These results indicate that intracellular REIC/Dkk-3 protein plays a pivotal role in biology, involved in cell differentiation and proliferation and the development of specific organs via the regulation of the Wnt and TGF-β signaling pathways. Notably, REIC/Dkk-3 protein acts as an inhibitor or inducer of the Wnt and TGF-β signaling pathways based on the cellular conditions of various tissues and organisms, from amphioxus to vertebrates (25). The binding partner of intracellular REIC/Dkk-3 protein has been investigated by several previous studies. It has been reported that Dkk-3 binds to other proteins, such as Kremen1/2 (28,29), β-TrCP (30) and TcTex-1 (31), and that these interactions occur in the cytoplasm. These proteins include substantial molecules that significantly affect and modify intracellular signaling pathways, including the Wnt and TGF-β (16,32). Therefore, the functional varieties of intracellular Dkk-3 protein in the Wnt and TGF-β signaling may be partially explained by the various interaction partners or behavior of the proteins.

4. Cytokine-like aspects of exogenous REIC/Dkk-3 protein in monocyte differentiation

The immunological aspects of exogenous REIC/Dkk-3 protein have been investigated in a previous study (33). Purified recombinant proteins were added to human monocytes obtained from peripheral blood and the cytokine-like actions were examined. To clarify the effects of exogenous REIC/Dkk-3 protein on monocyte differentiation, human $CD14^+$ monocytes were incubated with recombinant proteins at a concentration of 10 μg/ml. Recombinant REIC/Dkk-3 protein was found to induce monocyte differentiation to a DC phenotype. The morphological features of the REIC/Dkk-3-induced cell phenotype and its expression pattern of dendritic markers on the cell surface are similar to those of interleukin (IL)-4- and granulocyte macrophage-colony stimulating factor (GM-CSF)-induced DCs. Consistent with these observations, unpublished data indicates that recombinant REIC/Dkk-3 protein intraperitoneally administered in mice significantly upregulate the ratio of circulating DCs on flow cytometry. Recombinant REIC/Dkk-3 protein also possesses a cytokine-like function in the activation of STAT1 and STAT3 during dendritic phenotype differentiation *in vitro*. In terms of the expression of CD1a and CD14 surface markers, the REIC/Dkk-3-induced dendritic phenotype and IL-4- and GM-CSF-induced DCs are distinctly different. It is likely that these cells are categorized into DC subgroups. In addition, the direct cytotoxic effects of exogenous REIC/Dkk-3 protein were examined in several cancer cell lines. Even at a concentration of 20 μg/ml, no significant cytotoxic effects were associated with REIC/Dkk-3 protein treatment (33).

To date, the molecular mechanisms by which exogenous REIC/Dkk-3 protein differentiates monocytes into the DC phenotype have remained unclear. To clarify the immunomodulatory function of exogenous REIC/Dkk-3 protein, it is essential to determine the cell surface receptor. REIC/Dkk-3 is a secretory protein that is considered to act on cells via a cell surface receptor. However, the definitive cell surface receptors for this protein have not been identified. It has been previously reported that the expression levels of REIC/Dkk-3 modify intracellular Wnt and TGF-β signaling (24,25,27,29,30). There is a possibility that the REIC/Dkk-3 protein, secreted by the cells, interacts with unidentified cell surface receptors and exogenously affect these signaling pathways. Notably, several previous studies have demonstrated that Wnt and TGF-β signaling is involved in the differentiation of specific types of DC phenotypes (34-37). It is conceivable that modification of the signaling of the Wnt and TGF-β pathway by exogenous REIC/Dkk-3 protein triggers the differentiation to the DC phenotype in monocytes.

5. Adenovirus vectors expressing the human REIC/Dkk-3 gene (Ad-REIC) induce cancer cell-specific apoptosis

To examine the possible use of REIC/Dkk-3 as a tool for targeted gene-based therapy, we developed a replication-deficient adenovirus vector encoding the human REIC/Dkk-3 gene (Ad-REIC) (13). The CAG (CMV early enhancer/chicken β-actin) promoter was used to drive REIC/Dkk-3 expression, as this promoter enables strong gene expression (38,39). The overexpression of REIC/Dkk-3 induced by the Ad-REIC agent was found to stimulate apoptosis in a broad range of human cancer cell lines *in vitro* (13,18-20). By contrast, the ability of Ad-REIC to induce apoptosis was reduced in non-malignant cells (13,18,20). These observations indicate that the Ad-REIC agent selectively induces apoptosis in a cancer cell-specific manner. Since REIC/Dkk-3 expression is significantly downregulated in a broad range of human cancer cells, while being typically expressed in non-malignant or normal cells (9-17,22), the endogenous expression level of these proteins appear to correlate with sensitivity to the REIC/Dkk-3 overexpression induced by Ad-REIC.

The molecular mechanisms underlying the apoptosis induced by Ad-REIC have been previously investigated and the pathway is shown in Fig. 1. The phosphorylation (activation) of c-Jun N-terminal kinase (JNK) is a critical step in cancer cell death (13,18,19). REIC/Dkk-3 protein is a secretory protein and the overexpression of this protein induced by Ad-REIC treatment efficiently leads to ER stress-induced apoptosis in cancer cells (19,21,40). ER stress-induced apoptosis is triggered due to a failure in the folding of large amounts of REIC/Dkk-3 protein accumulated in the lumen of the ER. The phosphorylation of JNK occurs downstream of ER stress signaling in cancer cells. As the REIC/Dkk-3 gene expression is absent or lacking in cancer cells (9-17,41), the REIC/Dkk-3 expression and protein folding system in cancer cells does not function well when the protein is overexpressed by Ad-REIC. This implies that, due to the poor capacity for REIC/Dkk-3 gene expression, the cancer cells easily exhibit a failure to fold large amounts of REIC/Dkk-3 protein accumulated in the ER. The differences in the capacity for REIC/Dkk-3 gene overexpression between cancer and normal cells may explain the cancer cell-specific apoptosis induced by Ad-REIC.

Previously, differences in ER stress signaling following Ad-REIC treatment have also been studied in cancer and normal cells (13,19,21,40). As shown in Fig. 1, REIC/Dkk-3-sensitive cancer cells, IRE1α (an ER stress sensor), apoptosis signal-regulating kinase 1 (ASK1) and JNK activation by Ad-REIC, subsequently induce the phosphorylation of c-Jun. As a result of c-Jun activation, translocation

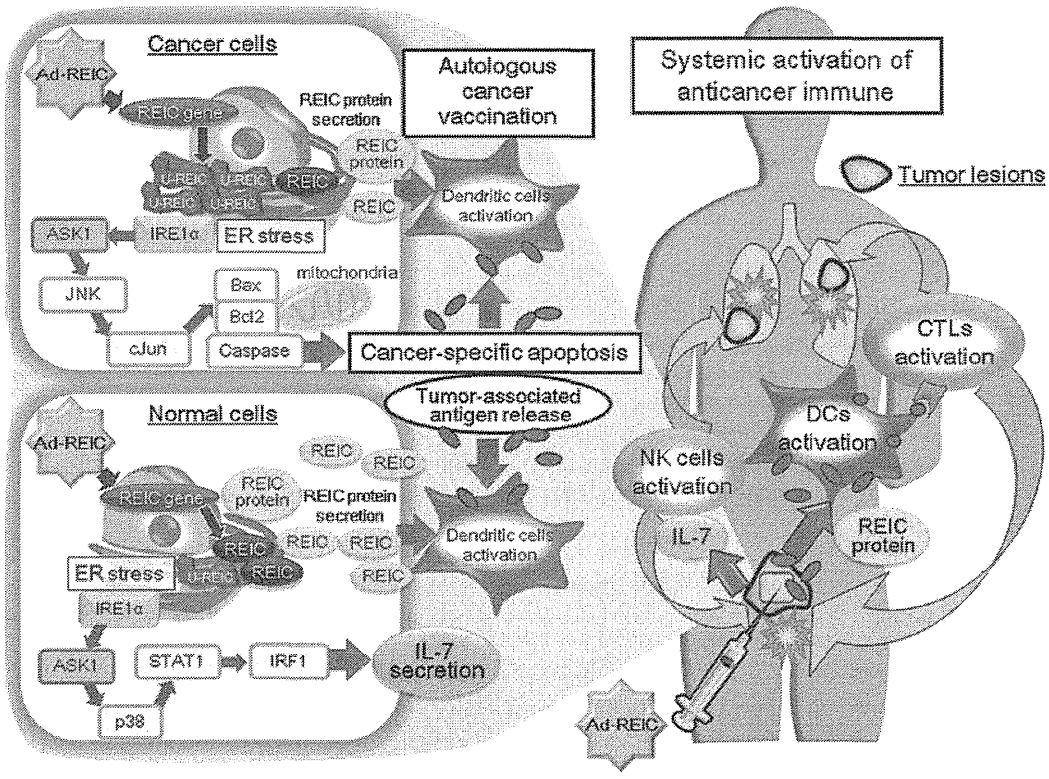

Figure 1. Therapeutic mechanisms of intratumoral Ad-REIC treatment. At the tumor site, Ad-REIC induces cancer cell-specific apoptosis in a phosphorylated JNK-dependent manner via ER stress signaling. Due to the poor capacity for the REIC/Dkk-3 gene expression, the cancer cells exhibit a failure to fold large amounts of REIC/Dkk-3 protein accumulated in the ER. This folding failure and the presence of U-REICs in the ER lead to the stress-induced apoptosis of cancer cells. The activation of JNK by Ad-REIC subsequently induces the phosphorylation of c-Jun, the translocation of Bax to the mitochondria and the downregulation of Bcl2, which subsequently leads to caspase-dependent apoptosis. By contrast, in non-cancer cells, which are typically resistant to Ad-REIC-induced apoptosis, a different ER stress response is observed following treatment. When REIC/Dkk-3 is overexpressed by Ad-REIC in normal cells, for example human fibroblasts, ER stress signaling of the p38, STAT1 and IRF1 pathways is activated. The activation of IRF1 then upregulates IL-7 expression and secretion in the cells. Autologous cancer vaccination with Ad-REIC treatment starts with cancer-specific apoptosis and the subsequent release of TAAs. The REIC/Dkk-3 protein, overexpressed and secreted by Ad-REIC transfection, differentiates monocytes into the DC phenotype at the tumor site. At the same time, abundant TAA fragments are released as a result of cancer cell-selective apoptosis and are supplied to the DCs induced by the secreted REIC/Dkk-3 protein. The activation of the DCs directly enhances cancer cell antigen presentation to CTLs, which upregulates systemic antitumor immunity. In addition, the enhanced IL-7 secretion observed in the normal cells activates NK cells, which also upregulates systemic anticancer immunity. The Ad-REIC-induced synergistic secretion of the REIC/Dkk-3 protein and IL-7 cytokines at the treated tumor site is important for the autologous cancer vaccination induced by the agent. These immunoactive proteins work together to mediate the phase of cancer-specific apoptosis to the anticancer immune effects observed at the injected and distant tumor sites. Ad, adenovirus; REIC, reduced expression in immortalized cells; JNK, c-Jun N-terminal kinase; ER, endoplasmic reticulum; Dkk-3, dickkopf-3; U-REICs, unfolded REIC/Dkk-3 protein; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; STAT1, signal transducer and activator of transcription 1; IRF1, interferon regulatory factor 1; IL, interleukin; TAAs, tumor-associated antigens; DC, dendritic cell; CTLs, cytotoxic T lymphocytes; NK, natural killer.

of Bcl-2-associated X protein to the mitochondria and the downregulation of B-cell lymphoma 2 occur, while the upregulation of caspases leads to the induction of apoptosis. By contrast, in non-cancer cells, which are typically resistant to Ad-REIC-induced apoptosis, a different ER stress response is observed following treatment. When the REIC/Dkk-3 gene is overexpressed by Ad-REIC in normal human fibroblasts, ER stress signaling with IRE1α, ASK1, p38, STAT1 and interferon regulatory factor 1 (IRF1) activation is observed, however, JNK activation is not detected. Furthermore, Ad-REIC treatment induces the upregulation of IL-7 expression and its significant secretion in human fibroblasts, based on IRF1 activation. These differences in the signaling of ER stress responses following REIC/Dkk-3 overexpression between cancer and normal cells may be involved in the various outcomes of apoptosis and the cancer cell-specific apoptosis induced by Ad-REIC agents.

6. Intratumoral Ad-REIC treatment robustly suppresses cancer growth in mouse tumor models

To demonstrate the therapeutic effects of Ad-REIC *in vivo*, mouse tumor models of several cancer types were established by transplanting the cancer cell line and treating the tumor-bearing mice with Ad-REIC (13,18-20,33,42,43). The tumor types of the model included prostate (13,33,42), breast (20), testicular (18) and gastric (43) cancer and malignant mesothelioma (19). Ad-REIC was administered intratumorally (prostate, breast and testicular cancer), intraperitoneally (gastric cancer) or intrapleurally (malignant mesothelioma) to inhibit the growth of cancer cells. In these *in vivo* experiments, significant inhibition of tumor growth was observed in the Ad-REIC-treated group, however, the tumors progressively grew in the control Ad-LacZ-treated groups. In specific experiments, the tumors that developed following Ad-REIC

treatment were resected and examined using TUNEL staining to evaluate the induction of apoptosis by Ad-REIC. Significant numbers of TUNEL-positive cells were observed in broad areas of the Ad-REIC-treated tumors, however, few apoptotic cells were noted in the tumors of the control groups. In an orthotopic prostate tumor model established with a murine prostate cancer RM9 cell line, the progression of orthotopic tumor development and spontaneous metastasis to the retroperitoneal lymph nodes were robustly suppressed by intratumoral Ad-REIC administration (42). In addition, adenoviral vectors encoding the murine REIC/Dkk-3 gene similarly suppressed the progression of RM9 prostate cancer in the mouse model as well as the Ad-REIC encoding the human REIC/Dkk-3 gene. A series of *in vivo* experiments definitively indicated that the REIC/Dkk-3 gene is a promising molecule for cancer gene therapy and that the therapeutic utility of Ad-REIC agents may be applied in a broad range of human cancer types.

7. Adenovirus-mediated REIC/Dkk-3 gene therapy induces autologous cancer vaccination

Adenovirus-mediated REIC/Dkk-3 overexpression has been demonstrated to induce significant apoptosis in treated tumor sites and robust antitumor effects in mouse models (13,18-20,33,42,43). The induction of cancer cell-specific apoptosis by intratumoral Ad-REIC injection is an important therapeutic mechanism. When orthotopic RM9 prostate tumors are injected with Ad-REIC, significant apoptotic induction and tumor growth inhibition are observed in the treated lesions (33,42). Furthermore, treatment of orthotopic prostate tumors with Ad-REIC suppresses the tumor growth of distant lung metastasis in a mouse model (33). Previous *in vitro* cytolytic assays have demonstrated that anticancer immunity against RM9 prostate cancer cells is significantly enhanced in Ad-REIC-treated mice (33). These results indicate that intratumoral Ad-REIC injection in one tumor lesion also suppresses the growth of other distant cancer lesions via the induction of anticancer immunity. Primarily, intratumoral Ad-REIC treatment induces local apoptotic cell death in treated cancer sites and then activates systemic anticancer immunity against cancer cells. In addition, at the Ad-REIC-treated tumor site, secreted REIC/Dkk-3 protein plays a cytokine-like role in inducing monocyte differentiation to a specific DC phenotype and appear to be involved in systemic anticancer immunity (33).

As shown in Fig. 1, there are two mechanisms underlying the anticancer immune activation induced by Ad-REIC gene therapy. The first mechanism is based on autologous cancer vaccination, which is specifically observed in Ad-REIC gene therapy. Cancer vaccination with Ad-REIC starts with the cancer-specific apoptosis and subsequent release of TAAs. Since the REIC/Dkk-3 protein differentiates $CD14^+$ monocytes into the DC phenotype (33), the REIC/Dkk-3 protein overexpressed and secreted by Ad-REIC transfection at the tumor site differentiates and activates DCs. At the same time, abundant TAA fragments are released as a result of cancer cell-selective apoptosis and supplied to the DCs induced by the secreted REIC/Dkk-3 protein. The activation of DCs directly enhances cancer cell antigen presentation to cytotoxic and helper T lymphocytes, which upregulate systemic antitumor immunity (1,2). Therefore, intratumoral Ad-REIC treatment activates the DCs via the actions of secreted REIC/Dkk-3 proteins and TAAs released at the Ad-REIC-injected tumor sites. Ad-REIC-based medicine is predicted to enhance systemic anticancer immunity and achieve antitumor effects in injected and distant lesions as a therapeutic cancer vaccine.

The second mechanism is based on the secretion of IL-7 observed in the normal stromal fibroblasts of the Ad-REIC-injected tumor sites (40). When the REIC/Dkk-3 gene is overexpressed by Ad-REIC in the fibroblasts of the treated tumor, significant levels of IL-7 are expressed and secreted in the cells. As shown in Fig. 1, this phenomenon is triggered by ER stress signaling via the actions of IRE1α and the activation of ASK1 and the p38 kinase system (40). The enhanced IL-7 expression and secretion observed following Ad-REIC treatment activate natural killer cells to play a role in the upregulation of systemic anticancer immunity (40,44). The Ad-REIC-induced synergistic secretion of REIC/Dkk-3 proteins and IL-7 cytokines at the treated tumor site is important for autologous cancer vaccination by the agent. Namely, these immunoactive proteins work together to mediate the phase of cancer-specific apoptosis to the anticancer immune effects observed at the injected and distant tumor sites. For these reasons, Ad-REIC-mediated medicine is predicted to provide autologous cancer vaccination therapy to be applied as an individualized tailor-made vaccine.

8. Future directions of Ad-REIC-mediated cancer vaccination therapy

The field of therapeutic cancer vaccination is currently undergoing a shift in focus, to individualize tailor-made vaccines and the targeting of multiple TAAs. Autologous tumor vaccines must be applicable vaccine formulations, as tumor cells are a clear source of TAAs for vaccination purposes and all relevant candidate TAAs must be contained within them (3). Ad-REIC-mediated cancer vaccination is based on the strategy of developing autologous cancer vaccines to achieve the individualized activation of antitumor immunity. The ultimate goal of Ad-REIC-mediated cancer vaccination is to cure not only the treated primary tumor, but also distant tumor lesions. Strategies of autologous cancer vaccination using intratumoral Ad-REIC treatment must also be available in the clinical setting.

Based on previous preclinical results and concepts, a phase I-IIa study of gene therapy using an Ad-REIC agent was initiated in January 2011 and is currently ongoing at Okayama University Hospital (Okayama, Japan). The aim of this clinical study is to verify the safety, efficacy and anticancer immunological effects of Ad-REIC-based gene therapy in prostate cancer patients. In particular, evidence of the concept of autologous cancer vaccination via Ad-REIC is being tested in clinical trials to clinically develop the agent for large-scale application. The safety and efficacy of Ad-REIC-mediated gene therapy have been verified in patients and the initial impression of the clinical trial has been good. We hypothesize that Ad-REIC-based medicine is likely to provide anticancer immunological effects via the application of autologous cancer vaccination, which is likely to become a promising therapeutic option for treating a wide range of human malignancies as a cancer vaccine.

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Establishment of HLA-DR4 Transgenic Mice for the Identification of CD4+ T Cell Epitopes of Tumor-Associated Antigens

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Abstract

Reports have shown that activation of tumor-specific CD4+ helper T (Th) cells is crucial for effective anti-tumor immunity and identification of Th-cell epitopes is critical for peptide vaccine-based cancer immunotherapy. Although computer algorithms are available to predict peptides with high binding affinity to a specific HLA class II molecule, the ability of those peptides to induce Th-cell responses must be evaluated. We have established HLA-DR4 (*HLA-DRA*01:01/HLA-DRB1*04:05*) transgenic mice (Tgm), since this HLA-DR allele is most frequent (13.6%) in Japanese population, to evaluate HLA-DR4-restricted Th-cell responses to tumor-associated antigen (TAA)-derived peptides predicted to bind to HLA-DR4. To avoid weak binding between mouse CD4 and HLA-DR4, Tgm were designed to express chimeric HLA-DR4/I-E^d, where I-E^d α1 and β1 domains were replaced with those from HLA-DR4. Th cells isolated from Tgm immunized with adjuvant and HLA-DR4-binding cytomegalovirus-derived peptide proliferated when stimulated with peptide-pulsed HLA-DR4-transduced mouse L cells, indicating chimeric HLA-DR4/I-E^d has equivalent antigen presenting capacity to HLA-DR4. Immunization with $CDCA1_{55-78}$ peptide, a computer algorithm-predicted HLA-DR4-binding peptide derived from TAA CDCA1, successfully induced Th-cell responses in Tgm, while immunization of HLA-DR4-binding Wilms' tumor 1 antigen-derived peptide with identical amino acid sequence to mouse ortholog failed. This was overcome by using peptide-pulsed syngeneic bone marrow-derived dendritic cells (BM-DC) followed by immunization with peptide/CFA booster. BM-DC-based immunization of $KIF20A_{494-517}$ peptide from another TAA KIF20A, with an almost identical HLA-binding core amino acid sequence to mouse ortholog, successfully induced Th-cell responses in Tgm. Notably, both $CDCA1_{55-78}$ and $KIF20A_{494-517}$ peptides induced human Th-cell responses in PBMCs from HLA-DR4-positive donors. Finally, an HLA-DR4 binding $DEPDC1_{191-213}$ peptide from a new TAA DEPDC1 overexpressed in bladder cancer induced strong Th-cell responses both in Tgm and in PBMCs from an HLA-DR4-positive donor. Thus, the HLA-DR4 Tgm combined with computer algorithm was useful for preliminary screening of candidate peptides for vaccination.

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Introduction

Tumor cells express various proteins different from those of normal somatic cells, or over-express proteins at higher levels than in normal cells. Thus, peptides derived from these proteins may be specifically recognized by T lymphocytes. Of the two tumor-specific T lymphocyte subsets, $CD8^+$ cytotoxic T lymphocytes (CTL) recognize tumor-associated antigen (TAA)-derived peptides in the context of MHC class I molecules (MHC-I), whereas $CD4^+$ helper T (Th) cells respond to peptide-MHC class II (MHC-II) complexes. Because of their ability to eradicate malignant cells directly, CTLs have long been defined as the critical effector cells in anti-tumor immunity, although Th cells can also induce robust anti-tumor immune responses [1-3]. It is well accepted that tumor-specific Th cells maintain the anti-tumor responses of CTLs by licensing dendritic cells (DC) to effectively prime CTLs [4,5] by generating effective memory CTL [6,7], or by direct stimulation of effector CTLs [8]. Thus, identification and vaccination of Th-cell epitopes that activate tumor-specific Th cells is might be a promising method to induce effective anti-tumor immunity in tumor-bearing hosts. Although it may be possible to identify such Th-cell epitopes using human peripheral blood mononuclear cells (PBMCs) isolated from healthy donors or cancer patients, it requires frequent bleedings and assays using a large number of overlapping peptides, which is in many cases a very long and labor-intensive procedure.

Utilizing a computer algorithm for the prediction of peptides with high binding affinity to a specific HLA/MHC molecule might be a convenient alternative. For example, SYFPEITHI (http://www.syfpeithi.de/) and BIMAS (http://www-bimas.cit.nih.gov) are powerful tools to predict MHC class I binding and therefore potential CTL-epitopes. On the other hand, the confidence of predicting peptides that bind to a specific HLA/MHC-II molecule is still under discussion and the ability of those peptides to induce Th-cell responses must be evaluated. Therefore, we established transgenic mice expressing HLA-DR4 (*HLA-DRA/HLA-DRB1*04:05*). of which allele frequency is 13.6% in Japanese population, to evaluate HLA-DR4-restricted Th-cell responses to tumor-associated antigen (TAA)-derived peptides predicted to bind to HLA-DR4. Using those mice, immunization with peptides known to be recognized by HLA-DR4-restricted human Th cells successfully induced peptide-specific and HLA-DR4-restricted mouse Th cell responses, demonstrating the validity of the HLA-DR4 Tgm for the preliminary screening of novel TAA-derived and HLA-DR4-restricted Th epitopes, which could be clinically applicable for peptide vaccine-based cancer immunotherapy in the future.

Materials and Methods

Ethics Statement

This study was approved by the animal research committee of Kumamoto University (Permission Number: B25-115). The mice were maintained at the Center for Animal Resources and Development of Kumamoto University, and were handled in accordance with the animal care guidelines of Kumamoto University.

The Institutional Review Board of Kumamoto University approved the research protocol for collecting and using PBMCs from healthy donors performed in this study with written informed consent.

Gene Constructs of HLA-DRA/I-$E^d\alpha$ and HLA-DRB1*0405/I-$E^d\beta$

To avoid possible problems caused by inter-species interactions between mouse CD4 and HLA-DR, a chimeric HLA-DR4/I-E^d molecule, of which translated α1 and β1 domains were derived from HLA-DR4 and the other domains were derived from respective I-E^d α and β molecules, was constructed [9]. The genomic fragments of exon 2 of *I-$E^d\alpha$* and β *genes* were replaced with those of *HLA-DRA*01:01* and *HLA*DRB1*04:05 genes*, respectively. The 8.5 kb HindIII and 16 kb KpnI fragments were used for the chimeric *HLA-DRA/I-$E^d\alpha$* and *HLA-DRB1*0405/I-$E^d\beta$* transgenes, which contain endogenous I-$E^d\alpha$ and I-$E^d\beta$ promoter regions spanning 3.2 kb and 5.2 kb of 5'-untranslated regions, respectively (Figure 1). The *HLA-DR4/I-E^d* transgenes were co-injected into C57BL/6 fertilized eggs [10] and were transferred into the oviduct of pseudopregnant ICR mice. A total of 25 pups were obtained and at 4-5 weeks after birth, their PBMCs were subjected to flow cytometric analyses using anti-HLA-DRα-chain monoclonal antibody (mAb) L243 and anti-HLA-DR4β-chain mAb TAL15.1. In addition, genomic PCR analyses was used using the following primer sets: 5'-CACCCAGACACTGTTTCTTC-3' and 5'-CAAAGCTGGCAAATCGTC-3' for *HLA-DRA/I-$E^d\alpha$* and 5'-CCCGTTAGTTGTGGTGACCT-3' and 5'-GCACTGTGAAGCTCTCACCA-3' for *HLA-DRB1*04:05/I-$E^d\beta$*, respectively after preparation of genomic DNA using DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands). The presence and expression of *HLA-DR4/I-E^d* transgenes were confirmed by PCR and flow cytometry and positive offspring were crossed with wild-type (WT) C57BL/6 mice.

Cell lines

L-DR4, genetically engineered mouse fibroblast L cells that express HLA-DR4 (*HLA-DRA*01:01/DRB1*04:05*), were maintained in DMEM supplemented with 10% fetal calf serum (FCS) and 50 U/ml penicillin/streptomycin in a 5% CO_2 atmosphere at 37°C.

Generation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BM-DCs) were generated as described by Inaba et al [11]. Briefly, bone marrow cells obtained from mouse thighbones and hind limbs were cultured at 1×10^7 cells per well in 10 ml of RPMI 1640 supplemented with mouse GM-CSF 10 ng/ml, 10% FCS, 50 μM 2-mercaptoethanol (2ME) and 50 U/ml penicillin/streptomycin. On day 14, the cells were recovered for antigen presentation.

Synthetic peptides

Peptides were synthesized by Biomatik (Cambridge, Ontario, Canada). The peptides used in this study were: CMV-derived

Figure 1

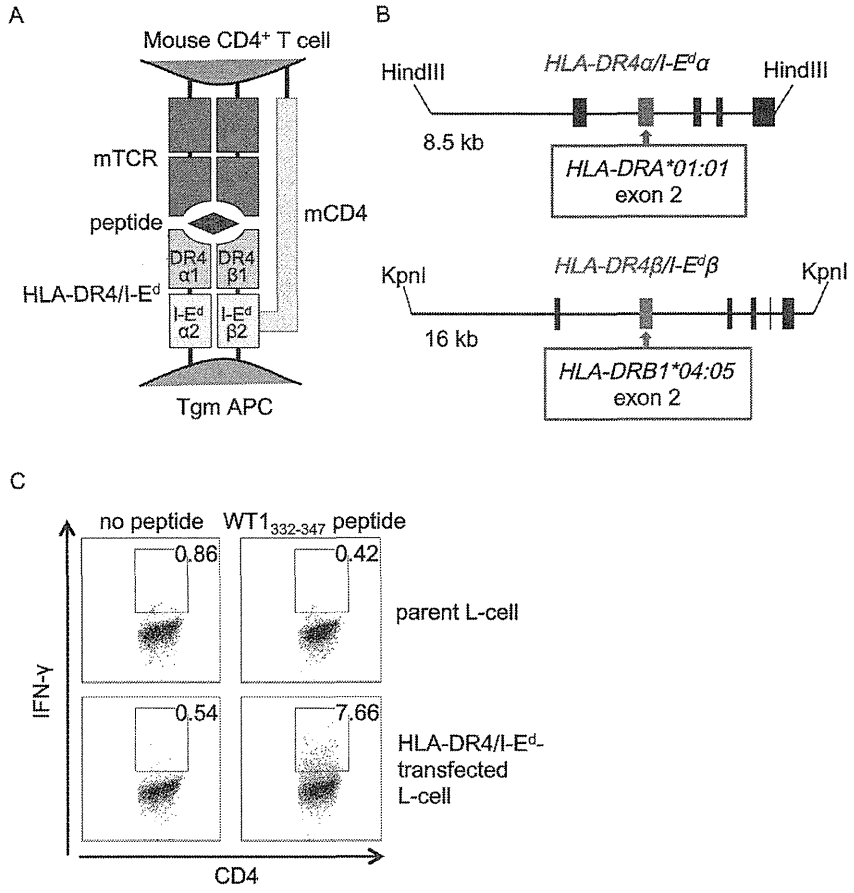

Figure 1. Transgenes encoding chimeric HLA-DR4/I-E^d molecule. (A) Schematic diagram of mouse T cell receptor (mTCR, green), mouse CD4 (mCD4, pale green) and HLA-DR4/I-E^d chimeric molecules (orange and yellow). (B) To avoid inter-species interactions between mCD4 and HLA-DR4, the second exons of *I-E^{d}α* and *I-E^{d}β* genes encoding α1 and β1 domains were substituted with those of HLA-DRA and *HLA-DR4B* genes (red boxes), respectively. The transgenes contain the endogenous I-E^{d}α and I-E^{d}β promoter regions spanning 3.2 kb and 5.2 kb of 5'-untranslated regions, respectively. (C) $WT1_{332-347}$ peptide-pulsed L-cells expressing HLA-DR4/I-E^d stimulated IFN-γ production by the $WT1_{332-347}$ peptide-specific and HLA-DR4-restricted human Th clone (gated on CD4).

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CMV-egH$_{290-302}$ peptide (SYLKDSDFLDAAL) [12], WT1-derived $WT1_{332-347}$ peptide (KRYFKLSHLQMHSRKH) [13], CDCA1-derived $CDCA1_{55-78}$ peptide (IVYGIRLEHFYMMPVNSEVMYPHL) [14], KIF20A -derived $KIF20A_{494-517}$ peptide (TLHVAKFSAIASQLVHAPPMQLGF) [15], DEPDC1-derived $DEPDC1_{191-213}$ peptide (RYVILIYLQTILGVPSLEEVINP) and a negative control $DEPDC1_{60-85}$ peptide (NSNFGPEVTRQQTIQLLRKFLKNHVI). The purity of the peptides was routinely >95%. Lyophilized peptides were dissolved in DMSO and stored at −20°C.

Flow Cytometry

After hemolysis, PBMCs were stained with various PE- or FITC-conjugated mAbs. The cells were analyzed by FACScan (BD Biosciences, Franklin Lakes, NJ, USA) using Cell Quest software. PE-conjugated anti-HLA-DR-α-chain mAb L243 was from BD and FITC-conjugated anti-HLA-DR-β-chain mAb TAL15.1, PE-conjugated anti-I-A^b mAb, PE-conjugated anti-mouse MHC-II mAb, FITC-conjugated anti-B220 mAb, PE- or FITC-conjugated anti-mouse CD4 mAb were from eBioscience (San Diego, CA, USA).

Intracellular IFN-γ staining

Parental L-cells and L cells expressing HLA-DR4/I-E^d were incubated with or without 10 μg/ml of $WT1_{332-347}$ peptide for 3 h, washed extensively, and then used as a stimulator. L cells were co-incubated with HLA-DR4-restricted $WT1_{332-347}$ peptide-specific $CD4^+$ T cell clones [13] in the presence of 2 μg/ml CD28/CD49d Costimulatory Reagent (BD Biosciences) and 10 μg/ml Brefeldin A (Sigma-Aldrich, St Louis, MO, USA) for 5 h. Intracellular staining of IFN-γ was performed using BD Cytofix/Cytoperm Buffer (BD Biosciences) according to the manufacturer's procedures after surface staining of CD4 molecules. PE-conjugated anti-IFN-γ mAb and FITC-conjugated anti-CD4 mAb were from BD Biosciences. The cells were analyzed with FACSAria (BD Biosciences). The data were analyzed with FlowJo software.

Immunization of mice

Mice were primed either in the tail base with 50 μl of peptides in PBS (1 μg/μl) emulsified with 50 μl of Complete Freund's Adjuvant (CFA, Sigma-Aldrich) or by intravenous injection with peptide-pulsed BM-DCs (5×10^5). Seven days after the first immunization, mice were boosted with 50 μl of peptide in PBS (1 μg/μl) emulsified with 50 μl of Incomplete Freund's Adjuvant (IFA, Sigma-Aldrich) or CFA. On day 14, the inguinal lymph nodes or spleen cells were collected and cultured with the peptides and IL-2 (20 U/ml) for 7 days. Th cells were isolated by MACS beads (Miltenyi Biotec, Bergish-Gladbach, Germany) according to the manufacturer's instruction and assayed as described below. In some experiments, the harvested lymph nodes cells on day 14 were subjected to *ex vivo* proliferation assay.

T cell proliferation assay and blocking experiment

Purified Th cells were co-cultured with peptide-pulsed L-DR4 cells for 48 h in RPMI 1640 medium containing 8% FCS, 50 U/ml penicillin/streptomycin and 2ME, then pulsed with 1 μCi [^{3}H-] thymidine and cultured for another 17 h. The cells were harvested and incorporated radioactivity was counted using a scintillation counter (l450 Microbeta, Trilux, PerkinElmer). To confirm the HLA-DR4 restriction, single cell-suspension of inguinal LN cells was cultured with or without peptide in the presence or absence of anti-HLA-DR blocking mAb L243 for 48 h. Then, incorporated radioactivity was counted as described above.

IFN-γ ELISPOT assay

The ELISPOT assay was performed as described previously [14]. Briefly, Th cells were incubated in triplicate in ELISPOT plates (BD Biosciences) under the presence of the indicated peptides (10 μg/ml) and L-DR4 as antigen presenting cells. According to the manufacturer's instructions, the plates were incubated for 18 h at 37°C and IFN-γ-positive spots were quantified using Eli photo (Minerva Tech, Tokyo, Japan).

Generation of antigen-specific human $CD4^+$ T cells

We obtained PBMCs from HLA-DR4-positive healthy donors (genotyped by the HLA Laboratory, Kyoto, Japan). Induction of antigen-specific $CD4^+$ T cells was performed as follows:. We isolated the PBMCs from the heparinized blood of Japanese healthy donors by means of Ficoll-Conray density gradient centrifugation. $CD4^+$ T cells and $CD14^+$ cells were purified from PBMCs. Monocyte-derived DCs were generated from $CD14^+$ cells and used as antigen-presenting cells (APCs) to induce antigen-specific $CD4^+$ T cells. $CD14^+$ cells co-cultured with human GM-CSF (100 ng/ml) and human IL-4 (10 ng/ml) in a 10 cm tissue culture dish for 7 days. On day 5, OK-432 (0.1 KE/ml) were added into the dish. On day 7, DCs (1×10^4 /well) were pulsed with 10 μg/ml peptide for 3 h, irradiated (45 Gy), and subsequently mixed with $CD4^+$ T cells (3×10^4 /well) in 200 μL AIM-V (Life Technologies) supplemented with 5% human decomplemented plasma in each well of a 96-well, flat-bottomed culture plate. After 7 days, half of the medium was removed from each culture, and fresh medium (100 μl/well) containing irradiated (50 Gy) autologous PBMCs (1×10^5) pulsed with peptide (10 μg/ml) and 5 ng/ml recombinant human IL-7 (rhIL-7) was added. Two days after the second stimulation with peptide, rhIL-2 was added to each well (10 IU/ml). A week later, the stimulated $CD4^+$ T cells in each well were analyzed for specificity in IFN-γ ELISPOT assays. The T cells showing a specific response to the cognate peptide were transferred to 24-well plates and re-stimulated at weekly intervals with irradiated autologous PBMCs (1×10^6 /well) pulsed with the peptide in medium supplemented with rhIL-2 (20 IU/ml) and rhIL-7 (5 ng/ml) [14].

Results

Expression and characterization of the chimeric HLA-DR4/I-E^d molecule *in vitro*

The *HLA-DRA/I-E^dα* and *HLA-DRB1*0405/I-$E^{d\beta}$* chimeric transgenes were first co-transfected into mouse fibroblast L cells and cell surface expression of HLA-DR4/I-E^d molecules were confirmed by positive staining with FITC-conjugated anti-HLA-DR antibody (TAL15.1) by flow cytometry (data not shown). HLA-DR4-restricted and $WT1_{332-347}$ peptide-specific T cell clones [13] produced IFN-γ in an HLA-DR4-restricted and a peptide-specific manner when T cells were co-cultured with the TAL15.1-positive L-cells pulsed with $WT1_{332-347}$ peptide (Figure 1C). These results suggested that the chimeric HLA-DR4/I-E^d molecules were expressed and could stimulate HLA-DR4-restricted T-cell responses by transduction of the *HLA-DRA/I-E^dα* and *HLA-DRB1*0405/I-E^dβ* chimeric transgenes into the mice.

Characterization of HLA-DR4 Transgenic Mice

To select HLA-DR-positive F_0 mice, their PBMCs were collected and analyzed by cell surface staining with anti-HLA-DR antibodies (anti-HLA-DRα mAb L243 and anti-HLA-DRβ mAb TAL15.1) by FACScan. Genomic DNA prepared from the cells was then subjected to PCR analysis 2 mice were positive for the expression of both HLA-DRα and -DRβ molecules (Figure 2A) and for the presence of *HLA-DRA/I-E^dα* and *HLA-DRB1*0405/I-E^dβ* transgenes (Figure 2B), and they were designated as #5 and #7 founder mice, respectively. These two mice were maintained by crossing with WT C57BL/6 mice, and

Figure 2

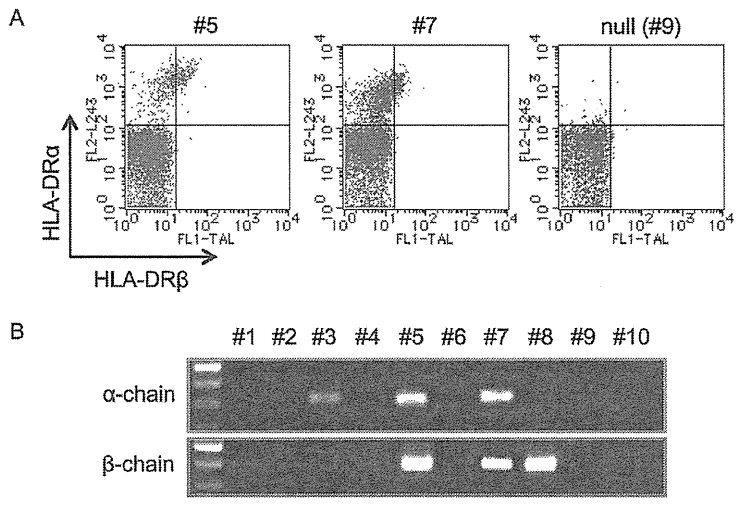

Figure 2. Selection of transgene-positive founder mice (F_0). Twenty five F_0 mice were arbitrarily numbered #1~#25. PBMCs were analyzed for HLA-DR4/I-E^d expression by flow-cytometric analyses (gated on lymphocytes) using anti-HLA-DRα and β mAbs (A) and genomic PCR analyses (B). A typical dot blot of PBMCs from other mice negative for transgenes is shown (null (#9) in A) and results from representative 10 mice from 25 F_0 mice are shown in B.

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the presence and expression of the transgenes were stably transmitted to their descendants (data not shown). Fluorescence in situ hybridization (FISH) analyses using fluorescent-labeled transgenes as probes revealed that the transgenes were inserted in the H2-H4 region of chromosome 3 in the line #5 Tgm (Figure 3A) and in the B-region of Y-chromosome in line #7 Tgm (Figure S1A), respectively.

To examine which subsets of cells expressed HLA-DR4/I-E^d molecules, PBMCs isolated from Tgm were analyzed by flow cytometry using fluorescent-labeled mAbs against HLA-DRα, mouse I-A^b and mouse B220. The majority of B220-positive cells were positive for HLA-DRα (Figure 3B, left panel), and most of the endogenous I-A^b-positive cells were positive for HLA-DRα (Figure 3B, right panel), indicating the expression profile of chimeric HLA-DR4/I-E^d was similar to the endogenous MHC-II, (I-A^b of C57BL/6 mice). Similar observation was also obtained in #7 Tgm (Figure S1B). We noticed that the expression level and positive ratio of HLA-DR4/I-E^d in I-A^b-positive cells were always slightly higher in the chromosome 3-linked line #5 Tgm compared with the Y chromosome-linked line #7 Tgm (Figure 3B and Figure S1B). Therefore, line #5 Tgm (hereafter called Tgm unless otherwise mentioned) were mainly used in the following immunological assays.

To examine the function of chimeric HLA-DR4/I-E^d molecules, Tgm that lacked expression of endogenous $^{MHC\text{-}II}$ gene were generated by crossing Tgm with $^{MHC\text{-}II}$ knock-out mice (B6.129S2-H2$^{dlAb1\text{-}Ea/J}$, Jackson Laboratory) and the presence of $CD4^+$ cells in PBMCs was examined. In PBMCs of $^{MHC\text{-}II}$ knock-out mice (*$DR^{-/-}$MHC-II$^{-/-}$*, Figure 4 left panels), both MHC-II positive cells (1.8%, lower panel) and $CD4^+$ cells (1.7%, upper panel) were almost absent. In PBMCs of Tgm (*$DR^{+/-}$ MHC-II$^{+/+}$*, Figure 4 center panels), both MHC-II positive (27.6%, lower panel) and HLA-DR4/I-E^d positive (16.4%, upper panel) cells were present and normal amount of $CD4^+$ cells were present (66.5%, upper panel). In PBMCs of *$DR^{+/-}$MHC-II$^{-/-}$* mice (Figure 4 right panels), although MHC-II positive cells were absent (0.6%, lower panel), the expression of HLA-DR4/I-E^d (upper panel) in the mice restored the presence of $CD4^+$ cells (59.3%, upper panel). This indicated that expression of either HLA-DR/I-E^d or I-A^b in mice were indispensable for $CD4^+$ cell differentiation and since a comparable proportion of $CD4^+$ cells in PBMCs of *$DR^{+/-}$MHC-II$^{-/-}$* mice with that of *$DR^{+/-MHC\text{-}II+/+}$* mice was observed (59.3% vs 66.5%, Figure 4, upper center and right panels), the chimeric HLA-DR4/I-E^d molecules had an equivalent function to I-A^b and could induce normal differentiation of $CD4^+$ cells. Thus, the chimeric HLA-DR4/I-E^d molecules were functionally expressed in the Tgm.

Induction of HLA-DR4-restricted and non-self peptide-specific Th cells in Tgm

To check that Tgm could induce peptide-specific and HLA-DR4-restricted Th-cell responses, Tgm were immunized with a CMV-derived peptide, CMV-eg$H_{290-302}$; SYLKDSDFLDAAL, that binds HLA-DR4 [12] using CFA and IFA as adjuvants. Th cells derived from immunized Tgm strongly proliferated when co-cultured with CMV-eg$H_{290-302}$ peptide-pulsed L-DR4 cells, but not with unpulsed cells (Figure 5A). In addition, splenocytes isolated from immunized WT C57BL/6 mice did not respond to either peptide-pulsed or unpulsed L-DR4 cells. Therefore, Tgm immunized with HLA-DR4-binding CMV-derived peptides

Figure 3

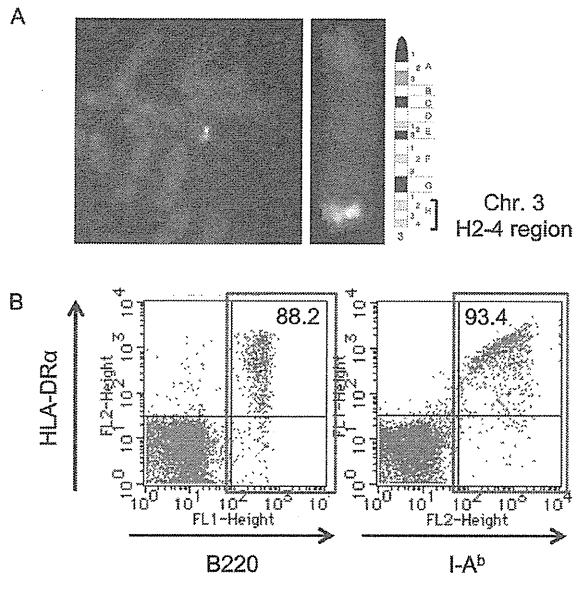

Figure 3. Chromosomal localization of transgene insertion site and cell-type specific expression of HLA-DR4/I-E^d. (A) Mapping of transgene insertion by fluorescence in situ hybridization (FISH) revealed integration in chromosome 3, H2-H4 region in line #5 Tgm. (B) PBMCs from line #5 Tgm were stained with anti-HLA-DR and anti-B220 mAbs (left) or anti-HLA-DR and anti-I-A^b mAbs (right). Numbers indicate the percentage of HLA-DR4/I-E^d-positive cells in B220-positive cells and MHC-II-positive cells indicated by the red boxes, respectively (gated on lymphocytes).

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successfully exhibited HLA-DR4-restricted and peptide-specific Th-cell responses. Thus, the Tgm were useful for screening of HLA-DR4-restricted Th-cell epitopes.

Induction of Th-cell responses specific for known HLA-DR4-binding TAA-derived peptides in Tgm

Next, we determined whether immunization of Tgm with TAA-derived peptides could induce HLA-DR4-restricted peptide-specific mouse Th-cell responses. CDCA1 (cell division cycle associated 1) is a TAA frequently overexpressed in lung cancer, head-and-neck cancer and other malignancies [16,17]. CDCA1$_{55-78}$ peptide (IVYGIRLEHFYMMPVNSEVMYPHL) is a CDCA1-derived peptide consisting of 15 amino acid residues (underlined) predicted to bind to HLA-DR4 with high affinity by a computer algorithm available at the Immune Epitope Data Base site (http://tools.immuneepitope.org/mhcii/). Tgm were immunized with CDCA1$_{55-78}$ peptide and CFA or IFA at the tail base as described in the Materials and Methods. As shown in Figure 5B, proliferation of Th cells was observed in Th cells stimulated with peptide-pulsed L-DR4 cells, but not with unpulsed L-DR4 cells or parental L-cells pulsed with or without the peptide. The data indicated that immunization of Tgm with TAA-derived peptides predicted to bind to HLA-DR4 successfully induced HLA-DR4-restricted and peptide-specific Th cells. As we previously reported [14], the CDCA1$_{55-78}$ peptide is naturally processed from CDCA1 protein by dendritic cells to induce HLA-DR4-restricted Th-cell responses in human PBMCs isolated from HLA-DR4-positive healthy donors and cancer patients. Thus, the screening of TAA-derived HLA-DR4 binding Th-cell epitopes could be performed by the combination of computer algorithm analyses and peptide immunization of Tgm.

The human CDCA1$_{55-78}$ peptide is different from the mouse ortholog peptide at 3 amino acid residues (VYGIRLEHFYMMPVNSEVMYPHL vs VYGVRLEHFYMMPMNIEVTYPHL), and therefore CDCA1$_{55-78}$ peptide is non-self and may be immunogenic in mice. However, the WT1$_{332-347}$ peptide, a known HLA-DR4 binding human Th-cell epitope derived from Wilms' Tumor 1 antigen (WT1) [13], has an identical amino acid sequence with the mouse ortholog peptide. As expected, immunization of Tgm with WT1$_{332-347}$ peptide using CFA and IFA as adjuvants failed to induce Th-cell responses (data not shown). DCs are superior antigen presenting cells and DC-based vaccination is thought to be a promising cancer immunotherapy [18-20]. Thus, to elicit stronger immune responses, we immunized Tgm with WT1$_{332-347}$ peptide-pulsed bone marrow-derived (BM)-DCs with a booster shot of WT1$_{332-347}$ peptide emulsified in CFA. In addition, to evaluate T-helper type 1 (Th1) cell-responses, which are important for the induction of potent anti-tumor immune responses [21-24], IFN-γ ELISPOT assay was performed to assess peptide-specific immune responses. As shown in Figure 5C, IFN-γ producing Th cells were increased in CD4$^+$ cells stimulated with peptide-pulsed L-DR4 cells compared with those stimulated with unpulsed L-DR4 cells. Therefore, BM-DC-based peptide immunization of Tgm in combination with a th-cell assay using IFN-γ ELISPOT is an effective way to screen TAA-derived and HLA-DR4-restricted Th-cell epitopes, especially if the peptides has low immunogenicity.

Using BM-DC-based peptide immunization, we sought to identify the Th-cell epitope in KIF20A, which is frequently overexpressed in gastric cancer [25], melanoma [26], lung cancer, pancreatic cancer [27], bladder cancer, breast cancer, and various other malignancies [28], and thus is a promising target for cancer immunotherapy [29]. The 24-mer KIF20A$_{494-517}$ peptide (TLHVAKFSAIASQLVHAPPMQLGF) consisting of overlapping 15-mer peptides with relatively high affinity binding to HLA-DR4 was predicted by a computer algorithm. In our recent study in humans, the KIF20A$_{494-517}$ peptide induced HLA-DR4-restricted Th-cell responses from PBMCs of an HLA-DR4-positive healthy donor [15].

To investigate the immunogenicity of the KIF20A$_{494-517}$ peptide in Tgm, mice were immunized with KIF20A$_{494-517}$ peptide-pulsed BM-DCs, and then with KIF20A$_{494-517}$ peptide $_{in}$ CFA emulsion. As shown in Figure 5D, Th-cells stimulated with KIF20A$_{494-517}$ peptide-$_{pulsed}$ L-DR4 cells showed a large number of IFN-γ positive spots compared with the Th-cells stimulated with unpulsed L-DR4.

Figure 4

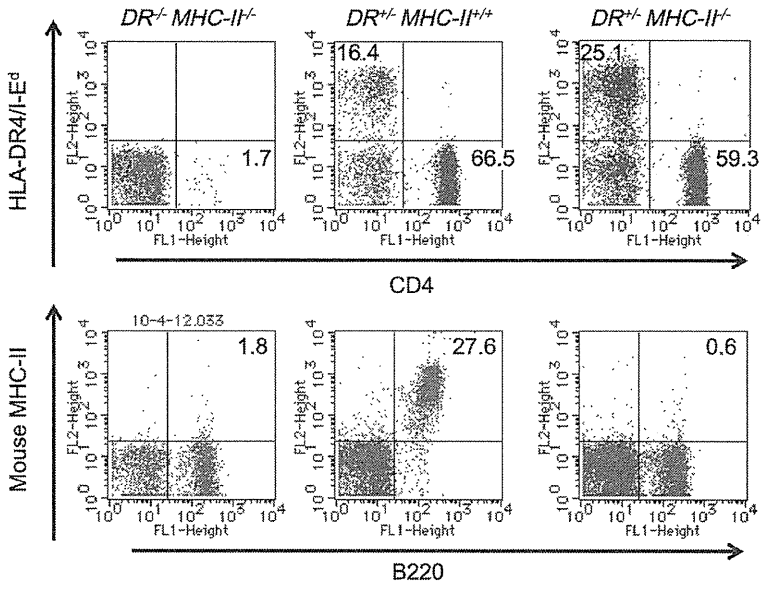

Figure 4. Chimeric HLA-DR4/I-E^d molecules induced the differentiation of mouse CD4$^+$ cells. (Upper panels) PBMCs (gated on lymphocytes) from *MHC-II* knock-out mice (DR$^{-/-}$MHC-II$^{-/-}$), Tgm (DR$^{+/+}$MHC-II$^{+/+}$) and Tgm that lack *MHC-II* (DR$^{+/+}$MHC-II$^{-/-}$) were stained with anti-HLA-DR and anti-mouse CD4 mAbs. (Lower panels) PBMC (gated on lymphocytes) from each mouse was stained with anti-B220 mAb and anti-mouse MHC-II mAb.

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Identification of a novel TAA-derived and HLA-DR4-restricted human Th-cell epitope using Tgm

DEP domain containing 1 (DEPDC1) is a novel TAA classified as cancer-testis antigen that is significantly overexpressed in a majority of bladder cancer specimens [30,31]. A clinical trial of DEPDC1-derived CTL epitope-vaccination was performed in six patients with advanced bladder cancers [32]. Four patients achieved stable disease or partial responses with the induction of CTL responses to DEPDC1$_{294\text{-}302}$ 9-mer peptide, while two cases were clinically non-responsive and negative for CTL responses. To induce more effective anti-tumor immune responses, we searched several candidate Th-cell epitope peptides in DEPDC1 using the computer algorithm and selected DEPDC1$_{191\text{-}213}$ 23-mer peptide (RYVILIYLQTILGVPSLEEVINP) consisting of overlapping 15-mer peptides with predicted high binding affinity to HLA-DR4. DEPDC1$_{60\text{-}85}$ 26-mer peptide (NSNFGPEVTRQQTIQLLRKFLKNHVI) consisting of overlapping 15-mer peptides with predicted relatively low binding affinity to HLA-DR4 was used as a control peptide. Immunization of DEPDC1$_{191\text{-}213}$ peptide emulsified in CFA and IFA in the tail base successfully induced the proliferation of CD4$^+$ cells from immunized Tgm even by *ex vivo* stimulation with the peptide, but not from WT mice (Figure 6A). Also, immunization of Tgm with control DEPDC1$_{60\text{-}85}$ peptide did not induce CD4$^+$ cell proliferation (Figure 6A), suggesting that the peptide with predicted low binding affinity to HLA-DR4 was less immunogenic than that with higher binding affinity. The responses observed in Tgm immunized with DEPDC1$_{191\text{-}213}$ peptide were blocked by the presence of anti-HLA-DR mAb (L243), while the presence of control immunoglobulin had no effect (Figure 6A). Thus, DEPDC1$_{191\text{-}213}$ peptide-specific Th-cell responses were HLA-DR4/I-E^d-restricted in Tgm.

Co-culture of CD4$^+$ cells from PMBCs of an HLA-DR4-positive healthy donor with DEPDC1$_{191\text{-}213}$ peptide-pulsed autologous CD14$^+$ cell-derived DCs and PBMCs induced anti-HLA-DR mAb-sensitive Th-cell responses to the peptide-pulsed L-DR4 cells but not to HLA-DR53-expressing L cells (L-DR53, Figure 6B). Thus, Tgm in combination with the computer algorithm-based analysis demonstrated its usefulness and effectiveness for the preliminary screening of HLA-DR4-restricted Th-cell epitopes.

Discussion

Many *HLA class II* Tgm have been established to identify Th-cell epitopes [33,34], as well as to analyze the pathogenesis of autoimmune diseases when susceptibility is associated with particular *HLA-II* alleles. In these Tgm, *HLA-II* alleles expressed in Tgm are those frequent in Caucasians but not in Japanese. To screen TAA-derived Th-cell epitopes for peptide-based cancer immunotherapy in as many Japanese as possible, we generated *HLA-DR4* Tgm expressing *HLA-*

Figure 5

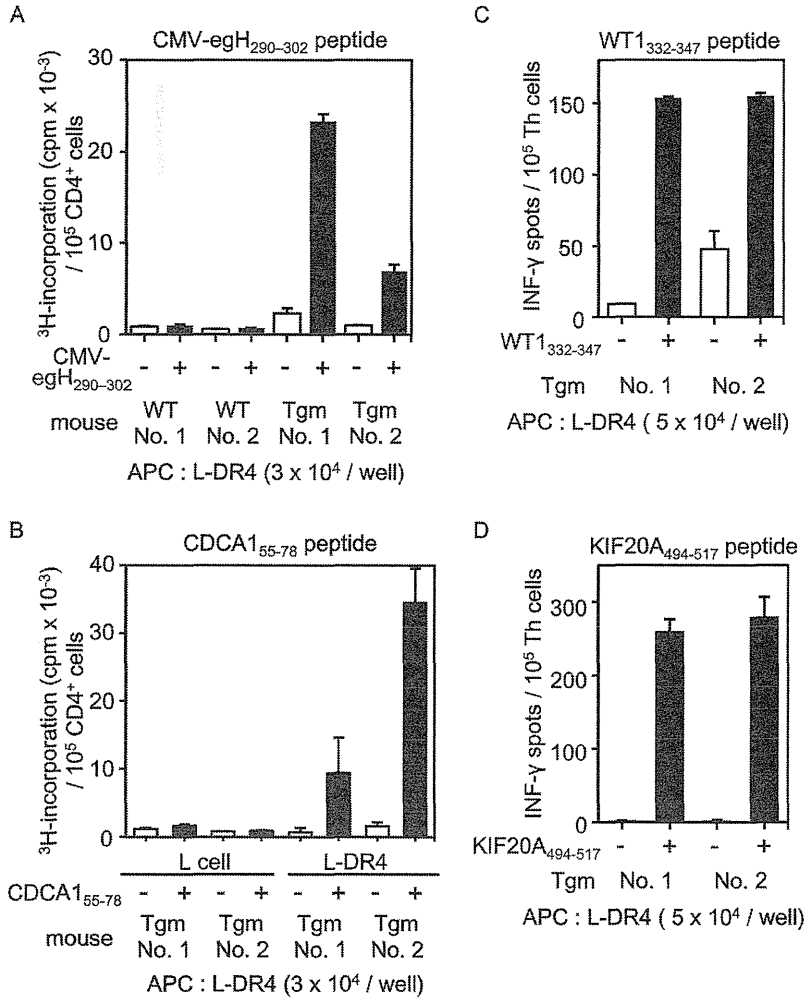

Figure 5. Immunization of TAA-derived peptides induced peptide-specific and HLA-DR4-restricted Th-cell responses in Tgm. Peptides emulsified in CFA and IFA were injected into the tail base of Tgm or C57BL/6 mice (WT) on day 0 and day 7. On day 14, the splenocytes were harvested and cultured *in vitro* for 7 days with the peptides (1 μg/ml). (A) Purified $CD4^+$ cells (1×10^5/well) were co-cultured with L-DR4 (3×10^4/well) pulsed with or without CMV-$egH_{290-302}$ peptide for 72 h and ^3H-thymidine uptake was measured. (B) Purified $CD4^+$ cells (1×10^5/well) were co-cultured with L cells (3×10^4/well) or L-DR4 (3×10^4/well) pulsed with or without $CDCA1_{55-78}$ peptide for 72 h and ^3H-thymidine uptake was measured. (C, D) Immunization of Tgm with syngeneic BM-DCs (5×10^5) pulsed with $WT1_{332-347}$ peptide (C) or $KIF20A_{494-517}$ peptide (D) followed by a booster shot of the peptide in CFA successfully induced IFN-γ production by Th cells (1×10^5/well) in response to the peptide-pulsed L-DR4 cells (5×10^4/well), but not to unpulsed L-DR4 cells.

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*DRA*01:01/HLA*DRB1*04:05* genes of which allele frequency is 13.6% in the Japanese population, that is, about one forth of Japanese have this HLA-DR molecule.

Although the overall amino acid sequence homology between human and mouse CD4 is about 80%, homology of the extracellular domain is only 55% and therefore, interspecies interactions between HLA-DR4 and mouse CD4 could be a potential problem for the induction of HLA-DR4-restricted mouse Th cells. To avoid this, Tgm were generated to express chimeric HLA-DR4/I-E^d molecules in which only the TCR-contacting and peptide-binding α1 and β1 domains were derived from HLA-DR4 but other-domains including CD4-interacting β2 domain were derived from I-E^d. The chimeric HLA-DR4/I-E^d molecules were successfully expressed on the

Figure 6

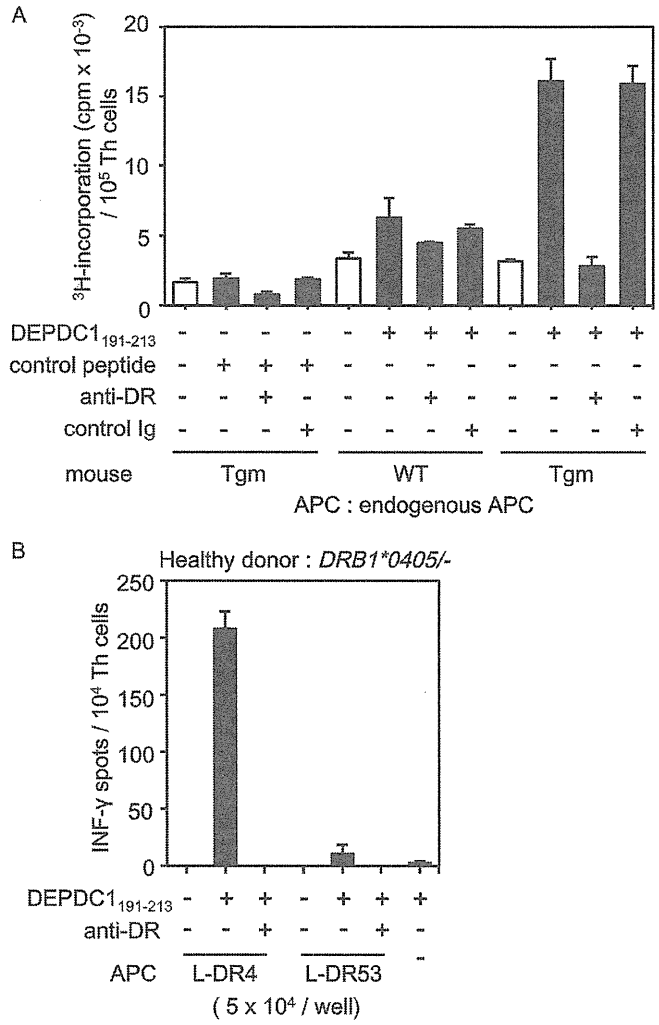

Figure 6. DEPDC1$_{191-213}$ peptide induced peptide-specific and HLA-DR4-restricted Th-cell responses in both Tgm and human PBMCs. (A) Tgm and C57BL/6 mice (WT) were immunized with DEPDC1$_{191-213}$ peptide or control peptide (DEPDC1$_{60-85}$) in CFA and IFA as described in Figure 6 legend. On day 14, inguinal lymph node cells were harvested and *ex vivo* ^{3}H-thymidine incorporation was measured. (B) Induced CD4$^+$ T cells (1 × 10^4/well) were co-cultured with peptide-pulsed L-DR4 (5 × 10^4/well) or L-DR53 cells (5 × 10^4/well) or unpulsed L-DR4 in the presence or absence of anti-HLA-DR blocking mAb L243. The CD4$^+$ T cells cultured with peptide only did not produce IFN-γ.

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surface of mouse L cells by co-transfection of the α and β transgenes as revealed by positive staining with anti-HLA-DR mAb, and L cell transfectants pulsed with WT1$_{332-347}$ peptide induced HLA-DR4-restricted IFN-γ-production and WT1$_{332-347}$ peptide-specific human Th cell clones *in vitro*. Conversely, mouse Th cells from Tgm immunized with WT1$_{332-347}$ peptide responded to HLA-DR4-expressing L-cells in a WT1$_{332-347}$ peptide-specific manner. Therefore, the chimeric HLA-DR4/I-E^d molecules were comparable with intact HLA-DR4 molecules when stimulating WT1$_{332-347}$ peptide-specific human or mouse Th-cell responses.

Although mice lacking endogenous I-A^b expression had very few, if any, CD4$^+$ T cells in the periphery, HLA-DR4/I-E^d expression without endogenous I-A^b expression restored a fraction of CD4$^+$ T cells in mice, comparable to mice expressing both endogenous I-A^b and HLA-DR4/I-E^d molecules. This indicated that chimeric HLA-DR4/I-E^d molecules were functionally equivalent to I-A^b molecules as they could

positively select $CD4^+$ thymocytes. Since the transgenes contained endogenous I-$E^d\alpha$ and I-$E^d\beta$ promoter regions spanning 3.2 kb and 5.2 kb of 5'-untranslated regions, respectively, the expression of HLA-DR4/I-E^d was expected to be cell type- and organ-specific similar to the endogenous I-A^b. Of total PBMCs from Tgm, more than 80% of B220-positive cells and approximately 90% of endogenous I-A^b-positive cells were positive for HLA-DR4/I-E^d, confirming the correct cell type-specific expression of the transgenes.

For FISH analyses, fluorescence-labeled α and β transgenes were used as probes to detect the positions of inserted transgenes. Both transgenes were co-localized in the telomeric H2-H4 region of chromosome 3 in line #5 Tgm or in the B region of the Y chromosome in line #7 Tgm with higher fluorescence intensity compared to the faint fluorescence detected on chromosome 17 where the mouse MHC region exists. This indicated that many transgenes were tandemly inserted in both Tgm lines. Nonetheless, the levels and cell-type specificity of expression of HLA-DR4/I-E^d and endogenous I-A^b were comparable between these two Tgm lines by the flow-cytometric analyses.

CMV-$egH_{290-302}$ peptide is a cytomegalovirus envelope glycoprotein H (egH)-derived peptide reported to bind to HLA-DR4 and induced CMV-$egH_{290-302}$ peptide-specific Th-cell responses[12]. Thus, CMV-$egH_{290-302}$ peptide is a natural HLA-DR4 binding Th-cell epitope that could be used as a positive control to induce specific and HLA-DR4-restricted Th-cell responses in Tgm. As expected, immunization of CMV-$egH_{290-302}$ peptide emulsified with CFA and IFA induced HLA-DR4-restricted and peptide-specific Th-cell responses. Again, mouse Th cells restricted by HLA-DR4/I-E^d molecules responded to CMV-$egH_{290-302}$ peptide presented by intact HLA-DR4, indicating that HLA-DR4/I-E^d and HLA-DR4 were interchangeable and that Tgm could be used for screening and identification of HLA-DR4-restricted Th-cell epitope peptides.

Since the $CDCA1_{55-78}$ peptide was predicted to be a strong HLA-DR4 binder but is a non-self peptide in mice, it was a good immunogenic peptide to induce mouse Th-cell responses in Tgm. A similar protocol to that used for CMV-$egH_{290-302}$ peptide immunization induced $CDCA1_{55-78}$ peptide-specific and HLA-DR4-restricted Th-cell responses. However, the $WT1_{332-347}$ peptide has an identical amino acid sequence to the mouse ortholog peptide, and immunization with CFA and IFA adjuvants was not successful, while immunization using peptide-pulsed DC was effective for the induction of peptide-specific and HLA-DR4-restricted Th-cell responses. Therefore, to screen the computer algorithm-predicted peptides that were expected to be less immunogenic for mice, the utilization of DCs prepared from syngeneic BM-DCs could be an alternative protocol for immunization. The $KIF20A_{494-517}$ peptide includes a 15-mer amino acid residue predicted to bind to HLA-DR4 with low affinity, and there are differences in three amino acid residues between the human peptide and mouse ortholog. However, the predicted core binding amino acid sequences to HLA-DR4 are similar between humans and mice (AKFSAIASQ vs AKFSALASQ), in which the only different amino acid residue, P6, is not in contact with the TCR but MHC-II. Thus, the $KIF20A_{494-517}$ peptide could be less immunogenic in mice to induce apparent Th-cell responses presented by HLA-DR4/I-E^d. However, immunization of $KIF20A_{494-517}$ $peptide_{-pulsed}$ BM-DCs followed by a booster shot using the same peptide in CFA emulsion successfully induced peptide-specific and HLA-DR4-restricted Th-cell responses. Therefore, the screening efficiency could be improved if peptide-pulsed DCs were used for immunization.

The $DEPDC1_{191-213}$ peptide has a different amino acid sequence to the mouse ortholog peptide (RYVILIYLQTILGVPSLEEVINP vs RYVIMIYLQTILSLPSIEELLNP), thus immunization using CFA and IFA as adjuvants was successful. DEPDC1 is over-expressed in various cancers, especially in bladder cancer and a clinical trial using DEPDC1-derived short CTL epitope peptide-vaccination was performed for 6 patients with advanced bladder cancer [30-32], although the clinical response was marginal. In a mouse model, co-vaccination of CTL- and Th-epitope peptides from HER-2 increased the numbers of specific CTL and Th cells and decreased the number of regulatory T cells compared with CTL-epitope vaccination alone [35]. In other clinical trials, Slingluff and colleagues recently reported that combination of CTL epitope- and Th cell epitope-vaccination in melanoma patients induced clinical benefit compared with CTL epitope-vaccination alone [36], and Woods and Cebon argued that tumor-specific T-cell help was associated with improved survival in melanoma [37]. Thus, co-vaccination of the DEPDC1 CTL peptide and Th epitope identified by using Tgm may induce additional or synergistic immune responses and clinical effects for bladder cancer patients.

In conclusion, the HLA-DR4 Tgm established in this study are useful for preliminary screening of HLA-DR4-restricted Th-cell epitope peptides derived from TAAs among candidate peptides predicted to bind to HLA-DR4 by the computer algorithm. If the Th cells from Tgm immunized with TAA peptides were reactive to syngenic BM-DCs incorporated whole TAA protein, the screened peptides could be proven to be naturally processed and such experimental system could be a powerful tool for the identification of Th-cell epitope peptides that could be applicable for vaccines for cancer immunotherapy.

Supporting Information

Figure S1. Chromosomal localization of transgene insertion site and Cell-type specific expression of HLA-DR4/I-E^d (line #7 Tgm). (A) Mapping of transgene insertion by FISH revealed integration in chromosome Y, B-region in line #7 Tgm. (B) PBMCs from line #7 Tgm were stained with anti-HLA-DR and anti-B220 mAbs (left) or anti-HLA-DR and anti-I-A^b mAbs (right). Numbers indicate the percentage of HLA-DR4/I-E^d-positive cells in B220-positive cells and MHC-II-positive cells indicated by the red boxes, respectively (gated on lymphocytes).
(TIF)

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Author Contributions

Conceived and designed the experiments: JY AI YN. Performed the experiments: JY AI KH YM NT FF. Analyzed the data: JY AI HT S. Senju YN. Contributed reagents/materials/analysis tools: YT AY MH MAS IS S. Sogo HS ME. Wrote the manuscript: JY AI YN.

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Identification of HLA Class I-binding Peptides Derived from Unique Cancer-associated Proteins by Mass Spectrometric Analysis

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Abstract. *Background/Aim: Since antigenic peptides of the cancer-associated antigens presented on human leukocyte antigen (HLA) molecules are recognized by specific cytotoxic T-lymphocytes, they have the potential to becoming effective peptide vaccines for cancer immunotherapy. Materials and Methods: Peptides binding to HLA-A*0201 and HLA-A*2402 obtained from human prostate cancer cells by acid-elution were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and source proteins of the peptides were determined based on the HLA-binding capacity listed on the Syfpeithi. Results: We identified TKLSA possibly derived from absent in melanoma 1-like protein (AIM1L), and RLRYT from trans-membrane protein-191C (TMEM 191C) or c20orf201. Messenger RNAs encoding these proteins were expressed in various cancer cell types but none or very few in non-cancerous tissues except for testis, cerebellum and ovary. Conclusion: HLA class I-binding peptides of unique cancer-associated proteins were identified by MS analysis, and might become a promising tool for the generation of novel cancer vaccines.*

Immune response to cancer-associated antigens expressed in cancer cells, but absent or scarcely expressed in non-cancerous cells, might play an important role for T-cell-mediated cancer cell elimination (1-3). Although immune attack on cancer cells alone is unlikely to suppress tumor progression in advanced stages of cancer, caused by the development of immune-escape by tumors, therapeutics based on the immune response against cancer-associated antigens might have the potential to eliminate tumors, providing that cancer-associated immune suppression can be properly regulated by treatment with bio-modulatory or immune-stimulating agents (4-7).

Cancer-associated antigens which could become suitable targets for T-cell-mediated antitumor immune response and their antigenic peptide, which could become synthetic peptide vaccines against cancer, have been enthusiastically sought (8). Essentially, antigenic peptides naturally present on major histocompatibility complex (MHC) class-I molecules on the surface of cancer cells would be the most relevant targets recognized by specific cytotoxic T-cells. Recently, high-throughput methods for a proteomics-based search for cancer-associated antigens have provided a new trend in the development of cancer immunotherapy (9, 10). Identification of HLA class I-presented cancer-associated antigens using immuno-proteomics has been reported, and antigenicity of the identified peptides, stimulating T-cells was demonstrated (11-13). Besides identification of cancer-associated antigens, proteomic tools are increasingly applied for the discovery of important molecules in cancer-associated immuno-biology or biomarkers for diagnosis and treatment of cancer (14, 15).

Immunoproteomics, using the liquid chromatography-tandem mass spectrometry (LC-MS/MS) platform is useful for the identification of naturally-processed antigenic peptide of cancer-associated antigens presented on MHC molecules (16). We previously reported the LC-MS/MS-based identification of MHC class-II binding antigenic peptide derived from cytochrome *P-450* 2J6 isoform expressed in murine hepatocellular carcinoma (HCC) cells by analyzing MHC class-II binding peptides from dendritic cells loaded with HCC cells (17). Synthetic analogue of the antigenic peptide was shown to be immunogenic and vaccination of mice with the peptide was able to stimulate cluster of differentiation (CD)4^+ T-cells. Morse *et al.* (13) described

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Key Words: HLA class-I, antigenic peptide, cancer associated antigen, prostate cancer, mass spectrometric analysis.

that vaccination of cancer patients with cancer-specific peptides identified by immunoproteomic analysis was successfully-able to augment the immune response towards solid tumors. Proteomics for generation of novel anticancer vaccines might have great value for the development of immune-based therapeutics.

In the late stage of metastatic prostate cancer showing resistance to hormone therapy, treatment modalities are limited and new therapeutic options are required for patients with such advanced disease. Prophylactic immunotherapy to prevent the progression to a hormone-resistant stage might contribute to the improvement of prognosis of prostate cancer (9, 14). In the present study, using immunoproteomics analysis, we identified HLA class I-binding peptides derived from unique cancer-associated proteins expressed in human prostate cancer cells, possibly leading to the generation of novel cancer vaccines for prostate cancer.

Materials and Methods

Cell lines. Human prostate cancer cell lines (LNCaP, PC3 and DU145), bladder cancer cell lines (T24, 5637 and TCCSUP) and bladder inverted papilloma cell line (RT4) were obtained from the American Type Culture Collection (Manassas, VA, USA). LNCaP-expressing HLA-A*24 (LNCaP-A*24) cell line was kind gift from Dr. Tanaka, Department of Urology, Sapporo Medical University, Sapporo, Japan (18). Ovarian cancer cell lines (SKOV3, Hac-2, OV-1063, JHOC-5 and JHOC-9), endometrial cancer cell line (Ishikawa), colon cancer cell lines (LOVO, SW48, LS180,, HCT116, SW480 and DLD-1) and chronic myelogenous leukemia cell lines (K562 and KU812) were kindly provided from Professor Okamoto, Dr. Sasaki (Department of Obstetrics and Gynecology Jikei University, Tokyo, Japan), Dr. Ito (Department of Oncology, Institute of DNA Medicine, Jikei University, Tokyo, Japan) and Dr. Kawano (Department of Molecular Genetics, Institute of DNA Medicine, Jikei University, Tokyo, Japan), respectively.

LNCaP, PC3, DU145, RT4, T24, 5637, TCCSUP, LOVO, SW48, LS180, HCT116, SW480, DLD-1, PANC-1, BxPC-3, SKOV-3, Hac-2, OV-1063, JHOC-5, JHOC-9, BT20, K562 and KU812 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, NICHIREI BIOSCIENCES INC., Tokyo, Japan) and Antibio-Antimyco (Life Technologies, Carlsbad, CA, USA). Ishikawa, AsPC-1, Capan-1, Capan-2, MIA-Paca-2 and MCF7 cells were cultured in DMEM supplemented 10% FBS and Antibio-Antimyco. LNCaP-A24 cell lines were cultured in RPMI 1640 supplemented 10% FBS and Antibio-Antimyco with 500 ng/ml puromycin (Life Technologies).

Acid treatment. LNCaP and LNCaP-A24 cells were grown sub-confluently in four 24-cm square dishes. After washing with Phosphate-buffer saline (PBS), cells were collected with a scraper and centrifuged for 5 min at 190 ×*g*. The cells were further washed with PBS twice, and treated with 10 ml of acid solution (0.13 M citric acid and 0.06 M Na_2HPO_4, pH 3.0) for one minute (19). After centrifugation, the supernatant was collected for MS/MS analysis.

LC-MS/MS analysis. The eluted peptide solution was analyzed *via* electrospray ionization (ESI) liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Q-TRAP triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with TurboionSpray source with information dependent acquisition (IDA). The mass spectrometer interfaced with an Agilent 1100 liquid chromatography and autosampler (Agilent Technologies, Wilmington, DE, USA). The ion source conditions and gas setting were as follows: ion spray voltage=5500 V, ion source heater temperature=500°C, collision gas setting=4, ion source gas 1 setting=50 and gas 2 setting=50, curtain gas setting=40. de-clustering potential=30 V, collision energy=40 V, collision exit potential=15 V. LNCaP and LNCaP-A24 cell-binding peptides were eluted at a flow rate of 0.2 ml/min from a Synergi MAX-RP 80A column (150×2.0 mm, 4 μm particle size) (Phenomenex, Torrance, CA, USA) using a linear gradient of 1.6% min^{-1} of 5-100% ACN (acetonitorile) containing 1% formic acid (FA). The LC-Q-TRAP mass spectrometer was controlled by the Analyst softoware 1.3.2. (Applied Biosystems/MDS SCIEX, Foster City, CA, USA). All the measured MS/MS data in an IDA file were analyzed by the Analyst software 1.3.2 Mascot script. The script can be used to send data over the Internet to the Matrix Science website. The unknown peptides were identified using Mascot MS/MS ion search engine (Matrix Science, Boston, MA, USA).

Protein identification. Possible source proteins containing the identified peptide sequence were searched using Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda MD, USA). Amino acid sequences of these candidate proteins were analyzed by Syfpeithi to determine whether the 9-mer peptide containing the identified peptide shows high binding affinity to HLA-A*2402, HLA-A*01 and HLA-A*0201. Proteins containing the identified peptide sequence with high binding affinity to specific HLA types were defined as candidate proteins.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Cancer cells (2.5×10^6) were seeded in a 10cm Petri dish. The following day, cells were collected and RNA was extracted using PARIS kit (Life Technologies). Normal tissue RNA was purchased from TAKARA BIO INC., Life Technoogies and Agilent (stomach: Human Stomach Total RNA, TAKARA BIO INC., Shiga, Japan; bladder: Life technologies; ovary, breast and pancreas: Agilent; and other tissues: Human Total RNA Master PanelII, TAKARA BIO INC.). One microgram of total RNA was used to synthesize cDNA with PrimeScript® RT reagent Kit with gDNA Eraser (TAKARA BIO INC.). Quantitative-PCR (qPCR) of *18S* ribosomal RNA and *c20orf201* was performed with Taqman gene expression assay (Life Technologies) and absent in melanoma 1-like protein (AIM1L), trans-membrane protein 191C (TMEM 191C) was performed with Solaris qPCR assay (Thermo Fisher Scientific Inc., Waltham, MA, USA). The expression of mRNA was analyzed by comparative Ct methods using *18S* ribosomal RNA as an internal control.

Results

Among peptides isolated from HLA molecules on the prostate cancer cells, representative 5- or 6-mer peptides were selected and their structural amino acid sequences were determined by LC-MS/MS. The number of candidate proteins and antigenic peptides presented in the context of HLA-A*0201, A*01 and A*2402 are shown in Table I.

Table I. *Identified peptide sequences from LNCaP and LNCaP-A24 cells.*

Peptide	Sequence	Cell line	No. of candidate proteins	No. of Syfpeithi-predicted proteins		
				A*0201	A*01	A*2402
1	TKLSA	LNCaP	29	26	8	-
2	TRAGD	LNCaP	3	2	0	-
3	PARSGA	LNCaP	2	1	0	-
4	RLRYT	LNCaP-A*2402	6	6	3	2
5	SMLAER	LNCaP-A*2402	1	0	1	0

Predictive scores of each peptide for binding affinity to each HLA type are shown in Table II. Possible cancer-associated molecules with high HLA-binding affinity were selected from each candidate. AIM1L was chosen as a possible source protein of TKLSA. TKLSA might be 925-933 amino acid of the AIM1L protein and the antigenic peptide should be PLGTKLSAL. TMEM191C and uncharacterized protein C20orf201 were chosen as possible source proteins of RLRYT. RLRYT might be 325-333 amino acid of the TMEM191C protein and the antigenic peptide should be TLRRLRYTL, or 125-133 amino acid of the C20orf201 protein and the antigenic peptide should be ALRLRYTRM.

AIM1L mRNA expression in several cancer cells lines and non-cancerous tissues are shown in Figure 1. Considerable high *ALM1L* mRNA expression was seen in several cancer cell lines but only placenta and testis exhibited high mRNA expression among noncancerous tissues. *TMEM191C* mRNA was expressed in various kinds of cancer cell, but characteristically high mRNA expression was seen in only testis among non-cancerous tissues (Figure 2). Expression of uncharacterized protein *C20orf201* mRNA was high in several cancer cell lines but only in testis and cerebellum among non-cancerous tissues (Figure 3).

Discussion

Five or six-mer peptides from the human prostate cancer cells obtained by a simple acid elution method were analyzed by LC-MS/MS. Since treatment of the cells with phosphate-citrate buffer at pH 3.3 decomposes the multi-subunit structure of the HLA molecule, antigenic peptides presented on HLA molecules should be released from the HLA (19). Although antigenic peptides recognized by specific Cytotoxic T-Lymphocytes (CTLs) are 8- or 9-mer in general, we did not find any proper candidate peptides composed of 8 or 9 amino acids by LC-MS analysis. Alternatively, 5- or 6-mer peptides were found as a possible decomposed product derived from HLA class I-binding antigenic peptide. These peptides might have been digested and truncated during the dissociation process. In fact, we identified a murine MHC class II-binding antigenic peptide from the peptide composed of four amino acids obtained from dendritic/HCC fusion cells. Based on the specific anchor structure in the context of MHC binding and phenotypic characteristics associated with HCC, we have assumed that this peptide might have been derived from cytochrome *P450* 2J isoform (17). A synthesized 16-mer peptide containing the four amino acid sequence with high affinity to the murine MHC class-II was actually immunogenic and vaccination of mice with this 16-mer antigenic peptide induced interferon-gamma production by stimulated T-cells (17). Accordingly, it seems to be possible to determine the structure of the original antigenic peptide and identify the source protein by analyzing the truncated peptide derived from the source protein.

Two peptides possibly derived from unique proteins, which are scarcely reported in the literature and seemingly a kind of cancer-associated phenotype, were obtained in the present study. AIM1, a novel non-lens member of betagamma-crystallin superfamily, was reported to be associated with chromosome-6 mediated tumor suppression (20, 21). Vainio *et al*. demonstrated that AIM1 was highly expressed in primary prostate cancer and cultured androgen-independent prostate cancer cells and suggested that AIM1 might be a potential drug target for treatment of prostate cancer (22). In the present study, *AIM1L* mRNA was found to be expressed in several cancer cell lines, but only in the placenta and testis among non-cancerous tissues. Although none of the characteristics of *TMEM191C* are known, it was found to be expressed in various cancer cell lines, but only testicular tissue exhibited high expression of *TMEM191C* among normal organs. *C20orf201* was identified by the Mammalian Gene Collection Program (23) without determination of functional property. Although no reports concerning association of *C20orf201* with cancer have been found so far, *C20orf201* was significantly expressed in various cancer cell lines but, here again, only in testis among normal tissues.

According to the expression signature of these three proteins in normal and malignan T-cells, it seems likely that they might have characteristics of a cancer-associated