short-lived empty complexes that reach the cell surface. Deficiency in TAP and  $\beta 2m$  genes leads to similar phenotype; impaired peptide association with MHC class I and reduced MHC class I expression on the cell surface (Table 3)(57, 58).

Consequently, TAP1 and  $\beta 2m$  were chosen as target molecules to inhibit the cell surface expression of MHC class I molecules in ES-DC.

**Table 3. Summary of TAP and  $\beta 2$ -microglobulin.**

	<b>TAP</b>	<b><math>\beta 2</math>-microglobulin</b>
<b>Gene locus and Protein length</b>	<b>Human</b> TAP1; 6q21.3, 808 a.a. TAP2; 6q21.3, 686 a.a.	<b>Human</b> 15q21-q22, 119 a.a.
	<b>Mouse</b> TAP1; chromosome 17, 724 a.a. TAP2; chromosome 17, 702 a.a.	<b>Mouse</b> chromosome 2, 119 a.a.
<b>Localization</b>	ER and cis-Golgi	Serum protein
<b>Function</b>	Delivery of peptides across ER membrane to MHC class I molecules.	Noncovalently association with the heavy chain of the polymorphic MHC class I.
<b>Phenotypes of Knockout mice</b>	<ul style="list-style-type: none"> <li>• Reduced MHC class I expression.</li> <li>• Defective antigen presentation.</li> <li>• No CD4-CD8<sup>+</sup> T cells.</li> </ul>	<ul style="list-style-type: none"> <li>• Reduced MHC class I expression.</li> <li>• Defective antigen presentation.</li> <li>• No CD4-CD8<sup>+</sup> T cells.</li> <li>• No functional Fc receptors.</li> </ul>
<b>Disease caused by mutation</b>	Bare lymphocyte syndrome type I	



**FIGURE 3. Overview of the MHC class I pathway of antigen presentation.**

MHC class I binding peptides derived from various sources of endogenous proteins and the peptides are translocated into the ER through TAP. The peptide transport is a multistep process; (a) ATP and peptide binding to TAP, (b) ATP hydrolysis and (c) peptide translocation. TAP most efficiently binds and transports peptides consisting of 8 to 12 amino acids. The binding affinity depends on the three N-terminal and the C-terminal residues. Then, additional processing by ERAAP can occur. The newly synthesized MHC class I heavy chain (HC) initially associates with the chaperone calnexin, which assists its bind folding and binding to β2-microglobulin (β2m) in the ER. The HC-β2m complex then incorporated into the loading complex composed of TAP, tapasin, calreticulin and PDI. The role of the loading complex is to help the binding of HC-β2m complex to high affinity peptides and to stabilize the HC-β2m-peptide complex. The peptide loaded MHC I complexes then leave the ER and are transported to the cell surface.

## 6.5. Aims of this work and experimental systems

The present study addressed the problem of histoincompatibility between patients and the ES-DC by employing the strategy of modification of the genes that control the cell surface expression of MHC class I, i.e.  $\beta$ 2-microglobulin ( $\beta$ 2m) and TAP.

To assess the strategy, an allogeneic mouse system was used (Fig. 4). In the present study, the haplotype of ES-DC was always b while that of the recipient mice (BALB/c) was d. Therefore, there was the histoincompatibility between ES-DC and the recipient mice in all of the experiments.  $TAP1^{-/-}$  and  $\beta 2m^{-/-}$  ES cell clones were generated from the ES cell lines derived from 129 mouse ( $H-2^b$ ) embryo. The expression vectors for  $H-2K^d$  and  $\beta 2m$ -linked form of  $H-2K^d$  ( $\beta 2m-K^d$ ) were then introduced into  $TAP1^{-/-}$  and  $\beta 2m^{-/-}$  ES cell clones, respectively. Subsequently, these genetically modified ES cells were subjected to a differentiation culture in order to generate ES-DC. The MHC class I molecules encoded by the genes in the  $H-2^b$  haplotype were either absent or at very low levels on the cell surface of these genetically modified ES-DC. The effect of the alteration of cell surface expression of MHC class I on activation of alloreactive ( $H-2^b$  haplotype-encoded MHC class I-reactive) CTL was analyzed in both *in vitro* and *in vivo* experiments. After loading ES-DC with the antigenic peptide having a capacity to bind  $H-2K^d$  molecule, these ES-DC were transferred into BALB/c mice ( $H-2^d$  haplotype) to determine whether the peptide-specific,  $H-2K^d$ -restricted CTL could be primed in the recipient mice and whether antigen-specific anti-tumor immunity could be induced.

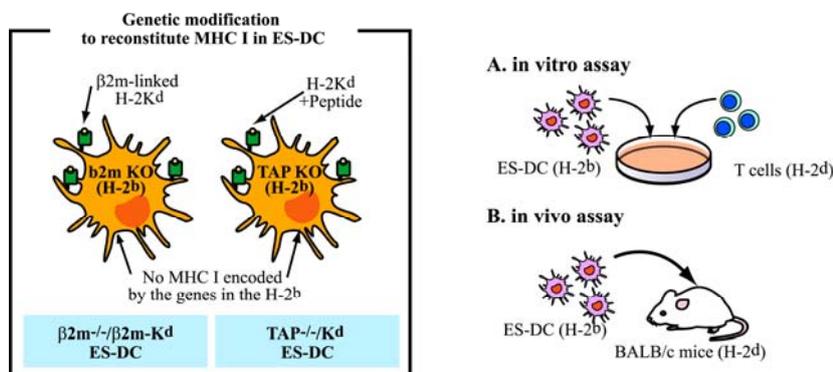


FIGURE 4. Experimental system used in this study.

## **7. Materials and Methods**

### **7.1. Mice**

Six- to eight-week-old female BALB/c and 129/Sv (129) mice were purchased from Japan SLC (Shizuoka, Japan) and Clea Japan (Tokyo, Japan), respectively. The mice were housed at the Center for Animal Resources and Development (CARD, Kumamoto University) under specific pathogen-free conditions. All studies were performed under the approval of the animal experiment committee of Kumamoto University.

### **7.2. Peptides and cell lines**

The respiratory syncytial virus (RSV) M2<sub>82-90</sub> epitope (SYIGSINNI) restricted to H-2K<sup>d</sup> has been described previously (59). H-2K<sup>d</sup>-restricted HIV gag protein-derived p24<sub>199-207</sub> epitope (AMQMLKETI) was used as an irrelevant control peptide (60). The peptides were commercially synthesized and supplied at > 98% purity (Anygen, Gwang-ju, Korea). Murine mastcytoma P815 cells were used as target cells for a <sup>51</sup>Cr release assay. A RSV-M2-transduced colon26/Luc cell line (colon26/M2-Luc) was established by the transfection of murine adenocarcinoma colon26 cells with an Influenza virus Hemagglutinin (HA)-tagged RSV M2 expression vector (pCAG-M2-IRES-neo<sup>R</sup>) and a firefly luciferase expression vector (pCAG-luc-IRES-puro<sup>R</sup>) by electroporation. The electroporation of the cells was performed at 750 μF and 200 V on Gene Pulsar (Bio-Rad, Hercules, CA). After the transfection, G418 and puromycin were added to the culture medium for selection and single clones were obtained by limiting dilution. The expression of firefly luciferase was verified by measuring the luciferase activity in the cell lysates as described below. The expression of the HA-RSV-M2 protein in the selected transfectant clones was confirmed by a flow cytometric analysis following intra-cellular anti-HA staining and also using ELISA detecting IFN-γ-production by M2<sub>82-90</sub> specific K<sup>d</sup>-restricted CTL co-cultured with the transfectants according to the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL).

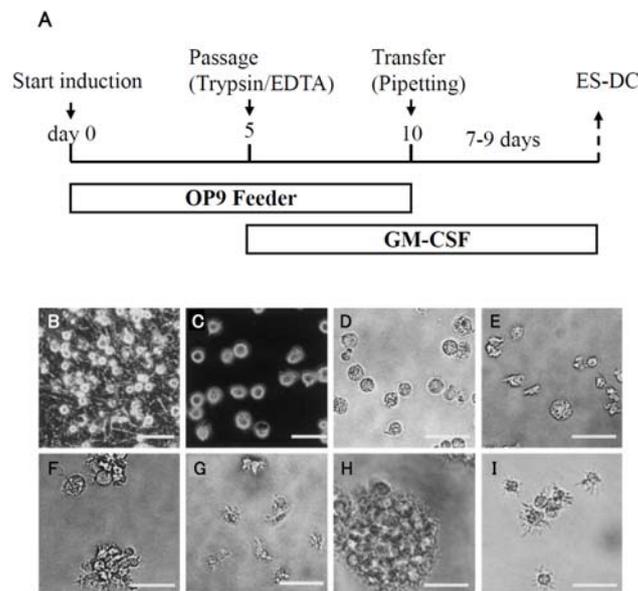
### 7.3. Generation of TAP1- or $\beta$ 2m-deficient ES cells

ES cells were cultured on primary mouse embryonic fibroblast feeder layers in complete ES cell medium, DMEM containing 20% KnockOut Serum Replacement (Gibco-Invitrogen), 2-mercaptoethanol (50  $\mu$ M), and mouse leukemia inhibitory factor (1000 U/ml). A  $\beta$ 2m<sup>+/-</sup> ES cell clone established from D3 cell line derived from a 129 mouse embryo (H-2<sup>b</sup>) was a generous gift from Dr. R. Jaenisch (Massachusetts Institute of Technology, Cambridge, MA) (61). To generate  $\beta$ 2m<sup>-/-</sup> ES cells,  $\beta$ 2m<sup>+/-</sup> ES cells ( $5 \times 10^5$  cells/ 90-mm culture dish) were cultured on feeder layers of neomycin-resistant primary embryonic fibroblast derived from GTPBP1<sup>-/-</sup> mouse embryos (62) in ES cell medium containing high dose G418 (1.5-2.0 mg/ml) for 10 days (63). After a further culture for 7 days without G418, the surviving ES cell colonies were picked up from the dishes, transferred to 24-well culture plates, and then expanded. For each isolated ES cell clones, a part of the expanded cells were cultured in gelatin-coated 6-well plates without PEF feeders. Genomic DNA extracted from the feeder-free ES cells was used for genotyping of the  $\beta$ 2m locus by genomic PCR and  $\beta$ 2m<sup>-/-</sup> ES clones were selected. TAP1<sup>+/-</sup> ES cells were generated from E14 ES cells derived from 129 mouse embryo (H-2<sup>b</sup>). E14 cells were transfected with 30  $\mu$ g of linearized targeting vector by electroporation (64). The electroporation of the cells was performed at 750  $\mu$ F and 200 V on Gene Pulsar. G418 and ganciclovir were added to the culture medium 24 h after the transfection and the surviving colonies were isolated during days 7-10 of selection. The isolated clones were analyzed by PCR and Southern blotting to identify cell clones with homologous recombination. Subsequently, one of the TAP1<sup>+/-</sup> clones was subjected to selection with high dose of G418 as in the case of  $\beta$ 2m<sup>-/-</sup> ES cells. The clones were expanded and analyzed by Southern blotting to select TAP1<sup>-/-</sup> clones. Expression vectors for H-2K<sup>d</sup> and  $\beta$ 2m-linked form of H-2K<sup>d</sup> ( $\beta$ 2m-K<sup>d</sup>) were introduced into TAP1<sup>-/-</sup> and  $\beta$ 2m<sup>-/-</sup> ES cell clones, respectively.

### 7.4. Induction of differentiation of ES cells into DC

The procedure for induction of differentiation of ES cells into DCs is shown in Figure 5 (44). ES cells were suspended in  $\alpha$ -MEM supplemented with 20% FCS and seeded ( $1.5 \times 10^4$  cells/2 mL medium/well) onto OP9 cell layers in 6-well plates.

On day 3, half of the medium was removed and 2 mL fresh medium was added to each well. On day 5, cells were harvested using phosphate-buffered saline (PBS)/0.25% trypsin/1 mM EDTA (ethylenediaminetetraacetic acid), reseeded onto fresh OP9 cell layers, and cultured in  $\alpha$ -MEM supplemented with 20% FCS and GM-CSF (1000 U/mL). At this step, cells recovered from 3 wells of 6-well culture plates were suspended in 20 mL medium and seeded into one 150-mm dish. On day 10 (5 days after the transfer), floating cells were recovered by pipetting. On average, 4 to  $8 \times 10^6$  cells were recovered from one 150-mm dish, thus indicating 100 to 200 times increase in cell number from undifferentiated ES cells. The recovered cells were transferred to bacteriologic Petri dishes ( $2.5 \times 10^5$  cells/90-mm dish) without feeder cells, and cultured in RPMI-1640 medium supplemented with 10% FCS, GM-CSF (500 U/mL), and 2-mercaptoethanol. On day 17-19 of cultures, the floating or loosely adherent cells were recovered from culture dishes by pipeting and then were used for the experiments.



**FIGURE 5. Differentiation and morphology of DC from ES cells.**

(A) Overview of the culture protocol for generation of DC from ES cells. (B-I) ES-DC on day 8 (B), day 12 (C, D), day 17 (E, F) and day 27 (G) of differentiation culture are shown. Cells on day 24 were recovered and stimulated for 2 days with IL-4, TNF- $\alpha$  plus agonistic anti-CD40 mAb (H) or with IL-4, TNF- $\alpha$  plus LPS (I), as described in the materials and methods. Panel (B) and (C) are phase contrast micrographs. Scale bars represent 20  $\mu$ m.

## **7.5. PCR**

The initial screening of ES cells was done by PCR using the following primers: the wild type TAP1 allele (5'-ATGGGACACATGCACGGC-3' and 5'-CCACAGTAGCAGGCTCAG-3'), the mutant TAP1 allele (5'-TGTAGC-TTTGGCTCTTCTGGAA-3' and 5'-GGGCCAGCTCATTCTCCACTC-3'),  $\beta$ 2m (5'-CCTCAGAAACCCCTCAAATTCAAG-3' and 5'-GCTTACCCCAGTAGACG-GTCTTGG-3'). The set of primers for  $\beta$ 2m was designed to amplify both the wild type and targeted loci. PCR amplifications of TAP1 and  $\beta$ 2m genes were carried out for 30 cycles with annealing temperatures of 65°C and 55°C, respectively.

## **7.6. Southern blot analysis**

To analyze the genotype of ES cells, genomic DNA isolated from ES cells was digested with Xho I and EcoR V. The DNA fragments were separated by electrophoresis in 0.8% agarose gels. Subsequently, the DNA was transferred onto nylon membranes. Probes for the Southern blot analysis were obtained by PCR with sets of primers for TAP1 locus (5'-GACCAGACTCTGGACAGCTCAC-3' and 5'-AAGGCAAGAGAGAATCAAGAG-3') from the genomic DNA of ES cells. Labeling of probe DNA with  $^{32}\text{P}$ -dCTP was done by using a Megaprime DNA Labeling Kit (GE healthcare, Buckinghamshire, UK) and the standard Southern blot procedure was carried out. Hybridization with this probe resulted in a 3.4-kbp band from the wild-type locus and a 2.5-kbp band from the mutant locus.

## **7.7. Antibodies and flow cytometric analysis**

FITC-conjugated anti-H-2K<sup>d</sup> (BD Pharmingen), H-2K<sup>b</sup> (Caltag Laboratories) and H-2D<sup>b</sup> (Caltag Laboratories) antibodies were purchased from the indicated sources. A flow cytometric analysis was done on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) and the data analysis was performed using the CellQuest software program (Becton-Dickinson).

## **7.8. Preparation of BM-DC**

Bone marrow cells prepared from BALB/c or 129 mice were cultured in RPMI-1640 medium supplemented with 10% FCS, 500 U/ml GM-CSF and 50  $\mu\text{M}$

2-mercaptoethanol. On day 7 of the culture, the cells were recovered and used as bone marrow-derived DC (BM-DC) for the experiments.

### **7.9. Analysis of stimulation of BALB/c -derived H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cell lines by DC**

To generate BALB/c (H-2<sup>d</sup>)-derived H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cell lines, 5 x 10<sup>6</sup> BALB/c spleen cells were cultured with 2 x 10<sup>6</sup> irradiated 129 (H-2<sup>b</sup>) spleen cells in 2 ml of RPMI-1640 medium supplemented with 10% FCS, 100 U/ml IL-2 and 50 μM 2-mercaptoethanol in a well of 24-well plates for 5 days (65) and after that CD8<sup>+</sup> T cells were isolated by using anti-CD8 magnetic beads (Miltenyi Biotech). The H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cells (1 x 10<sup>3</sup>) were cultured with the indicated stimulator DC (5 x 10<sup>3</sup>) for 16 h and the activation of T cells was detected by IFN-γ-production by using ELISPOT (BD bioscience). For the analysis of the priming of alloreactive (H-2<sup>b</sup>-reactive) CD8<sup>+</sup> T cells by ES-DC, BALB/c spleen cells (5 x 10<sup>6</sup>) were cultured with irradiated ES-DC (2 x 10<sup>6</sup>) for 5 days and then CD8<sup>+</sup> T cells were isolated as described above. The magnitude of priming of H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cells was analyzed by IFN-γ-production detected by ELISPOT upon co-culture with 129 mice-derived BM-DC as stimulators.

### **7.10. Analysis of frequency of alloreactive CTL in ES-DC-injected mice**

Spleen cells were isolated from naïve BALB/c mice or those that received multiple ES-DC injections and CD8<sup>+</sup> T cells were isolated by using anti-CD8 magnetic beads (Miltenyi Biotech). To analyze the frequency of auto (H-2<sup>d</sup>)- or allo (H-2<sup>b</sup>)-reactive CD8<sup>+</sup> T cells, the cells (5 x 10<sup>3</sup>) were co-cultured with BALB/c or 129 BM-DC (5 x 10<sup>3</sup>) for 16 h and IFN-γ producing cells were detected using the ELISPOT assay.

### **7.11. Detection of in vivo administered ES-DC in the draining lymph nodes**

The in vivo elimination of ES-DC was assessed according to the reported procedures with some modification (66). ES-DC were labeled with the 10 μM chloromethyl-benzoyl-amino-tetramethyl-rhodamine (CMTMR; Molecular Probes, Eugene, OR) and BALB/c BM-DC were labeled with 10 μM chloromethylfluorescein

diacetate (CMFDA; Molecular Probes) according to the manufacturer's instructions. The mice were injected s.c. in the forelimb with  $2 \times 10^6$  cells containing equal numbers of CMTMR-labeled ES-DC and CMFDA-labeled BM-DC. After 48 h, the draining axillary and brachial lymph nodes were removed, digested with 2.4 mg/ml collagenase type II (Gibco-Invitrogen) and DNase I (Sigma) for 90 min at 37°C. Lymph nodes were disrupted by repeated aspiration through a 18-gauge needle, sieved through gauze, washed in PBS, and resuspended in PBS containing 2% FCS and 0.01% sodium azide. Lymph node cell suspensions were analyzed by flow cytometry. The region containing DC was identified on the basis of FSC-SSC profile. The number of ES-DC was normalized to control syngeneic BALB/c BM-DC.

#### **7.12. Priming of RSV-M2 specific CD8<sup>+</sup> T cells by ES-DC**

ES-DC were incubated with RSV-M2<sub>82-90</sub> peptide (10  $\mu$ M) for 3 h and then washed three times with FCS-free DMEM. Antigen-loaded ES-DC were injected intraperitoneally ( $1 \times 10^5$  cells/injection/mouse) into the mice twice, with a 7-day interval. In some experiments, non-antigen-loaded ES-DC were injected five or ten times with 7-day intervals before the injection of antigen-loaded ES-DC. Seven days after the last injection of ES-DC, the mice were sacrificed and the spleen cells were isolated. After hemolysis, the spleen cells were cultured in RPMI-1640/10% horse serum/2-mercaptoethanol (50  $\mu$ M) containing M2<sub>82-90</sub> peptide (1  $\mu$ M) 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, P815 cells were labeled with sodium [<sup>51</sup>Cr]chromate for 1 h and washed. Subsequently, target cells were incubated in 24-well culture plates ( $1 \times 10^6$  cells/well) with or without 10  $\mu$ M M2<sub>82-90</sub> peptide for 3 h, washed, and seeded into 96-well round-bottomed culture plates ( $5 \times 10^3$  cells/well). The effector cells were added to the target cells according to the indicated E/T ratio and incubated for 4h at 37 °C. At the end of the incubation, supernatants (50  $\mu$ l/well) were harvested and counted on a gamma counter. The percentage of specific lysis was calculated as:  $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})]$ . The spontaneous release and maximal release were determined in the presence of medium alone and PBS-1% Triton X-100, respectively.

### **7.13. Tumor challenge experiments**

The mice were immunized with ES-DC and, 7 days after the immunization, colon26/M2-Luc ( $1 \times 10^6$ /mouse) cells were injected into the mice intraperitoneally. Ten days later, the mice were sacrificed and the luciferase activity of the lysates of the abdominal organs was measured to quantify tumor growth. The tissue specimens were homogenized in 3 ml of lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris pH 7.8) and the homogenates were cleared by centrifugation at 10,000 g for 5 min. Fifty  $\mu$ l of the supernatant was mixed with 50  $\mu$ l of dilution buffer (PBS containing 2.4 mM  $\text{CaCl}_2$  and 0.82 mM  $\text{MgSO}_4$ ) and 100  $\mu$ l of luciferase assay buffer (Steadyliteplus, PerkinElmer) and at 5 min after the mixing the light produced was measured for one second in a luminometer (Tristar LB941, Berthold Technologies).

### **7.14. Statistical analysis**

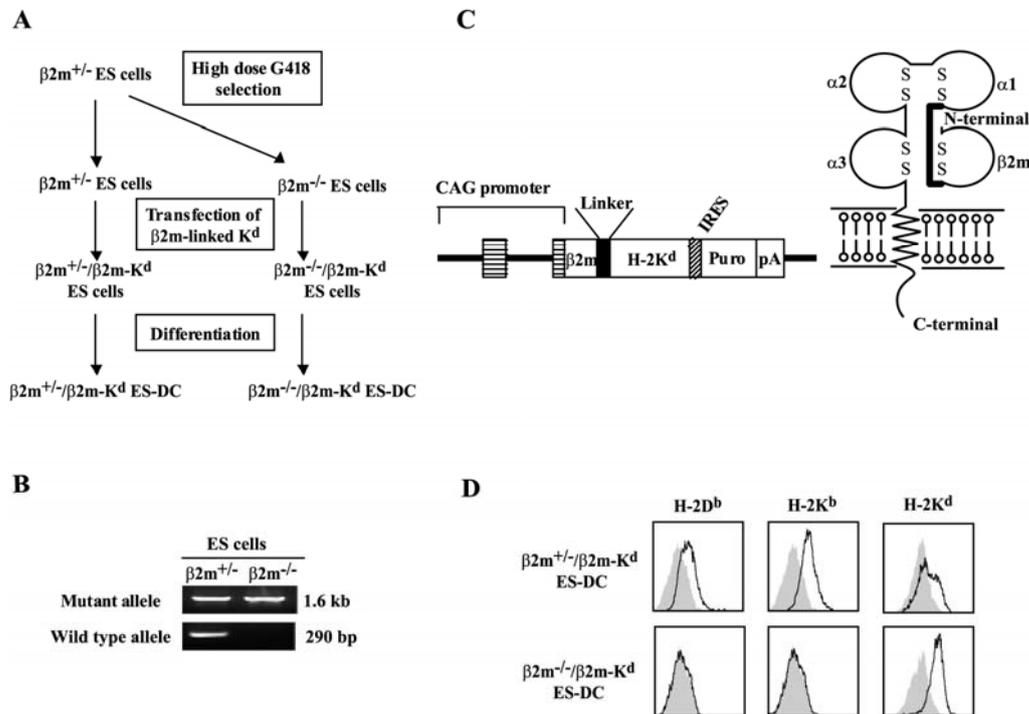
Student's t-test was used for the statistical analysis of data except for the data regarding the tumor invasion experiments. Because some of data in the tumor invasion experiments did not follow a normal distribution, the data were analyzed using the Mann-Whitney U test, a nonparametric test. A value of  $P < 0.05$  was considered significant.

## 8. Results

### 8.1. Generation of ES-DC expressing recipient-matched MHC class I but not intrinsic MHC class I.

The present study evaluated a strategy to prime antigen-specific CTL by transfer of genetically engineered 129 (H-2<sup>b</sup>)-derived ES-DC into BALB/c (H-2<sup>d</sup>) recipient mice, thus avoiding the recognition of ES-DC by allo (H-2<sup>b</sup>)-reactive CD8<sup>+</sup> T cells. To modify the cell-surface expression of MHC class I, two strategies were tested in parallel; 1) disruption of the  $\beta 2m$  gene in ES cells and introduction of  $\beta 2m$ -linked form of recipient-matched MHC class I ( $\beta 2m$ -K<sup>d</sup>) (Fig. 6A), 2) disruption of TAP1 gene, introduction of recipient-matched MHC class I (K<sup>d</sup>) and loading of K<sup>d</sup>-binding epitopes to the ES-DC (Fig. 7A).

$\beta 2m^{-/-}$  ES cells were generated by the selection of the previously established  $\beta 2m^{+/-}$  ES cells with high dose (1.5-2 mg/ml) of G418. Two out of the 39 clones were found to have a  $\beta 2m^{-/-}$  genotype by genomic PCR (Fig. 6B). Subsequently, an expression vector for  $\beta 2m$ -K<sup>d</sup> (Fig. 6C) was introduced into both  $\beta 2m^{+/-}$  and  $\beta 2m^{-/-}$  ES cells to generate  $\beta 2m^{+/-}/\beta 2m$ -K<sup>d</sup> and  $\beta 2m^{-/-}/\beta 2m$ -K<sup>d</sup> ES cells, respectively. The genetically modified ES cell clones were subjected to differentiation culture to generate ES-DC. Theoretically,  $\beta 2m$ -K<sup>d</sup> is the only MHC class I molecule expressed on the cell surface of  $\beta 2m^{-/-}/\beta 2m$ -K<sup>d</sup> ES-DC. In the flow cytometric analysis, the cell surface expression of MHC class I molecules of H-2<sup>b</sup> haplotype, H-2D<sup>b</sup> and K<sup>b</sup>, was detected in  $\beta 2m^{+/-}/\beta 2m$ -K<sup>d</sup> ES-DC but not in  $\beta 2m^{-/-}/\beta 2m$ -K<sup>d</sup> ES-DC. Expression of K<sup>d</sup> was detected in the both types of ES-DC, as expected (Fig. 6D).

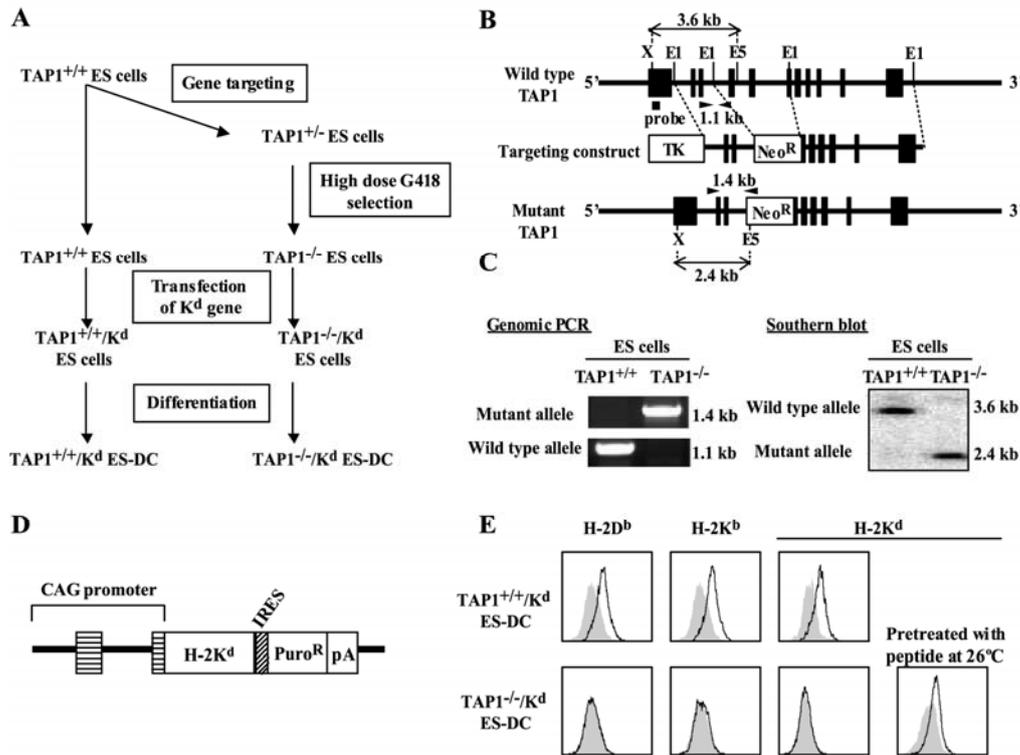


**FIGURE 6. Generation of ES-DC deficient in the intrinsic  $\beta 2m$  gene and expressing recipient-matched MHC class I linked to  $\beta 2m$ .**

(A) Overview of the method for generation of ES-DC expressing  $\beta 2m$ -linked  $K^d$ ,  $\beta 2m^{+/-}/\beta 2m-K^d$  ES-DC and  $\beta 2m^{-/-}/\beta 2m-K^d$  ES-DC.  $\beta 2m^{-/-}$  ES cells were generated from  $\beta 2m^{+/-}$  ES cells by high dose G418 selection.  $\beta 2m^{+/-}$  and  $\beta 2m^{-/-}$  ES cells were introduced with cDNA for the  $\beta 2m$ -linked form of  $K^d$  and subsequently differentiated to generate  $\beta 2m^{+/-}/\beta 2m-K^d$  ES-DC and  $\beta 2m^{-/-}/\beta 2m-K^d$  ES-DC. (B) The absence of wild type  $\beta 2m$  gene in the  $\beta 2m^{-/-}$  ES cell clone was confirmed by genomic PCR. (C) Structure of the expression vector for  $\beta 2m$ -linked H-2K<sup>d</sup> (left) and a schematic representation of the encoded molecule (right). The  $\beta 2m$  was fused to H-2K<sup>d</sup> via a flexible 15 amino acid-long linker ([Gly<sub>4</sub>-Ser]<sub>3</sub>). The vector is driven by CAG promoter (pCAG) and cDNA for  $\beta 2m$ -linked H-2K<sup>d</sup> are followed by the IRES-puromycin-resistance gene (Puro<sup>R</sup>)-polyadenylation signal sequence (pA). (D) Analysis of the cell surface expression of MHC class I on ES-DC by flow cytometry. The staining patterns with specific antibodies (thick lines) and isotype-matched controls (gray) are shown.

To mutate the TAP1 gene in ES cells, the targeting vector (Fig. 7B) was introduced into E14 ES cells to make several TAP1<sup>+/-</sup> ES cell clones. Subsequently, one of the TAP1<sup>+/-</sup> clones was subjected to selection with high dose of G418 as in the case of  $\beta$ 2m<sup>-/-</sup> ES cells and TAP1<sup>-/-</sup> ES cell clones were isolated. Eight of the 88 surviving clones were found to be of TAP1<sup>-/-</sup> genotype by genomic PCR (Fig. 7C, left) and Southern blotting (Fig. 7C, right). Next, an expression vector for K<sup>d</sup> (Fig. 7D) was introduced into both TAP1<sup>+/+</sup> and TAP1<sup>-/-</sup> ES cells to generate TAP1<sup>+/+</sup>/K<sup>d</sup> and TAP1<sup>-/-</sup>/K<sup>d</sup> ES cells, respectively. Intrinsic MHC class I molecules, D<sup>b</sup> and K<sup>b</sup>, as well as transgene-derived K<sup>d</sup>, were not detected on the cell surface of TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC (Fig. 7E). A low level of cell surface expression of K<sup>d</sup> on TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC was observed after incubation of the ES-DC with K<sup>d</sup>-binding peptide (RSV-M2<sub>82-90</sub>) at 26°C for 12 h followed by the incubation at 37°C for 4 h (Fig. 7E).

Collectively, ES-DC expressing only transgene-derived K<sup>d</sup> but not intrinsic (H-2<sup>b</sup> haplotype-derived) MHC class I molecules on the cell surface were generated by the 2 methods of genetic modifications of ES cells.

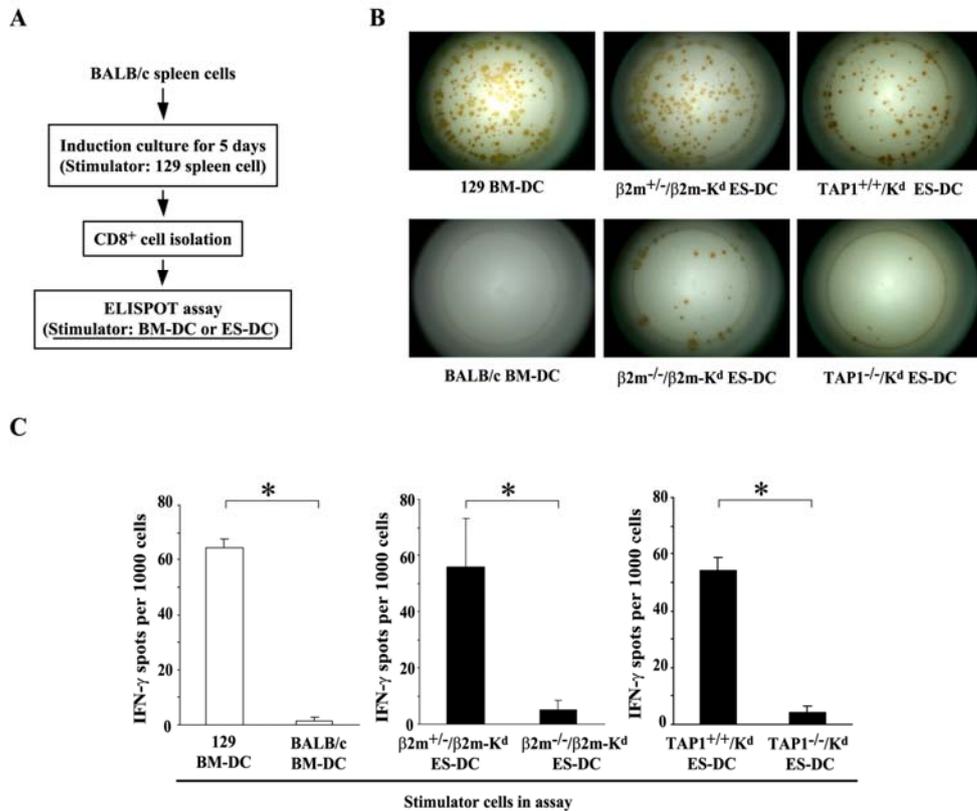


**FIGURE 7. Generation of TAP1-deficient, recipient-matched MHC class I gene-introduced ES-DC.**

(A) Overview of the method for generation of  $K^d$  expressing, TAP1-deficient ES-DC. TAP1<sup>+/+</sup> and TAP1<sup>-/-</sup> ES cells were transfected with cDNA for  $K^d$  and subsequently induced to differentiate into ES-DC. (B) Structure of the mouse TAP1 genomic locus, the targeting construct and the mutant allele. Closed boxes indicate exons. The targeting construct contains a neomycin resistant gene (Neo<sup>R</sup>) and herpesvirus thymidine kinase gene (TK). The sets of PCR primers for the wild type allele and mutant allele are shown as arrow heads. The position of the probe used for the Southern blot analysis is indicated as a small black box. The sizes of bands generated from the wild type and mutant allele by digestion with Xho I (X) and EcoR V (E5) are indicated. The EcoR I (E1) restriction site is also shown. (C) A genotype analysis of ES cells. Genomic DNA from ES cells was analyzed by genomic PCR and Southern blotting. (D) Structure of the H-2K<sup>d</sup> expression construct. The vector is driven by a CAG promoter (pCAG) and cDNA for H-2K<sup>d</sup> are followed by the IRES-puromycin-resistance gene (Puro<sup>R</sup>)-polyadenylation signal sequence (pA). (E) Surface phenotypes of genetically modified ES-DC. The surface expression of indicated MHC class I molecules on ES-DC were analyzed using flow cytometry. The expression of cell surface H-2K<sup>d</sup> by TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC were detected after cells were incubated with 10  $\mu$ M H-2K<sup>d</sup>-binding peptide (RSV M2<sub>82-90</sub>) at 26°C for 12 h, subsequently incubated at 37°C for 4 h and stained with anti-H-2K<sup>d</sup> antibody. The staining patterns with specific antibodies (thick lines) and isotype-matched controls (gray) are shown.

## 8.2. Avoidance of recognition by alloreactive CD8<sup>+</sup> T cells by genetic modification of ES-DC

H-2<sup>b</sup> MHC class I-reactive CD8<sup>+</sup> T cells were prepared by a 5-day-culture of BALB/c (H-2<sup>d</sup>) spleen cells with irradiated 129 (H-2<sup>b</sup>) spleen cells. They produced IFN- $\gamma$  in response to 129 mouse-derived BM-DC but not against BALB/c BM-DC, confirming that they responded specifically to H-2<sup>b</sup> MHC class I (Fig. 8C, left panel). The four types of ES-DC derived from H-2<sup>b</sup> ES cells with genetic modification described above were co-cultured with the H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cell line and the activation of the T cells was analyzed (Fig. 8). Figure 8C shows that  $\beta 2m^{+/-}/\beta 2m-K^d$  ES-DC (middle panel) and TAP1<sup>+/+</sup>/K<sup>d</sup> ES-DC (right panel), expressing MHC class I of H-2<sup>b</sup> haplotype along with K<sup>d</sup>, were recognized by the H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cells, thus resulting in IFN- $\gamma$  production at the magnitude similar to that observed in the case of 129 BM-DC. On the other hand, H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cells showed practically no response to  $\beta 2m^{-/-}/\beta 2m-K^d$  ES-DC or TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC. These results indicate that ES-DC that were not recognized by alloreactive CD8<sup>+</sup> T cells could be generated by the modification of  $\beta 2m$  or TAP1 gene to inhibit surface expression of intrinsic MHC class I molecules.

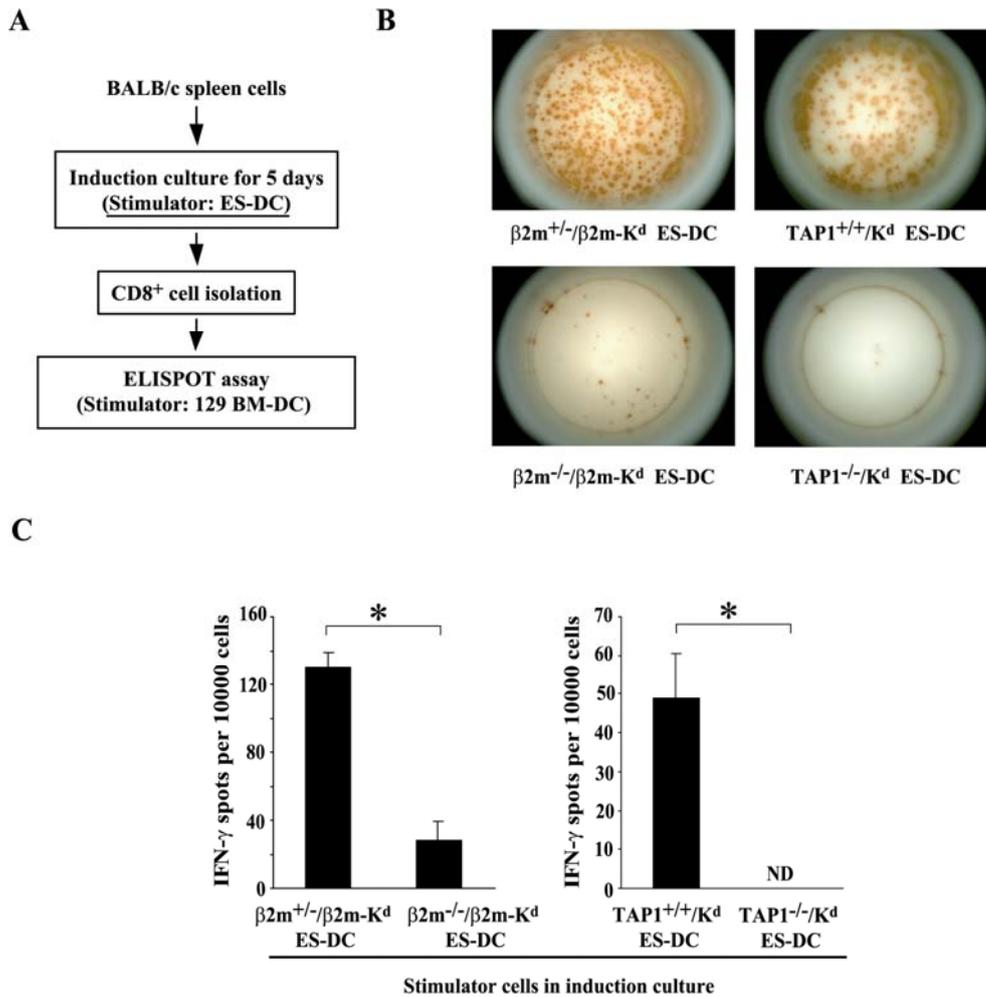


**FIGURE 8. Allogeneic MHC class I-deficient ES-DC avoided the recognition by alloreactive CD8<sup>+</sup> T cells *in vitro*.**

(A) Experimental system. To generate alloreactive (anti-H-2<sup>b</sup>) CTL,  $5 \times 10^6$  BALB/c (H-2<sup>d</sup>) spleen cells were cultured with  $2 \times 10^6$  irradiated 129 (H-2<sup>b</sup>) spleen cells for 5 days and after that the CD8<sup>+</sup> cells were purified from the culture. The alloreactive CD8<sup>+</sup> T cells ( $1 \times 10^3$ ) were cultured with the ES-DC ( $5 \times 10^3$ ) of the indicated genotype for 16 h. 129 or BALB/c BM-DC were used as additional controls. The number of IFN- $\gamma$  producing cells was measured using an ELISPOT assay. (B) Representative images of IFN- $\gamma$  ELISPOT wells. (C) Data are representative of at least two experiments with similar results. The data are the mean  $\pm$  SD of triplicate assays. The asterisks indicate significant ( $P < 0.05$ , Student's *t*-test) differences between the two groups. ND, not detectable.

### **8.3. Reduced priming of H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cells in vitro by genetically modified ES-DC**

Next, the in vitro priming of alloreactive CD8<sup>+</sup> T cells by ES-DC was examined. Spleen cells from BALB/c mice were co-cultured with either of the 4 types of genetically engineered ES-DC for 5 days. After that, CD8<sup>+</sup> T cells were isolated from the culture and their reactivity to 129-derived BM-DC (Fig. 9) was measured to assess the magnitude of priming in vitro of H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cells by the ES-DC. CD8<sup>+</sup> T cells cultured with  $\beta 2m^{+/-}/\beta 2m-K^d$  or TAP1<sup>+/+</sup>/K<sup>d</sup> ES-DC in the induction phase responded to 129-derived BM-DC, indicating that both  $\beta 2m^{+/-}/\beta 2m-K^d$  and TAP1<sup>+/+</sup>/K<sup>d</sup> ES-DC primed H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cells. In contrast, CD8<sup>+</sup> T cells cultured with  $\beta 2m^{-/-}/\beta 2m-K^d$  or TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC in the induction step exhibited reduced or no response to 129-derived BM-DC, thus indicating that the H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cells had not been well primed. These results suggest that the in vitro priming of allo-MHC class I-reactive T cells by ES-DC can be reduced through the genetic modification of  $\beta 2m$  or TAP1.



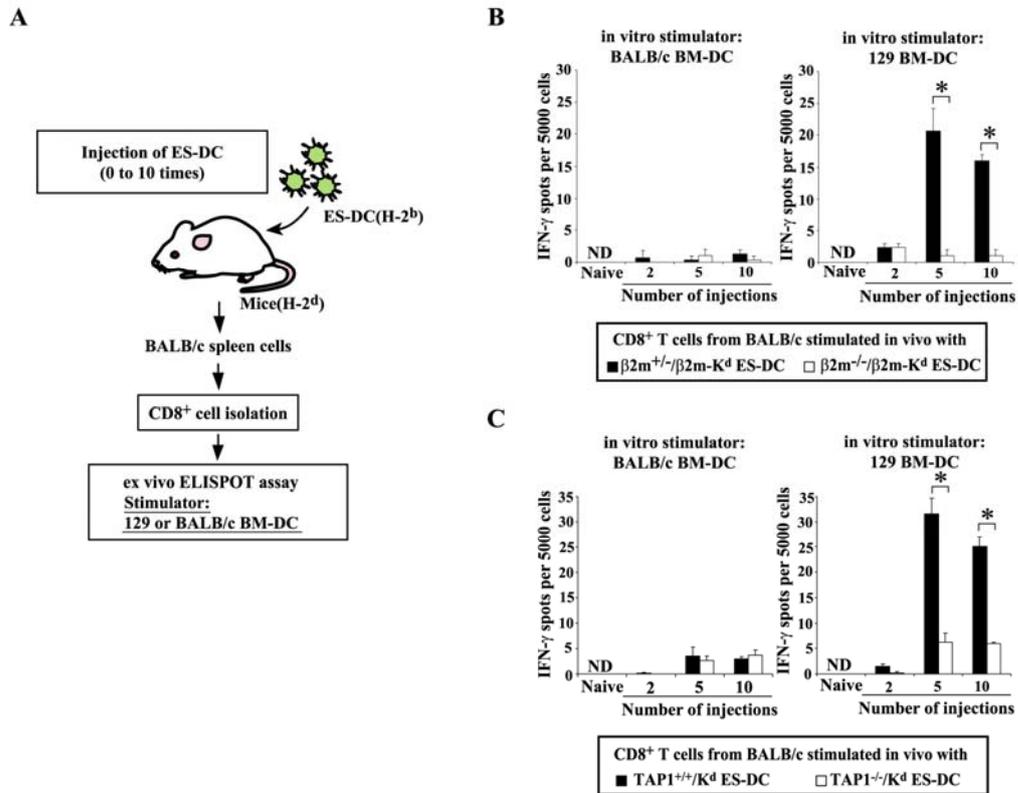
**FIGURE 9. Allogeneic MHC class I-deficient ES-DC showed reduced potency to prime alloreactive CD8<sup>+</sup> T cells *in vitro*.**

(A) Experimental system. Splenocytes ( $5 \times 10^6$ ) derived from BALB/c mice were cultured with irradiated ES-DC ( $2 \times 10^6$ ) of the indicated genotype for 5 days and after that CD8<sup>+</sup> T cells were purified from the culture. The IFN- $\gamma$  producing response of the CD8<sup>+</sup> T cells was measured with an ELISPOT with 129 BM-DC as stimulators. (B) Representative images of IFN- $\gamma$  ELISPOT wells. (C) The results of the experiments with  $\beta 2m$  deficient ES-DC (left panel) and TAP1 deficient ES-DC (right panel) are shown. Data are representative of at least two experiments with similar results. The data are the mean  $\pm$  SD of triplicate assays. The asterisks indicate significant ( $P < 0.05$ , Student's *t*-test) differences between the two groups. ND, not detectable.

#### **8.4. Reduced priming of allo-MHC class I-reactive T cells in vivo by TAP1 or $\beta 2m$ -deficient ES-DC**

The next experiments assessed whether or not priming of alloreactive CD8<sup>+</sup> T cells upon in vivo administration of ES-DC could be avoided by the current strategy. The frequency of primed H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cells in mice was quantified by an ex vivo ELISPOT assay detecting the production of IFN- $\gamma$  upon stimulation with 129-derived BM-DC (Fig. 10A).

CD8<sup>+</sup> T cells from naïve BALB/c mice showed little response to 129-derived BM-DC. CD8<sup>+</sup> T cells isolated from BALB/c mice injected 5 or 10 times with  $\beta 2m^{+/-}/\beta 2m-K^d$  (Fig. 10B) or TAP1<sup>+/+</sup>/K<sup>d</sup> ES-DC (Fig.10C) clearly responded, thus indicating the priming of H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cells. The magnitude of the response of the mice injected 10 times was lower than those injected 5 times, in both the  $\beta 2m^{+/-}/\beta 2m-K^d$  ES-DC and TAP1<sup>+/+</sup>/K<sup>d</sup> ES-DC-injected mice, thus suggesting that there is probably a limit in the frequency of alloreactive CD8<sup>+</sup> T cells. On the other hand, the frequency of H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cells in mice inoculated with  $\beta 2m^{-/-}/\beta 2m-K^d$  or TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC was very low, indicating that alloreactive CD8<sup>+</sup> T cells were hardly primed in vivo by these ES-DC.



**FIGURE 10. Reduced *in vivo* stimulation of alloreactive CD8<sup>+</sup> T cells by  $\beta 2m$  or TAP1 gene-modified ES-DC.**

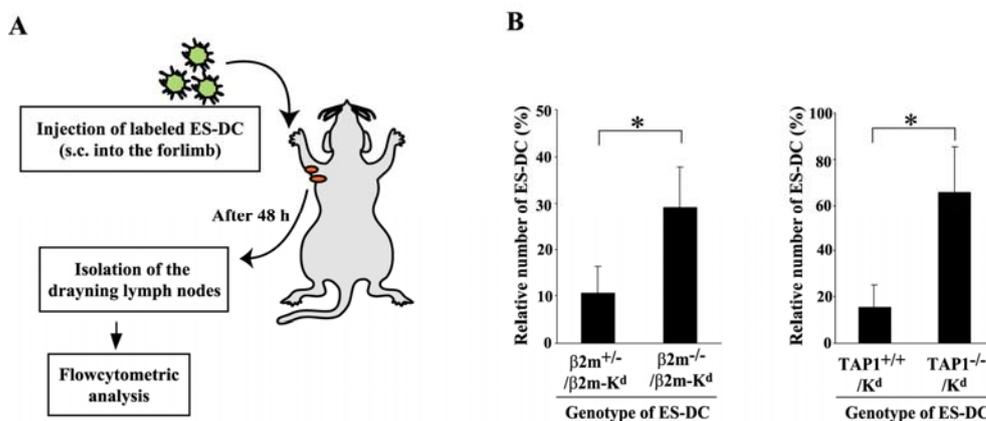
(A) Experimental system. (B and C) Splenic CD8<sup>+</sup> T cells were isolated from naïve BALB/c mice or those received multiple injections of  $\beta 2m$  deficient (B) or TAP1 deficient (C) ES-DC ( $1 \times 10^5$  cells/injection). Isolated CD8<sup>+</sup> T cells ( $5 \times 10^3$ ) were cultured with BALB/c BM-DC (left panels) or 129 BM-DC (right panels) ( $5 \times 10^3$ ) for 16 h. The numbers of IFN- $\gamma$  producing cells were measured using an ELISPOT assay. Data are representative of at least two experiments with similar results. The data are the mean  $\pm$  SD of triplicate assays. Asterisks indicate significant ( $P < 0.05$ , Student's *t*-test) differences. ND, not detectable.

### 8.5. Surviving advantage of $\beta 2m$ or TAP1 gene-modified ES-DC in vivo

According to a previous study by another group (50), BM-DC inoculated into allogeneic recipient mice are eliminated within a few days and the number of DC detected in the draining lymph node is lower than that of DC syngeneic to the recipient mice. In such circumstances, the rapid elimination of APC is mainly mediated by CD8<sup>+</sup> T cells reactive to allogeneic MHC class I (51). As described so far, the  $\beta 2m^{-/-}/\beta 2m-K^d$  ES-DC and TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC did not express intrinsic

H-2<sup>b</sup>-derived MHC class I molecule and escaped recognition by H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cells. Therefore, they were expected to have an advantage in surviving in allogeneic BALB/c mice, in comparison to ES-DC expressing H-2<sup>b</sup> gene encoded MHC on the cell surface.

To examine the effect of genetic modification on the survival of ES-DC upon injection into allogeneic mice, equal numbers of CMTMR-labeled ES-DC and CMFDA-labeled BALB/c derived BM-DC, as a control, were mixed and injected in the right forelimb footpad of BALB/c mice. After 48 h, a single cell suspension was made from the axillary and brachial lymph nodes and the fluorochrome-labeled DC were detected by using flow cytometry (Fig. 11A). The number of  $\beta 2m^{-/-}/\beta 2m-K^d$  ES-DC in the draining lymph nodes was about 3 times higher than that of  $\beta 2m^{+/-}/\beta 2m-K^d$  ES-DC (Fig. 11B). In the similar experiments, the number of detected TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC was about 4 times higher than that of TAP1<sup>+/+</sup>/K<sup>d</sup> ES-DC (Fig. 11C). These results suggest that ES-DC without cell surface expression of intrinsic MHC class I molecule can thus escape elimination by alloreactive CTL.



**FIGURE 11. The survival advantage of  $\beta 2m$  or TAP1 gene-deficient ES-DC in allogeneic recipient mice.**

(A) Experimental system. BALB/c mice were injected s.c. into the forelimb with CMFDA-labeled BALB/c BM-DC ( $1 \times 10^6$ ) and CMTMR-labeled ES-DC ( $1 \times 10^6$ ). After 48 h the mice were killed and the number of labeled BM-DC and ES-DC in the axillary and brachial lymph nodes was determined by flow cytometry. The number of detected ES-DC was normalized to control syngeneic BALB/c BM-DC [(CMTMR<sup>+</sup> ES-DC/CMFDA<sup>+</sup> BM-DC) x 100].

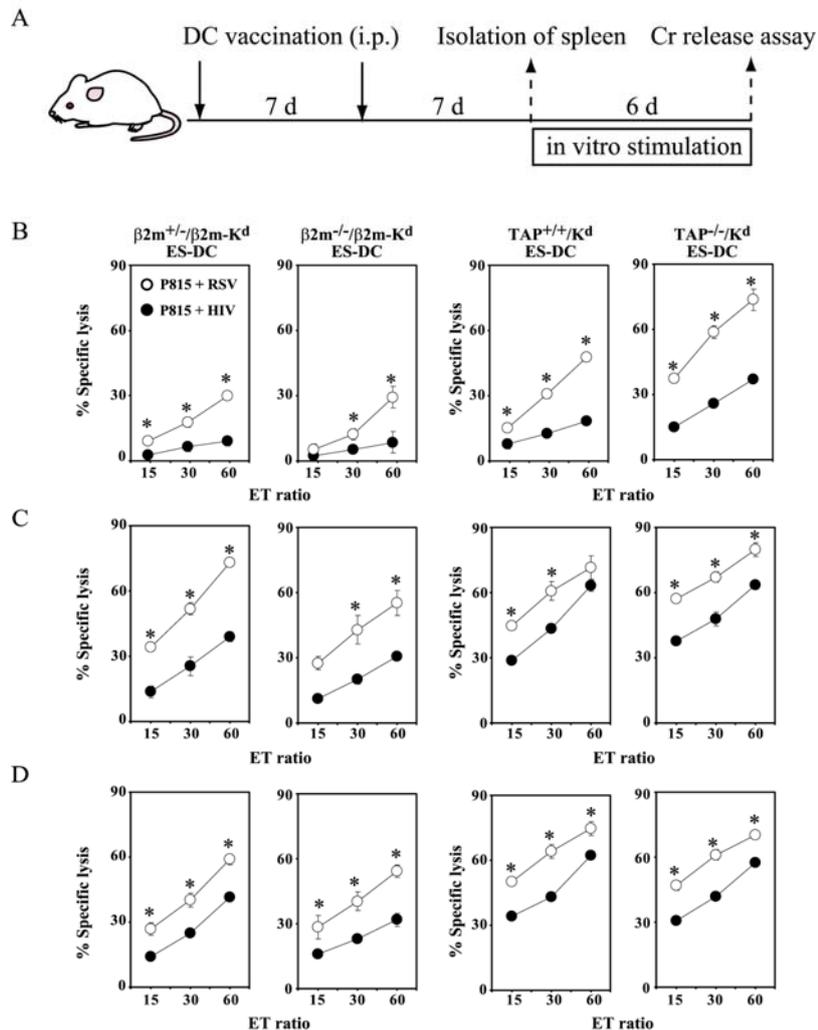
(Figure 11 legend continued) (B) The results of experiments with  $\beta$ 2m-deficient ES-DC (left) and TAP1-deficient ES-DC (right) shown. Data are representative of two experiments with similar results. The data are the mean  $\pm$  SD (5-6 mice per each group). Asterisks indicate significant ( $P < 0.05$ , Student's *t*-test) differences.

### **8.6. Priming of antigen-specific CTL by genetically modified ES-DC in allogeneic recipients.**

It has been noted that the CTL-mediated elimination of DC has a notable effect on the magnitude of immune responses in vivo (67) and the results so far described indicate that the  $\beta$ 2m or TAP1 gene-modified ES-DC expressing only recipient-matched MHC class I could thus avoid elimination by CTL upon transfer into allogeneic recipients. Therefore, the ability of such ES-DC to elicit more robust antigen-specific immune responses in allogeneic recipients than ES-DC expressing intrinsic MHC class I was examined.

The priming of a RSV M2 protein epitope (M2<sub>82-90</sub>)-specific and H-2K<sup>d</sup>-restricted CTL by ES-DC administered into BALB/c mice was examined. M2<sub>82-90</sub> peptide-loaded ES-DC were injected i.p. into BALB/c 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 M2<sub>82-90</sub> peptide. After 6 days, the cultured spleen cells were recovered and assayed for their capacity to kill P815 mastcytoma cells (H-2<sup>d</sup>) pre-pulsed with the M2 peptide (Fig. 12A). M2 peptide-specific and K<sup>d</sup>-restricted CTL was primed in BALB/c mice immunized with either of the 4 types of genetically modified ES-DC (Fig. 12B). Therefore, ES-DC expressing K<sup>d</sup> could prime K<sup>d</sup>-restricted antigen-specific CTL, irrespective of cell surface expression of intrinsic MHC class I encoded by the H-2<sup>b</sup> haplotype.

To determine whether or not ES-DC could prime M2<sub>82-90</sub> specific CTL in the presence of pre-primed H-2<sup>b</sup>-reactive CTL, ES-DC without peptide loading were injected in BALB/c mice 5 (Fig. 6C) or 10 times (Fig. 6D) with 7-day intervals and then the same ES-DC loaded with M2<sub>82-90</sub> peptide were injected. In addition, in this case, the specific CTL were primed by all of the 4 types of ES-DC. These results indicate that even in the presence of pre-activated alloreactive CTL, ES-DC expressing recipient-matched MHC class I are able to prime the antigen-specific CTL, whether or not the ES-DC express intrinsic MHC class I on their cell surface.



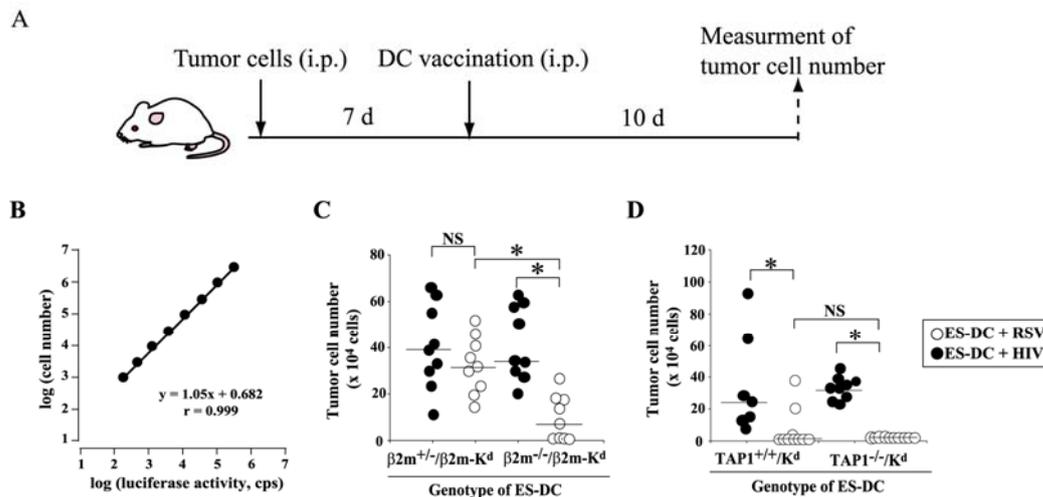
**FIGURE 12. Priming of exogenous antigen-specific CTL by injection of genetically modified ES-DC loaded with antigenic peptide.**

(A) Experimental system. (B) RSV antigenic peptide-loaded ES-DC ( $1 \times 10^5$ /injection/mouse) were injected i.p. into the naive mice twice with a 7-day interval. (C and D) Non-Ag loaded ES-DC were injected 5 (C) or 10 (D) times with 7 days intervals before immunization with peptide-loaded ES-DC. The mice were sacrificed 7 days after the last injection of ES-DC and the spleen cells were isolated. The spleen cells were cultured in the presence of RSV peptide ( $1 \mu\text{M}$ ) for 6 days and then analyzed on the RSV peptide-specific cytolytic activity by  $5 \text{ h}^{-51}\text{Cr}$  release assay. As target cells, P815 cells either pulsed with M2<sub>82-90</sub> peptide (open circles) or control H-2K<sup>d</sup>-restricted HIV gag p24<sub>199-207</sub> peptide (closed circles) were used. Data are representative of three experiments with similar results. The data are the mean specific lysis  $\pm$  SD of triplicate assays. The asterisks indicate significant ( $P < 0.05$ , Student's t-test) differences.

### **8.7. Induction of antitumor immunity by genetically modified ES-DC in allogeneic recipients.**

Next, anti-tumor immunity induced by the genetically modified ES-DC was assessed. To this end, a tumor cell line colon26/M2-Luc, a BALB/c-derived colon carcinoma cell line colon26 expressing RSV-M2 along with firefly luciferase, was generated. After the inoculation of the tumor cells, it was possible to quantify the number of cancer cells in mouse tissues by measuring the luciferase activity of tissue homogenates (Fig. 13A) as reported (68). The luciferase activity in the homogenates of colon 26/M2-Luc cells was linearly correlated with the number of the cells in the range from 150 to 350,000 counts per second (Fig. 13B). In a pilot study, when the tumor cells were injected into the mice intraperitoneally, most tumor cells were detected in the greater omentum and the mesenterium, and the luciferase activity of these 2 organs were in parallel (data not shown). Therefore, the luciferase activity of the greater omentum was chosen to be measured in the following studies.

BALB/c mice were injected i.p. with ES-DC loaded with M2<sub>82-90</sub> peptide and other mice were injected with ES-DC pre-pulsed with irrelevant control peptide (HIV gag p24<sub>199-207</sub>) with K<sup>d</sup>-binding affinity. One week after the ES-DC injection, the mice were challenged i.p. with colon26/M2-Luc. After 10 days, the growth of tumor cells was evaluated by measuring the luciferase activity in the homogenate of the greater omentum. In the mice injected with either of the  $\beta 2m^{-/-}/\beta 2m\text{-K}^d$  ES-DC (Fig. 13C) or TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC (Fig. 13D) loaded with M2 peptide, tumor growth was significantly reduced in comparison to the mice injected with the same ES-DC loaded with the control peptide. Therefore, the 2 types of genetically modified ES-DC could induce antigen-specific anti-tumor immunity. The anti-tumor effect induced by  $\beta 2m^{-/-}/\beta 2m\text{-K}^d$  ES-DC was significantly stronger than that induced by  $\beta 2m^{+/-}/\beta 2m\text{-K}^d$  ES-DC, indicating that the disruption of intrinsic  $\beta 2m$  gene and introduction of  $\beta 2m$ -linked MHC class I in ES-DC may provide an advantage in the induction of antigen-specific anti-tumor immunity.



**FIGURE 13. Induction of protective immunity by antigen-loaded genetically modified ES-DC against peritoneally disseminated tumor cells in allogeneic recipients.**

(A) Experimental system. (B) Homogenates were made from the indicated numbers of in vitro cultured colon26/M2-Luc cells and the luciferase activity was measured.  $r$ , correlation coefficient. (C and D) BALB/c mice were injected with M2<sub>82-90</sub> peptide- or control HIV p24<sub>199-207</sub> peptide-loaded ES-DC ( $1 \times 10^5$ /mouse) i.p. on day  $-7$  and challenged with colon26/M2-Luc ( $1 \times 10^6$ /mouse) i.p. on day 0. On day 10, the mice were sacrificed and luciferase activity of the greater omentum was measured. Luciferase activity of tissue lysates was converted to tumor cell number in the greater omentum based on the standard curve shown in B. The results of mice treated with  $\beta 2m^{+/-}/\beta 2m-K^d$  ES-DC or  $\beta 2m^{-/-}/\beta 2m-K^d$  ES-DC are shown in C. The results of mice treated with TAP1<sup>+/+</sup>/K<sup>d</sup> ES-DC or TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC are shown in D. All data are representative of at least two experiments with similar results. Values for individual mice injected with M2<sub>82-90</sub> peptide-loaded ES-DC (open circles) and HIV p24<sub>199-207</sub> peptide-loaded ES-DC (closed circles) are shown; bars indicate median values. The asterisks indicate significant ( $P < 0.05$ ) differences between two groups based on the Mann-Whitney U test. NS, not significant.

## 9. Discussion

### **Clinical application of ES-DC and related problems.**

To induce T cell-mediated anti-cancer immunity, vaccination with DC loaded with tumor antigen-derived peptides or tumor cell lysates are being clinically tested (69, 70). For such purposes, DC are generated from monocytes obtained from peripheral blood of the patients. However, monocytes are not easily propagated *in vitro* and apheresis, a procedure sometimes invasive for the patients, is necessary to obtain a sufficient number of monocytes as the source of 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 too labor-intensive and costly to be broadly applied.

ES cells exhibit the remarkable properties of self-renewal and pluripotency. This capacity allows for the production of sizeable quantities of therapeutic cells of the hematologic lineage, including DC (71). If the ES-DC method is clinically applied, it will be possible to generate genetically engineered DC expressing target antigens or immuno-stimulatory molecules, without use of virus-based vectors. However, considering the medical application, one drawback of the ES-DC method is the unavailability of human ES cells genetically identical to the patients to be treated (46). Specifically, an HLA allele mismatch between ES cells and patients is a crucial problem.

### **The strategy to overcome the histoincompatibility between ES-DC and patients.**

In previous studies, it was shown that allogeneic BM-DC are rapidly eliminated from the draining lymph nodes during the course of a primary alloreactive responses (50, 72). Another study revealed that the elimination of transferred APC in allogeneic recipients is mainly mediated by T cells reactive to allogeneic MHC class I but not MHC class II. Therefore, if the expression of the intrinsic MHC class I by ES-DC could be blocked, then ES-DC inoculated into allogeneic recipients would escape elimination by the alloreactive T cells of the recipients. However, the genomic region including the MHC class I genes spans more than 1,000 kb in both the mouse and human genome and complete elimination of such a large genomic

region by gene-targeting technique is currently infeasible. The feasible candidates for genetic modification are the genes that encode  $\beta 2m$  and TAP, which regulate MHC class I expression (57, 58). Therefore, the present study adopted a strategy to block the cell surface expression of the MHC class I molecules by elimination of the  $\beta 2m$  or TAP1 gene.

Both alleles of the TAP1 or  $\beta 2m$  gene were disrupted in 129-derived ES cells (H-2<sup>b</sup>) and subsequently expression vectors for the recipient (BALB/c)-matched K<sup>d</sup> or  $\beta 2m$ -linked form of K<sup>d</sup> were introduced. The genetically modified ES cells were subjected to the differentiation culture to generate TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC and  $\beta 2m^{-/-}$ / $\beta 2m$ -K<sup>d</sup> ES-DC. As intended, the  $\beta 2m^{-/-}$ / $\beta 2m$ -K<sup>d</sup> ES-DC expressed only K<sup>d</sup> molecule as MHC class I molecules on the cell surface. TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC hardly expressed any classical MHC class I and a low level of cell surface expression of K<sup>d</sup> was observed after incubation with K<sup>d</sup>-binding peptide. *In vitro*,  $\beta 2m^{-/-}$ / $\beta 2m$ -K<sup>d</sup> and TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC were not recognized by pre-activated H-2<sup>b</sup>-reactive CD8 T<sup>+</sup> cells and the ES-DC did not prime H-2<sup>b</sup>-reactive CD8<sup>+</sup> T cells (Fig. 8). When these cells were inoculated into BALB/c mice, they did not prime H-2<sup>b</sup> reactive CD8<sup>+</sup> T cells *in vivo* (Fig.10).

Consistent with these results,  $\beta 2m^{-/-}$ / $\beta 2m$ -K<sup>d</sup> ES-DC and TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC had a survival advantage in comparison to  $\beta 2m^{+/-}$ / $\beta 2m$ -K<sup>d</sup> ES-DC and TAP1<sup>+/+</sup>/K<sup>d</sup> ES-DC, when transferred into BALB/c mice (Fig. 11). The results suggest that ES-DC deficient in  $\beta 2m$  or TAP1 and expressing only recipient-matched MHC class I were resistant to elimination by alloreactive CTL. It has been shown that CTL-mediated elimination of DC has a notable effect on the magnitude of immune responses *in vivo* (67, 73). Therefore,  $\beta 2m$ - or TAP1-deficient ES-DC should be able to elicit more robust priming of antigen-specific CTL in allogeneic recipients than ES-DC expressing intrinsic MHC class I. When loaded with RSV-derived peptide and inoculated into BALB/c mice, not only  $\beta 2m^{-/-}$ / $\beta 2m$ -K<sup>d</sup> and TAP1<sup>-/-</sup>/K<sup>d</sup> ES-DC but also  $\beta 2m^{+/-}$ / $\beta 2m$ -K<sup>d</sup> and TAP1<sup>+/+</sup>/K<sup>d</sup> ES-DC primed K<sup>d</sup>-restricted, RSV peptide-specific CTL (Fig. 12). Unexpectedly, there was no significant difference in the magnitude of priming of antigen-specific CTL among these ES-DC. CTL-mediated allogeneic DC elimination is mainly dependent on the perforin/granzyme B pathway (51, 72). Therefore, the result shown in Figure 6 may

be due to resistance of ES-DC to killing by CTL that is attributed partly to the high level of expression of SPI-6, the granzyme B-specific protease inhibitor, in ES-DC (45). In addition, in the experiments shown in Fig.6, we cultured spleen cells isolated from immunized mice for 5 days in the presence of RSV-M2 peptide to amplify RSV-specific CTL before the cytotoxicity assay, because we could not detect RSV-specific CTL activity in a direct *ex vivo* killing assay. Probably, in the data shown in Fig. 6, difference in the CTL activity induced *in vivo* by the different genotype of ES-DC may have been masked by this culture procedure.  $\beta 2m^{-/-}/\beta 2m-K^d$ ,  $TAP1^{-/-}/K^d$  and  $TAP1^{+/+}/K^d$  ES-DC also could induce antigen specific antitumor immunity (Fig.13). However, No difference in the anti-tumor effect between  $\beta 2m^{+/-}/\beta 2m-K^d$  ES-DC loaded with RSV peptide and those loaded with HIV peptide (Fig. 13C) indicates that no significant antigen-specific anti-tumor effect was elicited by  $\beta 2m^{+/-}/\beta 2m-K^d$  ES-DC. As shown in Fig. 5, very few  $\beta 2m^{+/-}/\beta 2m-K^d$  ES-DC survived for 48 h in the recipient BALB/c mice as compared with  $\beta 2m^{-/-}/\beta 2m-K^d$  ES-DC. Although the small number of surviving  $\beta 2m^{+/-}/\beta 2m-K^d$  ES-DC primed some RSV-reactive CTL and those were detectable in an *in vitro* cytotoxicity assay (Fig. 12), those may be insufficient to elicit a significant antigen-specific anti-tumor effect.

In the present study, the MHC class II haplotype of ES-DC was always b while that of the recipient mice (BALB/c) was d. Therefore, there was mismatch in MHC class II haplotype between ES-DC and the recipient mice in all of the experiments. Since the mismatch of the MHC class II allele has been reported to not cause any acute elimination of transferred APC (51), the class II mismatch may not have negatively affected the priming of antigen-specific CTL in the present study. Instead, alloreactive helper T cells are expected to enhance the CTL response via cytokine production, although we did not experimentally address this issue in the present study.

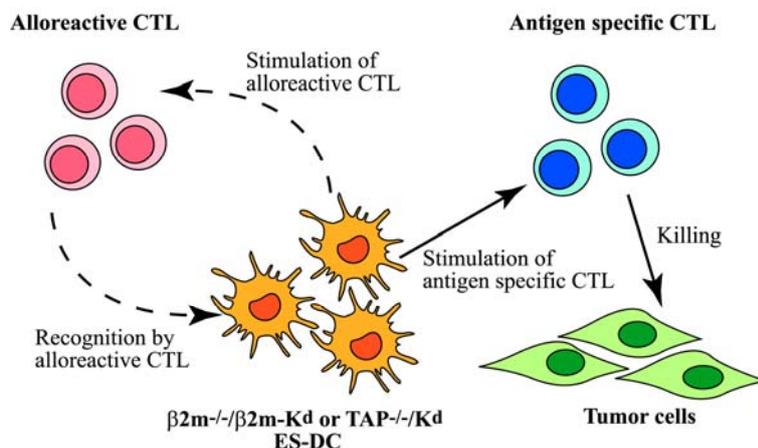
### **Application of the current strategy to induced pluripotent stem cells.**

Theoretically, the issue of histocompatibility related to the ES cell-based medical technology may be resolved by the recent development of induced pluripotent stem (iPS) cells that can be generated by introduction of several defined

genes into somatic cells (74-77). However, the medical application of iPS cells nevertheless has some drawbacks. The use of virus vectors is necessary to generate iPS cells and generation of iPS cells for individual patients may be too costly, time consuming and labor-intensive to be broadly applied. The genetic modification of ES cells or iPS cells to modify cell surface HLA class I by the presently reported methods may be more economical, faster and thus, more realistic than the individual generation of “fully personalized iPS cells”.

Although targeted gene disruption of OCT4 and HPRT in human ES cells has been reported (78), the methodology of gene targeting for human ES cells has not been well established at present. Therefore, as an alternative strategy, we are planning to generate iPS cells from patients with Type I bare lymphocyte syndrome caused by mutation of the TAP 1 or TAP 2 gene (79, 80). Once a clone of TAP- or  $\beta$ 2m-deficient human ES or iPS cells is established, a pre-made library of pluripotent stem cell clones expressing various types of HLA class I can be generated by the introduction of various HLA class I genes. Such a pluripotent stem cell bank may serve as a source of not only DC but also of various kinds of differentiated cells that may be useful in the field of regenerative medicine.

## 10. Conclusions



**FIGURE 14. Conclusions of this study.**

The present study attempted to establish a strategy to overcome the issue of histoincompatibility between patients to be treated and ES-DC. To this end, ES-DC expressing only transgene-derived  $\text{K}^d$  but not intrinsic MHC class I molecules on the cell surface ( $\beta 2m^{-/-}/\beta 2m\text{-K}^d$  and  $\text{TAP}1^{-/-}/\text{K}^d$  ES-DC) were generated by the genetic modifications of ES cells. These ES-DC showed (i) reduced potency to be recognized by alloreactive  $\text{CD}8^+$  T cells, (ii) reduced potency to prime allo-reactive  $\text{CD}8^+$  T cells and (iii) capability to activate the antigen-specific immune response *in vitro* and *in vivo*. The modification of the  $\beta 2m$  or TAP gene may therefore be an effective strategy to resolve the problem of HLA class I allele mismatch between human ES or iPS cells and the recipients to be treated.

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