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Effective induction of anti-tumor immune responses with oligomannose-coated liposome targeting to intraperitoneal phagocytic cells

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Abstract

We recently established a novel drug delivery system (DDS) using oligomannose-coated liposomes (OMLs) which are probably taken up by macrophages (Mϕ) to carry anti-cancer drugs to milky spots known as preferential metastatic sites of gastric cancers [Y. Ikehara, T. Niwa, L. Biao, S.K. Ikehara, N. Ohashi, T. Kobayashi, Y. Shimizu, N. Kojima, H. Nakanishi, A carbohydrate recognition-based drug delivery and controlled release system using intraperitoneal macrophages as a cellular vehicle, Cancer Res. 66 (2006) 8740–8748]. In the present study, we applied this intraperitoneal DDS for systemic cancer immunotherapy employing ovalbumin (OVA) as a model antigen. The cells taking up the OMLs containing FITC-OVA injected into the peritoneal cavity were predominantly Mϕ, as they showed adhesive characteristics and expressed F4/80 and CD11b almost exclusively. The phagocytic cells also took up bare OVA directly to the same extent as OML-enclosed OVA (OML-OVA), as it is a highly mannosilated protein. The phagocytic cells taking up OML-OVA, however, could activate OVA-specific $CD8^+$ (from OT-I: H-$2K^b$/$OVA_{257-264}$-specific) and $CD4^+$ (from OT-II: H-$2A^b$/$OVA_{323-339}$-specific) T cells much more effectively *in vitro* than those taking up bare OVA. Furthermore, only the mice pre-immunized with OML-OVA rejected E.G7-OVA (OVA-transfected EL4) but not EL4. These results indicate that

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the OMLs can also be used as an effective antigen delivery system for cancer immunotherapy activating both CTL and Th subsets.

Keywords: Drug delivery system; Cancer vaccine; Immune responses to cancer; Oligomannose liposome

1. Introduction

While recent advances in tumor immunology enable us to identify tumor antigens recognized by T cells and understand the molecular and cellular bases of T cell-mediated anti-tumor responses, the clinical realization of effective immunotherapy for solid tumors has not yet been convincingly achieved [1,2]. Many $CD8^+$ and $CD4^+$ T cells recognizing tumor antigen in the context of MHC class I and II, respectively, have been reported, and the former are known to be a major effector of the adaptive anti-tumor immune responses [3–5]. $CD4^+$ T cells play an important role for the expansion and persistence of $CD8^+$ T cells, while some of them are known to function as regulatory cells [5–7]. Optimal anti-tumor immune responses are therefore considered to require the concomitant activation of both $CD8^+$ and $CD4^+$ T cells and the selective activation of $CD4^+$ T cells with helper but not regulatory functions [8]. Endogenous and exogenous antigens are presented as peptides preferentially by MHC class I and II, respectively, and most tumor antigen peptides are derived from the proteins expressed endogenously. Novel methods to make tumor antigens presented simultaneously by both MHC class I and II molecules are therefore needed for the concomitant activation of antigen-specific $CD8^+$ and $CD4^+$ T cells, and many attempts have been made for this purpose [2,3,8].

We recently developed a novel drug delivery system (DDS) using oligomannose-coated liposomes (OMLs) [9,10] which are effectively taken up by $F4/80^+$ intraperitoneal cells to carry anti-cancer drugs to milky spots known as a preferential metastatic site of gastric and ovarian cancers [9,11,12]. We demonstrated that this system could control the formation of overt metastasis of seeded gastric cancer cells at the extra-nodal lymphoid tissues such as the omentum [10].

In the present study, we applied this OML-based intraperitoneal DDS for cancer immunotherapy using ovalbumin (OVA) as a model antigen, aiming at the concomitant activation of antigen-specific $CD8^+$ and $CD4^+$ T cells. Peritoneal phagocytic cells took up OML containing OVA and then migrated into milky spots as previously reported. In addition, they activated both OVA-specific $CD8^+$ [13,14] and $CD4^+$ [15] T cells effectively *in vitro*. Spleen cells from OML-enclosed OVA (OML-OVA)-injected mice showed an effective killing activity against E.G7-OVA (OVA-transfected EL4) [16] but not EL4 [17] *in vitro*, and only the mice pre-immunized with OML-OVA rejected E.G7-OVA but not EL4 *in vivo*. In light of these results obtained *in vitro* and *in vivo*, the potential of our novel OML-based immunization method for the prevention of tumor metastasis is discussed.

2. Materials and methods

2.1. Mice

Female C57BL/6 (B6) mice ($H\text{-}2^b$) at 8–12 weeks of age were obtained from Charles River Japan Inc. (Yokohama, Japan) and kept under standard housing conditions. T cell receptor transgenic mice OT-I (specific for $H\text{-}2K^b/OVA_{257\text{–}264}$) [13,14] and OT-II ($H\text{-}2A^b/OVA_{323\text{–}339}$) [15] were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions. All animal experiments were performed under the experimental protocol approved by the Ethics Review Committee for Animal Experimentation of Aichi Cancer Center.

2.2. Cell lines

EL4 [17], a B6-derived thymoma cell line, was maintained in RPMI1640 medium (Invitrogen, Carlsbad, CA) supplemented with 8% fetal bovine serum, 0.2% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1% Hepes, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 50 μM 2-ME (complete RPMI). EG.7-OVA (EL4 transfected with *OVA* gene) [16] was obtained from ATCC (Manassas, VA) and maintained in complete RPMI supplemented with 400 μg/ml G418 (Wako, Osaka, Japan) in a humidified 5% CO_2 incubator at 37 °C.

2.3. Man3-DPPE and liposome preparation

Dipalmitoylphosphatidylcholine (DPPC), cholesterol, and dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Sigma–Aldrich (St. Louis, MO). Mannotriose (Man3: Manα1-6(Manα1-3)Man) was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Man3-DPPE was prepared by conjugation of the mannotriose with DPPE by reductive amination as described in previous papers [10,18]. The purity of Man3-DPPE was confirmed by high-performance thin-layer chromatography (Silica gel 60 HPTLC plate, MERCK, Darmstadt, Germany) and time-of-flight mass spectrometry (Auto FLEX, Bruker Daltonics, Bremen, Germany). The purified Man3-DPPE was quantified by determination of the phosphate contained.

Liposomes were prepared as described previously [10]. Briefly, a chloroform–methanol (2:1, v/v) solution containing 1.5 μmol of DPPC and 1.5 μmol of cholesterol was placed in a conical flask and dried by rotary evaporation. Subsequently, 2 ml ethanol containing 0.15 μmol of Man3-DPPE was added to the flask and evaporated to prepare a lipid film containing neoglycolipids. Procedures for protein-encasing of oligomannose-coated liposomes (OMLs) were performed as described previously [10]. The multilamellar vesicles were generated with either 200 μl of FITC-labelled or non-labelled OVA (5.0 mg/ml, Sigma–Aldrich), Alexa Fluor 680 (Molecular Probes, Eugene, OR)-labelled bovine serum albumin (BSA, 5 mg/ml, Sigma–Aldrich), or PBS in the dried lipid film by intense vortex dispersion. The multilamellar vesicles were extruded 10 times through polycarbonate membranes of 1 μm pore (Nucleopore, Pleasanton, CA). Liposomes entrapping proteins were separated from free untrapped proteins by four successive rounds of washing in PBS with centrifugation (20,000*g*, 30 min) at 4 °C. The amounts of entrapped proteins were measured using a modified Lowry protein assay reagent (Pierce, Rockford, IL) in the presence of 0.3% (w/v) sodium dodecyl sulfate using BSA as the standard.

2.4. Flow cytometry

One hour after intraperitoneal injection, peritoneal exudate cells (PEC) were recovered from B6 mice with 5 ml ice cold PBS. PEC were incubated on ice for 30 min with fluorescein-labelled antibodies against mouse hematopoietic cell lineage markers after blocking with mouse Fc Blocker (BD Biosciences, San Jose, CA) and then analysed on a FACS Calibur (BD Biosciences). The following monoclonal antibodies used in this study were purchased or kindly provided: anti-F4/80 (A3-1, Serotec Ltd., Oxford, UK), anti-MHC class II (M5/114.15.2, e-Bioscience, Boston, MA), anti-CD11b (M1/70.15, Caltag Laboratories, Burlingame, CA), anti-CD3ε (145-2C11, BD Biosciences), anti-CD19 (1D3, BD Biosciences), and anti-H-2K^{b}D^b (20-8-4S, Dr. E. Nakayama, Okayama University).

2.5. Macrophage depletion by plastic adhesion

PEC suspension (2×10^7 cells in 10 ml of complete RPMI) was poured into a 75 cm^2 tissue culture flask and incubated at 37 °C for 2 h in a humidified 5% CO_2 incubator. Non-adherent cells were collected with serum-free DMEM and subjected to FACS analysis.

2.6. In vitro activation of OVA-specific T cells

One hour after injection of either OML-encased OVA or bare OVA into the peritoneal cavity of B6 mice, PEC were recovered with 5 ml ice cold PBS. The PEC suspended in complete RPMI were seeded into a 96-well culture plate (5×10^5 cells in each well) and incubated at 37 °C for overnight in a humidified 5% CO_2 incubator. On the next day, non-adherent cells were washed out with complete RPMI, and co-cultured with 5×10^5 cells of either CD8$^+$ or CD4$^+$ T cells from the spleen of OT-I and OT-II mice, respectively. CD8$^+$ and CD4$^+$ T cells were prepared with the isolation kits for corresponding subsets (Miltenyi Biotec Inc., Auburn, CA). The supernatants were collected at 24, 48, and 72 h and assayed for IFN-γ production with Mouse IFN-γ ELISA kit (Pierce Biotechnology, Inc., Rockford, IL).

2.7. CTLs assay

B6 mice were immunized biweekly three times by intraperitoneal injection of 1 μg OVA in liposome/mouse with or without oligomannose coating. Spleen cells were isolated from the mice 1 week after the last challenge, and 1×10^6 spleen cells were stimulated with 10 μg OVA in 1 ml for 72 h. The effector cells thus prepared were co-cultured with target cells (E.G7-OVA or EL4) at various effector/target ratios for 8 h at 37 °C, and the cytotoxicity was measured with CytoTox96 Non-Radioactive Cytotoxicity assay kit (Promega, Madison, WI).

2.8. Tumor assay

Tumor cells (in 0.2 ml) were injected intradermally into the backs of mice with a 27 gauge needle. The diameter of the tumors was measured with Vernier calipers twice at right angles to calculate the mean diameter, and the survival time after tumor challenges was followed.

3. Results

3.1. OMLs are taken up preferentially by intraperitoneal macrophages

We showed that neoglycolipid-coated liposomes are ingested by intraperitoneal cells much more effectively than non-coated liposomes [10]. Of those, OMLs are incorporated most effectively, and the cells ingesting

OMLs are preferentially F4/80$^+$ and migrate into extranodal lymphoid tissues in the omentum after the uptake. We have also shown that the OML-ingesting cells are very useful drug delivery vehicles for cancer chemotherapy in the previous study [9,10]. To verify whether the OMLs are applicable also for cancer immunotherapy, we first analyzed in detail the peritoneal cells incorporating OMLs. Bovine serum albumin (BSA, Sigma–Aldrich) was labelled with Alexa Fluor 680, encased in OML and then injected into the peritoneal cavity of B6 mice. One hour after the injection of OMLs containing Alexa Fluor 680-labelled BSA, PEC were collected and analyzed. As shown in Fig. 1A, PEC were divided into three groups based on the incorporation of OMLs. When adherent cells were removed by plastic dish adherence, only the population with higher OML uptake (R1) disappeared (Fig. 1B). In addition, most cells of R1 express F4/80 and CD11b but not CD3 and CD19 (Fig. 1C), suggesting that R1 population preferentially consists of macrophages (Mϕ). The PEC with lower OML uptake (R2) did not express F4/80, and nearly 2/3 of them were considered to be B cells because of their CD19 expression. These results together confirmed that OMLs injected into the peritoneal cavity were ingested preferentially by Mϕ, and also indicate that OML is a good vehicle for the phagocytosis of non-glycosylated proteins.

3.2. Phagocytic cells ingesting OMLs activate both CD8 and CD4 T cells in vitro in an antigen-specific manner

We next analyzed the antigen-presenting capacity of the phagocytic cells ingesting OMLs containing ovalbumin (OVA) as an antigen. CD8$^+$ T cells from OT-I (a transgenic strain of T cell receptor (TCR) recognizing $OVA_{257-264}$ peptide presented by H-2K^b) and CD4$^+$ T cells from OT-II (a transgenic strain of TCR recognizing $OVA_{323-339}$ peptide presented by H-2A^b) were used as responder cells. When these T cells were co-cultured with adherent cells enriched from PEC of the mouse intraperitoneally injected with OML-encased OVA (OML-OVA), both CD8$^+$ and CD4$^+$ T cells produced large amounts of IFN-γ (Fig. 2). Though adherent cells from the mice injected with soluble OVA also stimulated both CD8$^+$ and CD4$^+$ T cells, much higher amounts of OVA were needed compared to those from the mice injected with OML-OVA. Mϕ ingesting OML-OVA are supposed to

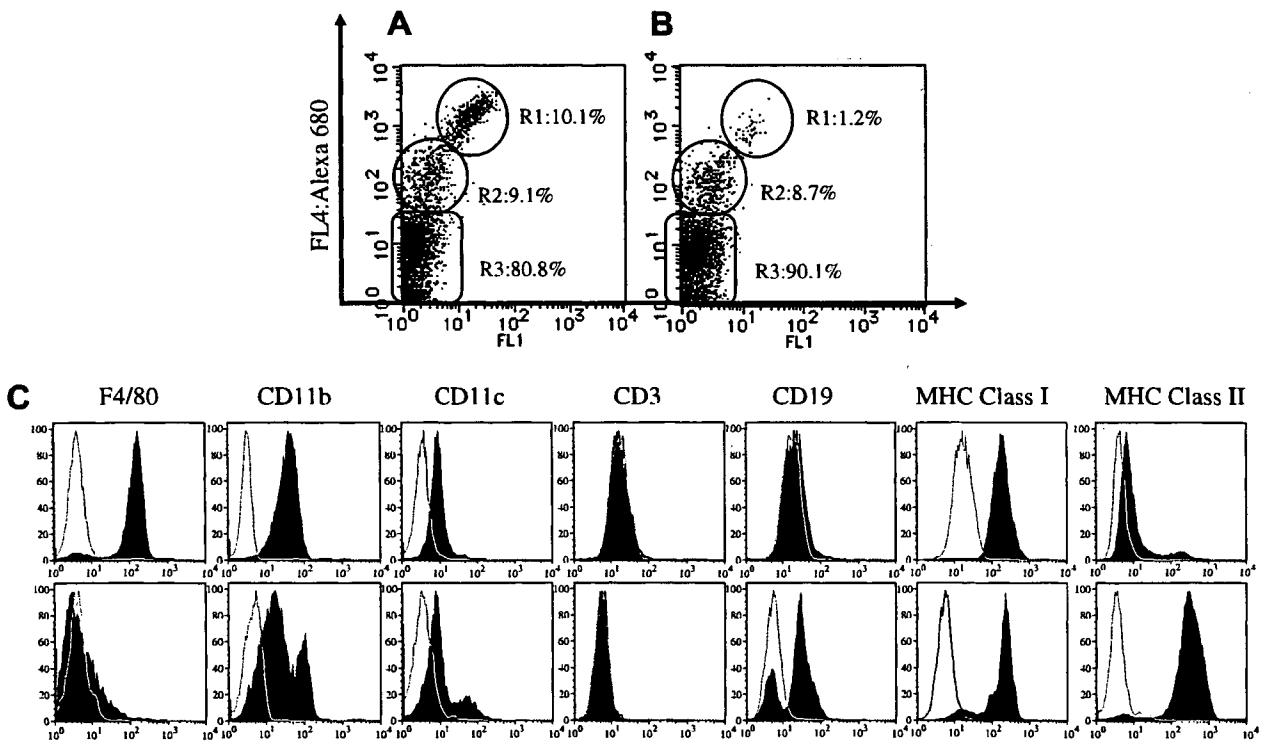

Fig. 1. OMLs injected into the peritoneal cavity were ingested preferentially by adhesive cells. One hour after injection of OMLs containing Alexa Fluor 680-labelled BSA, PEC were collected and their fluorescence was analyzed by flow cytometry (A). Non-adherent PEC were further isolated by plastic adherence for 2 h and analyzed (B). (C) Phenotypic analysis of PEC derived from OML-injected mice. One hour after injection of OMLs containing Alexa Fluor 680-labelled OVA, PEC were collected and stained with mAbs indicated. As shown in (A) and (B), PEC were divided into three groups based on their fluorescence intensity of Alexa Fluor 680, and the surface phenotypes of R1 and R2 were further analyzed.

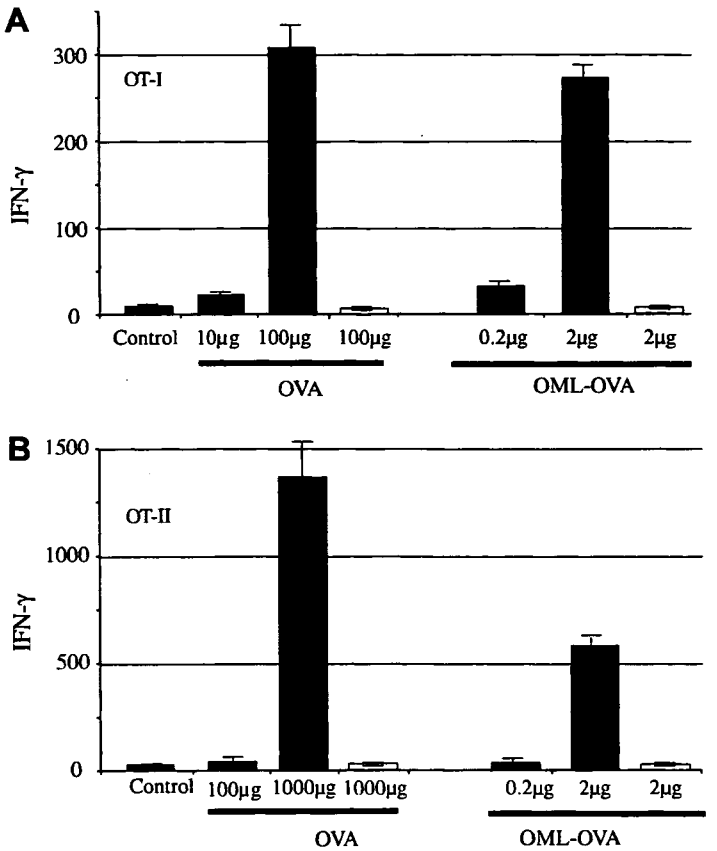

Fig. 2. Mϕ ingesting OVA encased in OML activate OVA-specific $CD8^+$ and $CD4^+$ T cells much more effectively than those ingesting soluble OVA. One hour after intraperitoneal injection of antigens, PEC were prepared from mice, and adherent cells were enriched by plastic adherence. $CD8^+$ and $CD4^+$ T cells were purified from spleen of OT-I (A) and OT-II (B), respectively, co-cultured (closed bar) with adherent PEC or cultured adherent PEC alone (open bar) for 24 h, and then production of IFN-γ in supernatants was tested by ELISA. PEC recovered from mice without any treatment was used as control. OML-OVA, OVA encased in OML; OVA, OVA; control, OML containing PBS. The graph shows the average and standard error from three independent experiments.

present antigen effectively also *in vivo*, as they effectively induced proliferation responses of OVA-specific $CD8^+$ T cells in the spleens of OT-I mice (Supplement Figure 1).

We next analyzed the uptake efficiency of OML-encased and soluble OVA and found that peritoneal phagocytic cells effectively uptake OVA irrespective of sugar encapsulation (Fig. 3). The uptake of soluble OVA is probably mediated by mannose receptors, as it is known as a highly mannosylated protein [19]. These results together indicate that OML-mediated ingestion promotes the presentation of OVA peptides by both MHC class I and II molecules by enhancing the antigen processing but not the uptake efficiency.

3.3. Induction of antigen-specific cytotoxic T lymphocytes (CTL) in vitro by OML-mediated immunization

We next performed CTL assay to detect OVA-specific T cells in the spleen. Only the spleen cells from mice immunized with OML-OVA but not bare liposome (BL)-encased OVA showed cytotoxicity against E.G7-OVA. The spleen cells from neither group showed cytotoxicity against EL4, confirming that OVA-specific CTL can be effectively induced *in vivo* by OML-OVA immunization (Fig. 4).

3.4. OML-mediated immunization induces antigen-specific anti-tumor immunity in vivo

We finally examined whether intraperitoneal immunization with OMLs also induces antigen-specific anti-tumor immunity *in vivo*. Mice were immunized intraperitoneally with OVA with or without OML encasing and then challenged with E.G7-OVA or EL4. As shown in Fig. 5, only the mice immunized with OML-OVA survived for more than 70 days when challenged with E.G7-OVA, while naïve and bare OVA-immunized mice died within 55 days. All the mice including those immunized with OML-OVA died within 30 days when challenged with EL4, indicating that the rejection of

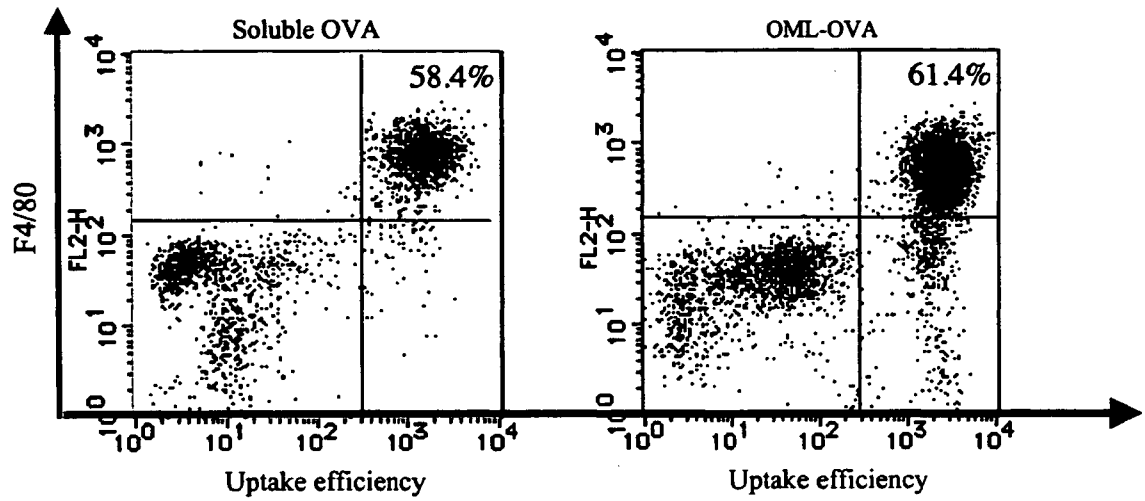

Fig. 3. Peritoneal F4/80$^+$ cells uptake OVA effectively irrespective of carbohydrate encapsulation. One hour after injection of either soluble FITC-OVA (20 μg) or OML-encapsulated FITC-OVA (20 μg) into the peritoneal cavity of B6 mice, uptake efficiency of FITC-OVA by peritoneal cells was analyzed by flow cytometry together with F4/80 expression.

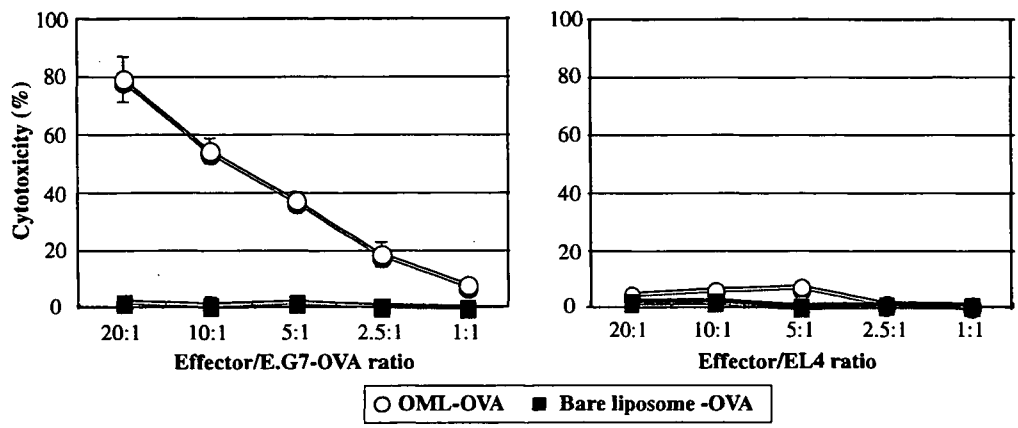

Fig. 4. OML-OVA-generated OVA-specific cytotoxicity. B6 mice were immunized biweekly three times by intraperitoneal injection of 1 μg OVA encased in oligomannose-coated (OML-OVA, open symbols) or bare liposomes (bare liposome-OVA, closed symbols). Spleen cells were isolated from mice one week after the last challenge, and 1×10^6 cells were stimulated with 10 μg OVA in 1 ml for 72 h. The graph shows the average and standard error from three independent experiments.

E.G7-OVA is OVA-specific. These results together showed that OML-mediated immunization can induce systemic immune response robust enough to protect mice from tumor challenge in an antigen-specific manner.

4. Discussion

In this study, we demonstrated that our novel OML-based drug delivery system (DDS) targeted to intraperitoneal phagocytic cells can also be used for the induction of systemic immune responses. After ingesting OML-encased OVA (OML-OVA), intraperitoneal phagocytic cells to extra-nodal lymphoid tissues in abdominal cavity and presented OVA-derived peptides in the context of both MHC class I and II molecules. Only the mice pre-immunized with OML-OVA rejected E.G7-OVA but not EL4 challenged subcutaneously. These results together indicate that the OMLs can be used as an effective antigen delivery system for immunotherapy activating both CTL and Th subsets. Fig. 6 shows the plausible induction process of anti-tumor immunity starting from phagocytic cells triggered by OML injection.

OMLs are very useful not only for the promotion of non-glycosylated protein uptake by antigen-presenting cells but also for the enhancement of antigen-processing of encased antigens. Endogenous

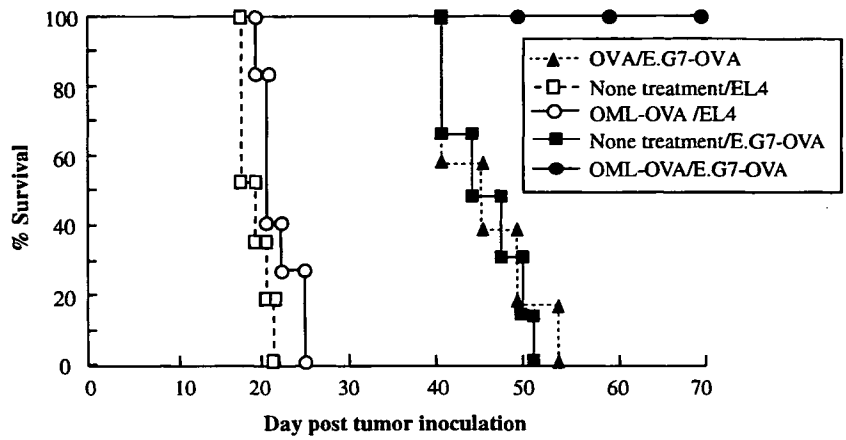

Fig. 5. OML-mediated immunization induces antigen-specific anti-tumor immunity *in vivo*. OML-OVA-immunized (circles) and naïve (squares) mice were challenged with E.G7-OVA (closed circles and squares) or EL4 cells (open circles and squares). As a control, mice were immunized with soluble OVA and challenged with E.G7-OVA (closed triangles).

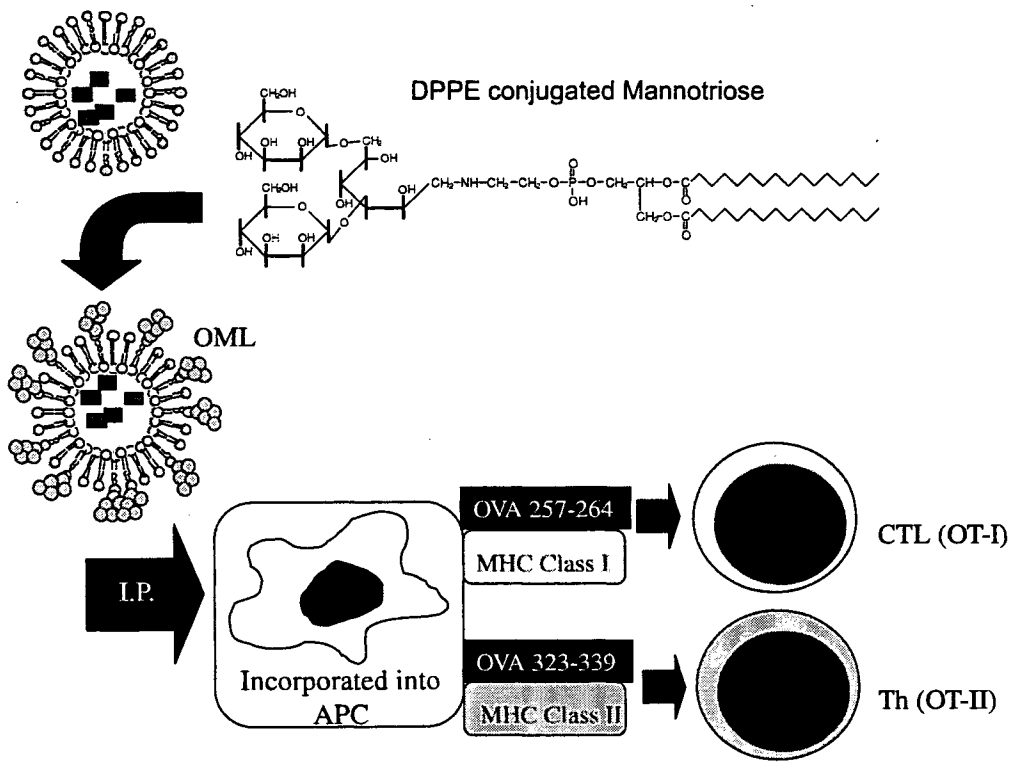

Fig. 6. Possible mechanism of OML-based vaccine delivery. Coating of bare liposomes with DPPE-conjugated mannotriose facilitates functions of intraperitoneal macrophages, resulting in antigen-specific activation of both CTL and Th populations.

and exogenous antigens are presented preferentially by MHC class I and II, respectively. OML-OVA, however, were effectively directed to both pathways, even when added exogenously. This advantage of OML-mediated immunization will hopefully facilitate the simultaneous activation of tumor antigen-specific $CD4^+$ and $CD8^+$ T cells as shown here with OVA. It is also very interesting to study the mechanism by which OML-mediated ingestion of antigens enhances the antigen presentation by both MHC molecules [20–22]. So far, we observed the up-regulation of CD80 and CD86 on OML-ingesting cells (in press on Cytokine, H. Takagi et al.), but it seems very important to know the additional signals to

make antigen presenting cells more immunogenic [23]. Additional adjuvant effects of various cytokines and/or toll-like receptor ligands on OML-mediated immunization are now being investigated.

Another advantage of OML-mediated immunization is Th1-skewing of the cytokine profiles. Indeed, OT-I and OT-II T cells stimulated with antigen-presenting cells ingesting OML-OVA produced IFN-γ but not IL-4 or IL-10 (unpublished observation). Moreover, our previous study demonstrated that the OML-mediated immunization protects BALB/c mice against *Leishmania major* infection, possibly due to the Th1-skewing of immune responses [24]. We observed that phagocytic cells ingesting OML preferentially produce IL-12 (unpublished observation), suggesting this cytokine is a key of Th1-skewing as reported previously [25]. Further investigation of the mechanism of this Th1-skewing of immune responses induced by OML-mediated immunization is currently underway.

Cells belonging to the monocyte-Mϕ lineage have been known to be heterogeneous, reflecting the plasticity and versatility of these cells in response to various microenvironmental signals [26]. Mϕ are now roughly categorized into M1 and M2 based on their functional properties, and several studies revealed that M1 and M2 promote type I and type II Th responses, respectively [27–29]. It is also reported that M1 and M2 are prone to induce inflammatory and immunoregulatory responses, respectively [29]. A possible concern of our DDS system is therefore the protumoral effects by M2 with antigen-encased OMLs, as they are supposed to express macrophage mannose receptors induced by IL-4 [30]. Although at least our *in vitro* study clearly showed OML-mediated skewing to type I immune responses, more precise investigation including the conditions for M1 polarization should be done especially in tumor-bearing mice. In addition, characteristics of the small population of non-Mϕ cells ingesting OMLs should be investigated as well.

In order to use our DDS in clinical study, the best administration routes should be determined to pursue repetitive vaccination while avoiding possible side effects. As generally acknowledged, intraperitoneal administration is accompanied with a high risk of side effects such as catheter-related complications, and abdominal pain [31]. In this connection, we have already obtained anti-tumor effects by subcutaneous injection of OML-OVA similar to those by intraperitoneal injection. However, side effects induced by subcutaneous injection of OMLs should be further investigated to assure their safe clinical application.

In the previous study, we reported that the formation of intraperitoneal metastasis of seeded gastric cancer cells in milky spots can be controlled with OMLs containing anti-cancer drugs [10]. In the present study, we have further extended the possibility of OMLs for the immunotherapy of systemic metastasis and existing tumor cells aside from milky spots. Oligomannose coating of liposomes showed the best uptake efficiency by intraperitoneal Mϕ among the neoglycolipids so far tested, and the encased antigen was effectively presented by both MHC class I and II molecules. However, the additional effects for immune responses by other neoglicolipids (shown here) have not been studied at all so far. We have a great interest in their effects on immune responses and are seeking sugar materials with immunoregulatory properties. If such materials are found, we believe that further study of our sugar-coated liposome technology will find it also to be applicable for antigen-specific regulation of autoimmune diseases and allergy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2007.10.038.

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Alternative splicing due to an intronic SNP in *HMSD* generates a novel minor histocompatibility antigen

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Here we report the identification of a novel human leukocyte antigen (HLA)-B44–restricted minor histocompatibility antigen (mHA) with expression limited to hematopoietic cells. cDNA expression cloning studies demonstrated that the cytotoxic T lymphocyte (CTL) epitope of interest was encoded by a novel allelic splice variant of *HMSD*, hereafter designated as *HMSD-v*. The immunogenicity of the epitope was generated by differential protein expression due to alternative splicing, which was completely controlled by 1 intronic single-nucleotide polymorphism located in the consensus 5′ splice site adjacent to an exon. Both *HMSD-v* and *HMSD* transcripts were selectively expressed at higher levels in mature dendritic cells and primary leukemia cells, especially those of myeloid lineage. Engraftment of mHA$^+$ myeloid leukemia stem cells in nonobese diabetic/severe combined immunodeficient (NOD/SCID)/γc^{null} mice was completely inhibited by in vitro preincubation with the mHA-specific CTL clone, suggesting that this mHA is expressed on leukemic stem cells. The patient from whom the CTL clone was isolated demonstrated a significant increase of the mHA-specific T cells in posttransplantation peripheral blood, whereas mHA-specific T cells were undetectable in pretransplantation peripheral blood and in peripheral blood from his donor. These findings suggest that the *HMSD-v*–encoded mHA (designated ACC-6) could serve as a target antigen for immunotherapy against hematologic malignancies. (Blood. 2007; 110:1055-1063)

Introduction

Minor histocompatibility antigens (mHAs) are major histocompatibility complex (MHC)-bound peptides derived from cellular proteins encoded by polymorphic genes. Following human leukocyte antigen (HLA)-matched allogeneic hematopoietic cell transplantation (HCT), donor-recipient disparities in mHAs can induce a favorable graft-versus-leukemia (GVL) effect that is often associated with graft-versus-host disease (GVHD).[1-3] Significant efforts have been made to identify mHAs, particularly those specific for hematopoietic cells, since such mHAs are speculated to contribute to the GVL effect. The first report on the identification of a hematopoietic lineage-specific mHA, HA-1, was generated by the Goulmy group in 1998 (den Haan et al[4]) as a result of biochemical analysis of peptides eluted from HLA-A*0201 molecules. The only other mHAs with selective expression in hematopoietic cells described to date are HA-2[5]; ACC-1 and ACC-2[6]; and DRN-7,[7] HB-1,[8,9] and PANE1,[10] the latter 2 of which are B-cell lineage-specific. Thus, identification of more mHAs should facilitate a better understanding of the biology of GVL and the development of effective immunotherapy to induce GVL reactions.

Immunogenicity of most autosomal mHAs identified to date results from single-nucleotide polymorphisms (SNPs) that cause amino-acid substitutions within epitopes, leading to the differential display/recognition of peptides between HCT donor and recipient via several mechanisms: peptide binding to MHC observed in HA-1/A2-,[4] HA-2-,[5] and *CTSH*-encoded mHAs[11]; proteasomal cleavage in HA-3[12]; peptide transport in HA-8[13]; and altered recognition of MHC-peptide complex by cognate T cells in HB-1,[8,9] HA-1/B60,[14] ECGF1/B7,[15] and SP110/A3.[7] Other examples of mechanisms of mHA generation include differential protein expression due to a nonsense mutation in *PANE1*[10] and a frame-shift mutation in *P2X5*.[16] *UGT2B17*[17] is the sole example of differential protein expression due to gene deletion instead of an SNP. Because SNPs are scattered throughout the genome, it has been speculated that mHAs caused by those other than coding SNPs should be present.

In this study, we report the identification of a novel gene encoding an HLA-B44–restricted mHA that is recognized by the 2A12 cytotoxic T lymphocyte (CTL) clone and selectively expressed in primary hematologic malignant cells, especially those of myeloid lineage, multiple myeloma (MM) cells, and normal mature dendritic cells (DCs). The antigenic peptide recognized by 2A12-CTL was encoded by a novel allelic splice variant of *HMSD*,

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hereafter designated as *HMSD-v*, due to an intronic SNP located in the consensus 5′ splice site adjacent to an exon. The leukemic stem cell (LSC) engraftment assay using severely immunodeficient mice demonstrated that the engraftment of primary acute myeloid leukemia (AML) cells was completely abolished by coincubation with the CTL clone before injection. These findings suggest that this novel mHA epitope may be an attractive therapeutic target for immunotherapy.

Patients, materials, and methods

Cell isolation and cell cultures

This study was approved by the Institutional Review Board of Aichi Cancer Center. All blood or tissue samples were collected after written informed consent was obtained in accordance with the Declaration of Helsinki. B-lymphoid cell lines (B-LCLs) were derived from donors, recipients, and healthy volunteers. B-LCLs and all cell lines of hematologic malignancy were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 1 mM sodium pyruvate (referred to as complete medium). CD40 ligand-activated B (CD40-B) cells were generated as previously described.[18]

Immature DCs were generated by culturing $CD14^+$ cells isolated from peripheral-blood mononuclear cells (PBMCs) with 500 U/mL GM-CSF and 500 U/mL interleukin 4 (IL-4) in AIM-V medium (Invitrogen, Carlsbad, CA) for 2 days, and then DCs were matured by cultivating the immature DCs for 2 additional days with 10 ng/mL IL-1β, 20 ng/mL IL-6, 10 ng/mL tissue necrosis factor α (TNF-α; all cytokines were from R&D Systems, Minneapolis, MN), and 1 μg/mL PGE2 (Cayman Chemical, Ann Arbor, MI). When necessary, cells were retrovirally transduced with restricting HLA cDNA by a method described previously.[18,19]

Generation of CTL lines and clones

CTL lines were generated from PBMCs ($\sim 10^6$) obtained at day 197 after HCT by primary stimulation with irradiated (33 Gy) pre-HCT recipient PBMCs ($\sim 10^6$), thereafter stimulated weekly with irradiated (33 Gy) recipient CD40-B cells (2×10^6) twice in RPMI 1640 supplemented with 10% pooled human serum and 2 mM L-glutamine (referred to as CTL medium).[11] IL-2 was added on days 1 and 5 after the second and third stimulation. CTL clones were isolated by standard limiting dilution and expanded in CTL medium as previously described.[11,20]

Chromium release assay

Target cells were labeled with 3.7 MBq of ^{51}Cr for 2 hours, and 10^3 target cells/well were mixed with CTLs at the effector-target (E/T) ratio indicated in a standard 4-hour cytotoxicity. All assays were performed at least in duplicate. Some target cells were pretreated with interferon γ (IFN-γ; 500 U/mL) and TNF-α (10 ng/mL; both from R&D Systems) for 48 hours as indicated. Percent specific lysis was calculated as follows: ([experimental cpm − spontaneous cpm]/[maximum cpm − spontaneous cpm]) × 100, where cpm indicates counts per minute.

cDNA library construction

The cDNA library used in the present study was the same one that had been used to identify HLA-A31- and HLA-A33-restricted *cathepsin H*-encoded mHAs (ACC-4 and ACC-5) previously.[11] The cDNA library was constructed from mRNA of a B-LCL derived from an AML patient (UPN-027) using the SuperScript Plasmid System (Invitrogen). The library contained 1.5×10^6 cDNA clones with an average insert size of approximately 2500 bp. cDNA pools, each consisting of approximately 120 and 5 clones for initial and second screens, respectively, were expanded for 24 hours in 96 deep-well plates, and plasmid DNA was extracted with the QIAprep 96 Turbo Miniprep kit (Qiagen, Valencia, CA).

Transfection of 293T cells and ELISA

Twenty thousand 293T cells retrovirally transduced with HLA-B*4403 were plated in each well of 96-well flat-bottomed plates, cultured overnight at 37°C, then transfected with 0.12 μg of plasmid containing a pool of the cDNA library using Trans IT-293 (Mirus, Madison, WI). Ten thousand CTL-2A12 cells were added to each well 20 hours after transfection. After overnight incubation at 37°C, 50 μL of supernatant was collected and IFN-γ was measured by enzyme-linked immunosorbent assay (ELISA).

Genotyping of polymorphisms

Genomic DNA was isolated from each B-LCL with a QIAamp DNA blood kit (Qiagen). Total RNA was extracted using an RNeasy Mini Kit (Qiagen), and cDNA was synthesized by standard methods. Genomic DNA or cDNA was amplified using KOD-plus-DNA polymerase (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The polymerase chain reaction (PCR) temperature profile was 30 cycles of 94°C for 15 seconds, 58°C for 20 seconds, and 68°C for 40 seconds on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA).

The primer sequences used to amplify from exon 1 to exon 4 of *HMSD* cDNA were as follows: sense, 5′-CCTCTCCGACCCGGTCTC-3′; antisense, 5′-GGGAAAAGCTAAAGCTAGAGAAAA-3′. Exonic sequence and intronic sequence adjacent to *HMSD* exon 1 and 2 were amplified with primers as follows: exon 1 sense, 5′-GACTGAAAACTCCCGGACAG-3′; exon 1 antisense, 5′-GAAAGGTCTGGAGCAACAGG-3′; exon 2 sense, 5′-GCAGACATTCACTCACAGCA-3′; exon 2 antisense, 5′-AAGCACCCACATGAGTGACC-3′. PCR products were purified and directly sequenced with the same primer.

Construction of minigenes and truncated genes for *HMSD-v*

Mammalian expression plasmids containing the full-length or truncated forms of the *HMSD-v* cDNA were constructed by reverse transcriptase (RT)-PCR using the isolated cDNA clone as a template. The constructs all encoded a Kozak sequence and initiator methionine (CCACC-ATG) and a stop codon (TAA). All products were ligated into *Hin*dIII-*Not*I-cut pEAK10 vector (Edge Bio Systems, Gaithersburg, MD) and verified by sequencing.

Epitope reconstitution assay

The candidate *HMSD*-encoded epitopes were synthesized by standard Fmoc chemistry. ^{51}Cr-labeled donor B-LCLs were incubated for 30 minutes in complete medium containing 10-fold serial dilutions of the peptides and then used as targets in standard cytotoxicity assays.

Real-time PCR assay for *HMSD* and *HMSD-v* expression

cDNAs were prepared from various hematologic malignant cell lines, primary cell cultures, freshly isolated $CD34^+$ bone marrow (BM) and peripheral-blood hematopoietic cells and their subpopulations, immature and mature DCs, activated B and T cells, $CD34^+$ subsets of primary leukemic cells, and $CD138^+$ subsets of primary MM cells. Cell sorting was performed using magnetic-activated cell separation (MACS) immunomagnetic beads (Miltenyi Biotec, Bergish Gladbach, Germany). A panel of cDNA made from different human adult and fetal tissues was purchased (MTC panels human I and II; BD Biosciences, San Diego, CA). Real-time PCR analysis was performed using the TaqMan assay as described previously.[11] Because of uncertainty of which allele(s) were included in each cDNA pool from the MTC panels, quantitative PCR primers and a probe were designed to detect the exon 3-4 boundary, which is shared by both alleles. The following sequences spanning the exon 3-4 boundary were used as primers with TaqMan probe to detect both *HMSD* and *HMSD-v* transcripts simultaneously: sense, 5′-AGAACTGCCAACGGGCTCTT-3′; antisense, 5′-TTGGTAGAATTTGCCACAGGAAT-3′; probe, 5′-(FAM)-CTTATGATTTCCTCACAGGTT-(MGB)-3′. To selectively detect *HMSD-v* transcripts, the following oligonucleotides specific for the exon 1-3 boundary were used: sense, 5′-CTCCGACCCGGTCTCACTT-3′; antisense, 5′-TCTCCATCTTCACCTCCGATTT-3′; probe, 5′-(FAM)-CAAAGTGCCCCAGTTC-(MGB)-3′.

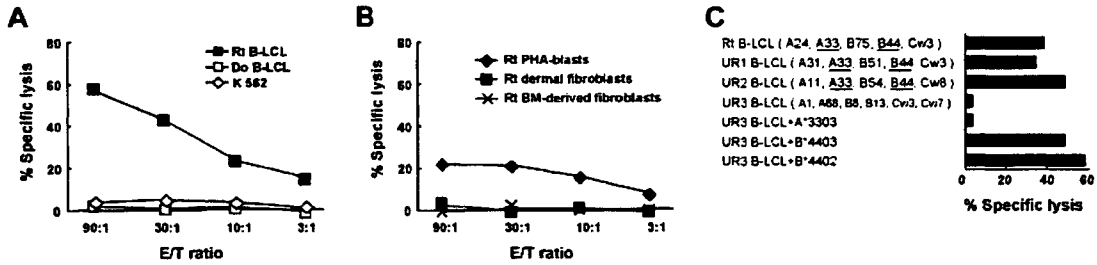

Figure 1. Specificity of the HLA-B44–restricted CTL clone 2A12. The cytolytic activity of CTL-2A12 was evaluated in a standard 4-hour ^{51}Cr release assay at the indicated E/T ratios. (A) CTL-2A12 recognition of target cells derived from recipient (Rt) but not donor (Do) B-LCLs. NK-sensitive K562 cells were used to determine nonspecific lysis. (B) CTL-2A12 recognition of Rt PHA-stimulated T cells (PHA blasts) but not of Rt dermal fibroblasts and bone marrow (BM)-derived fibroblasts pretreated with 500 U/mL IFN-γ and 10 ng/mL TNF-α for 48 hours before ^{51}Cr labeling. (C) CTL-2A12 recognition of an HLA-B*4403- and -B*4402-restricted mHA epitope. The following target cells were tested: Rt B-LCL, B-LCLs of 2 unrelated individuals (UR1 and UR2) sharing an HLA-A33, B44 haplotype with the recipient, and B-LCLs of an HLA class I-mismatched individual (UR3) that were transduced with either HLA-A*3303, B*4403, or B*4402 (E/T ratio, 30:1).

CD45 mRNA expression was detected as described previously.[21] A primer and probe set for human *GAPDH* (Applied Biosystems) was used as an internal control. PCR was performed according to the manufacturer's instructions in the ABI PRISM 7700HT Sequence Detector System (Applied Biosystems). Samples were quantified using relative standard curves for each experiment. All results were normalized with respect to the internal control and are expressed relative to the levels found in recipient B-LCLs.

LSC engraftment assay of AML cells in immunodeficient NOG mice

BM cells were obtained from patients with AML at diagnosis and then positively selected for $CD34^+$ subsets using MACS immunomagnetic beads (Miltenyi). NOD/Shi-scid, IL-2Rγc^{null} (NOG) mice[22] were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan). All mice were maintained under specific pathogen-free conditions in the Aichi Cancer Center Research Institute. The Ethical Review Committee of the Institute approved the experimental protocol. The ACC-2^D mHA-specific CTL clone 3B5[6] restricted by the same HLA-B*4403 allele as CTL-2A12 was used as a control CTL clone for this assay. AML cells (7.0×10^6) were preincubated for 16 hours in CTL medium supplemented with 25 units/mL recombinant human IL-2 at 37°C with 5% CO_2 either alone or in the presence of CTL-2A12 or CTL-3B5 at a T-cell/AML cell ratio of 5:1. Thereafter, the cultures were harvested and resuspended in a total volume of 300 μL and were inoculated via the tail vein of 8- to 12-week-old NOG mice (3 mice per group). Five weeks after inoculation, mice were killed, peripheral blood was aspirated from the heart, and BM cells were obtained by flushing the femora with complete medium. Nucleated cells were prepared for flow cytometry by incubation at 4°C for 20 minutes in PBS and 2% FCS with antihuman CD45 and CD34 (all from BD Biosciences) and were analyzed with a FACSCalibur flow cytometer and CellQuest 3.3 software (BD Biosciences). Percentage of engraftment was examined by 1-way analysis of variance (ANOVA) test.

Real-time PCR assay for detecting CTLs specific for ACC-6, a newly identified mHA

Complementary DNAs for a standard curve were prepared from mixtures of ACC-6–specific CTL clone (CTL-2A12) at various ratios with $CD3^+$ cells from healthy donors, and cDNAs of peripheral blood $CD3^+$ cells from the donor and patient before and after HCT were prepared from the AML patient (UPN-027). Real-time PCR analysis was performed using a TaqMan assay as described in "Real-time PCR assay for *HMSD* and *HMSD-v* expression." The primers and fluorogenic probe sequences spanning the CTL-2A12 complementarity-determining region 3 (CDR3) were used to detect T cells carrying the CDR3 sequences identical to that of CTL-2A12. Samples were quantified with the comparative cycle threshold (C_T) method. The delta C_T value was determined by subtracting the average GAPDH C_T value from the average CTL-2A12 CDR3 C_T value. The standard curve for the proportion of CTL-2A12 among $CD3^+$ cells (Figure 7A) was composed by plotting mean delta C_T values for each ratio, and the percentages of T cells carrying the CDR3 sequence identical to CTL-2A12A were calculated by using this standard curve.

Results

Characterization of a CTL clone

The $CD8^+$ CTL clone 2A12 (CTL-2A12) was 1 of 24 putative CTL clones isolated from day-197 post-HCT PBMCs of a male with refractory AML with multilineage dysplasia (UPN-027) receiving an HLA-identical HCT from his brother (A*2402, A*3303, B75, B*4403, Cw3, DR4, DR6).[11] The patient developed grade 1 acute GVHD in the first 2 years after transplantation and then suffered from glomerular IgG deposition and mild bronchiolitis obliterans organizing pneumonia. He is alive and in good condition and has been disease free for more than 3 years.

Cytotoxicity assays revealed that CTL-2A12 lysed the recipient B-LCL and less efficiently phytohemagglutinin (PHA)-stimulated T-cell blasts but not donor B-LCL or natural killer (NK)-sensitive K562 cells (Figure 1A,B). No cytotoxicity was observed against the recipient's dermal fibroblasts and BM-derived fibroblasts even after treatment with IFN-γ and TNF-α (Figure 1B). Cytotoxicity against recipient B-LCL was blocked by anti-HLA class I antibody (Ab) but not by anti-HLA-DR Ab, suggesting HLA class I-restricted recognition of mHA (data not shown). Based on the screening results of a panel of B-LCLs derived from individuals partially sharing HLA class I alleles with the recipient (Figure 1C UR1 and UR2; data not shown), those from HLA-mismatched individuals that were transduced with either HLA-A*3303 or -B*4403 were further tested. CTL-2A12 lysed UR3 B-LCLs when transduced with HLA-B*4403. In addition, UR3 B-LCLs transduced with HLA-B*4402 were also recognized, indicating that the mHA peptide can be presented by both HLA-B*4403 and -B*4402 (Figure 1C).

Identification of the gene encoding the mHA and elucidation of the mechanism of antigenicity

cDNA expression cloning using a cDNA library was conducted as described in "Patients, materials, and methods, cDNA library construction." In the first round of screening, 1 of 96 plasmid pools induced IFN-γ production by CTL-2A12. Two-step subclonings (~5 cDNAs and 1 cDNA) of this pool finally resulted in the isolation of a cDNA clone (data not shown).

The cDNA clone was sequenced and a BLAST search[23] revealed that this cDNA clone was previously unreported, but partially identical to XM_209104. XM_209104 was designated histocompatibility (minor) serpin domain containing (*HMSD*) by

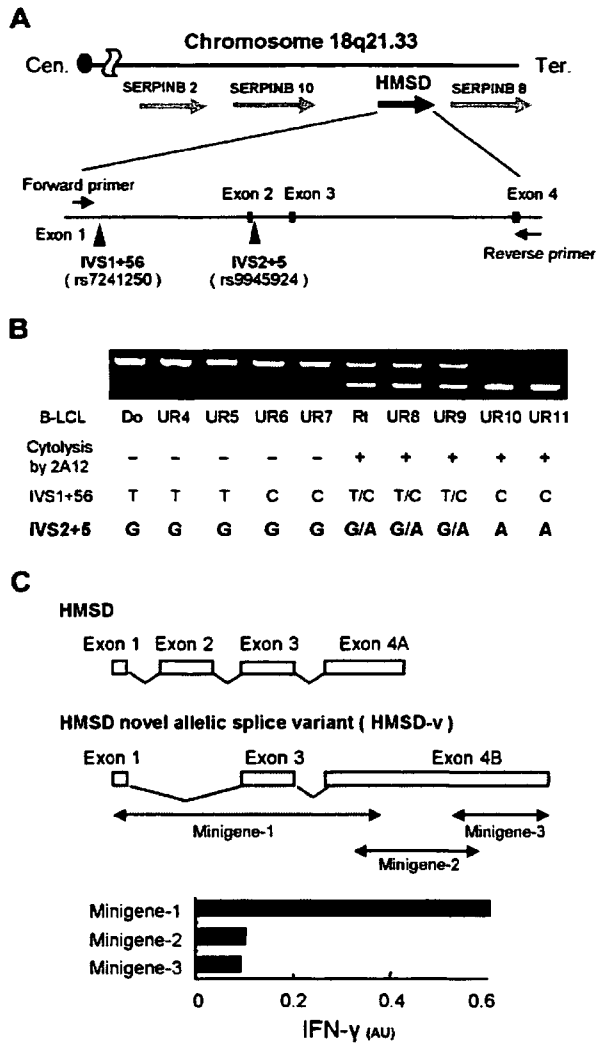

Figure 2. Identification of a novel splice variant transcript of *HMSD* encoding the mHA. (A) Summary of genome mapping around chromosome 18q21.33 showing relative positions of *HMSD*. Two identical cDNA clones were homologous to exons 1 and 3 plus exon 4 but lacked exon 2. This novel allelic splice variant of *HMSD* was designated *HMSD-v* (panel C). Search for potential SNPs responsible for the alternative splicing revealed 2 potential SNPs at IVS1+56 and IVS2+5 (arrowheads). Cen indicates centromere, Tel, telomere. (B) The correlation between sequence polymorphisms of the 2 SNPs and susceptibility of B-LCLs to CTL-2A12. Detection of allelic polymorphisms in B-LCLs was conducted by RT-PCR. Primers were set in exon 1 and the 5′ part of exon 4 of *HMSD* (horizontal arrows in panel A). Due to the lack of exon 2, the mHA^+ allele produced a smaller PCR product. Genotyping of the 2 SNPs mentioned above and cytolysis of B-LCLs by CTL-2A12 are summarized below the results of electrophoresis. The correlation between the genotyping results of SNPs at IVS2+5, CTL-2A12 cytolysis, and the bands of electrophoresis produced by mHA^+ and mHA^- allele showed complete concordance. (C) Schematic representation of *HMSD* and *HMSD-v* and mapping of the region encoding the CTL-2A12 mHA epitope by minigenes. The *HMSD-v* cDNA was divided into 3 minigenes, and mammalian expression plasmids containing individual minigenes were constructed. 293T/B*4403 cells were transfected with individual plasmids and cocultured with CTL-2A12. Supernatants were then harvested and assayed for IFN-γ production by ELISA. Release of IFN-γ is expressed in arbitrary units (AUs) corresponding to optical density at 630 nm.

the Human Genome organization Nomenclature Committee (Figure 2A). *HMSD* is a gene predicted by RefSeq[24] based on previously reported expressed sequence tags (ESTs). We speculated that this novel cDNA clone was a splice variant of *HMSD* (Figure 2C) because it had exons 1 and 3 plus exon 4B but lacked exon 2. The first third of exon 4B was identical to exon 4A of *HMSD*. Primers were set in exon 1 and the 5′ part of exon 4 (Figure 2A), and RT-PCR was carried out using cDNA from B-LCLs typed by CTL-2A12. Interestingly, these PCR products from mHA^- samples consisted of 1 longer band (674 bp), whereas those from mHA^+ samples consisted of the longer band and a shorter band (500 bp) or a single shorter band. This association was concordant with all 34 samples we examined (Figure 2B; data not shown), which revealed that differential expression of *HMSD* and its splice variant is responsible for antigenicity. Exon 1, exon 2, and introns adjacent to exons 1 and 2 were sequenced to account for the alternative splicing, and we found 2 sequence polymorphisms of intronic SNPs, the intervening sequence 1+56 (IVS1+56; rs7241250) and IVS2+5 (rs9945924), in our samples. The correlation between these 2 SNPs and susceptibility to CTL-2A12 was studied, which demonstrated that IVS2+5G>A, but not the SNP at IVS1+56, was completely concordant with cytolysis by CTL-2A12 (Figure 2B). Because the alternatively spliced cDNA clone isolated was generated as an allelic splice variant due to SNP, it was designated *HMSD-v*.

Identification of an HLA-B*4403-restricted epitope of *HMSD-v* and epitope reconstitution assay

To identify the epitope recognized by CTL-2A12, *HMSD-v* cDNA was divided into 3 minigenes overlapping each other by around 100 bp (Figure 2C) and then transfected into 293T/B*4403 cells. CTL-2A12 recognized 293T/B*4403 transfected with minigene-1, which expressed the first 809 bp of *HMSD-v* (Figure 2C). After searching all frames, 2 reading frames in the *HMSD-v* transcript were found to be able to encode polypeptides starting with an ATG codon, which was at least 9 amino acids (aa's) long (Figure 3A). The longest 53-mer polypeptide was divided into 16- or 17-aa peptides with 9 aa's overlapping each other, and downstream 3 peptides were expressed as minigenes starting with ATG (methionine) in 293T/B*4403 cells and tested. The construct encoding the first polypeptide, MEIFIEVFSHFLLQLT, was clearly recognized by CTL-2A12 (Figure 3B). To determine the mHA epitope, the minigene was serially deleted from its C-terminus and tested. An undecameric peptide was sufficient to induce IFN-γ production from CTL-2A12 (Figure 3A underlined; Table 1).

Subsequently, a peptide reconstitution assay was conducted. Undecameric peptide (MEIFIEVFSHF), its C-terminal deleted decameric peptide (MEIFIEVFSH), and N-terminal deleted decameric peptide (EIFIEVFSHF) were synthesized and titrated by adding to the mHA^- donor B-LCL, and among these, only undecameric peptide showed dose-dependent cytolysis with a half-maximal lysis at 20 nM (Figure 4A). This undecameric peptide contains the HLA-B*4403 anchor motif—a glutamic acid at position 2 and a phenylalanine at the C-terminus[25,26]—although undecameric peptide is not common as a T-cell epitope. We designated the mHA as ACC-6 (Aichi Cancer Center No. 6).

HMSD and *HMSD-v* mRNA expression in various hematopoietic and nonhematopoietic cells

To determine the expression of *HMSD* and *HMSD-v* mRNA in a more comprehensive manner, real-time PCR was performed. Individual real-time PCR analysis specific for the *HMSD-v* transcript and for both *HMSD* and *HMSD-v* transcripts revealed that both were equally present in cDNA samples from B-LCLs heterozygous for the defined mHA (data not shown). Thus, further real-time PCR analysis was performed to quantify the total expression of both transcripts partly because mHA allelic status of commercial

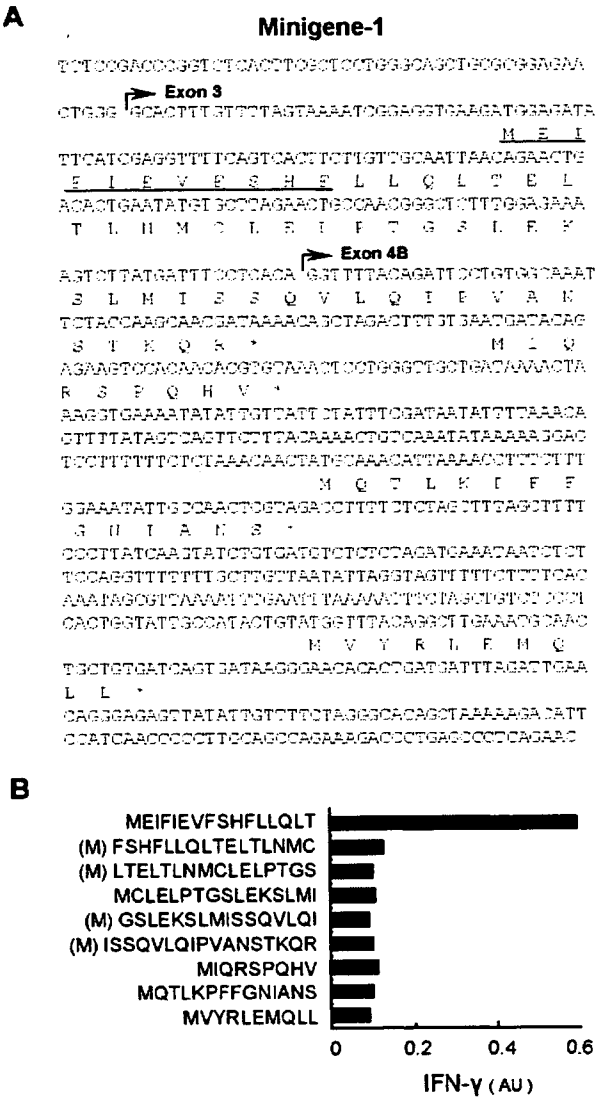

Figure 3. The nucleotide and deduced amino-acid sequences of minigene-1 encoding the CTL-2A12 mHA epitope. (A) Exon 2 encoding the original start codon in *HMSD* was deleted. After searching all frames, 2 reading frames in the *HMSD-v* transcript shown here were found to be able to encode polypeptides longer than 9 aa's starting with an ATG codon. Polypeptides longer than 9 aa's are all indicated. Asterisks indicate a stop codon. The start of exon 3 and exon 4B are indicated with horizontal arrows. The epitope recognized by CTL-2A12 is underlined (see Figure 4). (B) Six small minigenes with 9 aa's overlapping derived from the longest 53-mer polypeptide and downstream 3 minigenes (shown in panel A) were expressed in 293T/B*4403 cells and cocultured with CTL-2A12. Production of IFN-γ was similarly measured by ELISA. Release of IFN-γ is expressed in arbitrary units (AUs) corresponding to optical density at 630 nm. (M) indicates an artificially added methionine as a start codon.

tissue cDNAs was unknown. High levels of expression were observed in primary AML and MM cells, mature DCs, CD40-B cells and PHA blasts (Figure 5A top panel), and malignant hematopoietic cell lines (especially those of myeloid lineage; Figure 5A bottom panel). In contrast, most normal tissues (Figure 5B top panel), including resting primary hematopoietic cells (Figure 5A top panel), showed lower or no expression, except for testis, which expressed a moderate amount of transcript. Weak expression observed in commercial cDNA from nonhematopoietic tissues including brain, lung, and placenta could be caused at least in part by contaminating hematopoietic cells or resident cells of

Table 1. Fine epitope mapping with minigenes

Minigene sequence	Length, bp	CTL response
M E I F I E V F S H F L L Q L T	16	+
M E I F I E V F S H F L L Q L	15	+
M E I F I E V F S H F L L Q	14	+
M E I F I E V F S H F L L	13	+
M E I F I E V F S H F L	12	+
M E I F I E V F S H F	11	+
M E I F I E V F S H	10	−
M E I F I E V F S	9	−

To determine the mHA epitope, a minigene encoding 16 amino acids, which stimulated CTL-2A12, was serially deleted from its C terminus and then tested by ELISA. An undecameric but not decameric peptide was sufficient to induce IFN-γ production from the CTL-2A12.

hematopoietic origin such as pulmonary macrophages, because relatively high levels of *CD45* transcript were detected in those tissues (Figure 5B bottom panel).

It is possible that *HMSD-v* is differentially expressed from *HMSD* in cell types other than B-LCLs, where both transcripts were generated at similar levels. Thus, we examined both total *HMSD* and *HMSD-v* transcripts in various primary cells that were heterozygous for the ACC-6 allele. As shown in Figure 5C, the *HMSD-v* levels were approximately half of total *HMSD* levels in all cell types tested.

Inhibition of human AML-cell engraftment in severely immunodeficient NOG mice by CTL-2A12

We first confirmed that the positively selected CD34$^+$ fraction of primary AML cells positive for HLA-B*4403 and the ACC-6$^+$ allele (all heterozygous) by genotyping was efficiently lysed by

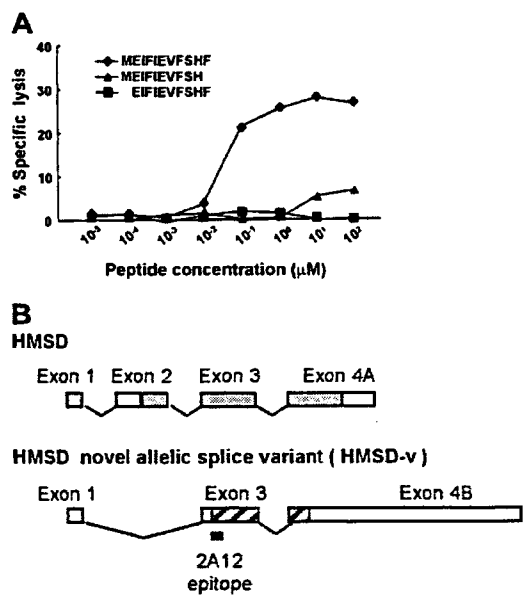

Figure 4. Identification of the CTL-2A12 minimal mHA epitope. (A) A peptide reconstitution assay was conducted to determine the concentration of peptides needed to stimulate CTL-2A12. Undecameric peptide (MEIFIEVFSHF), its C-terminal deleted decameric peptide (MEIFIEVFSH), and N-terminal deleted decameric peptide (EIFIEVFSHF) were synthesized and titrated by adding to the antigen-negative donor B-LCL. (B) Transcript of *HMSD* (encoding a 139-mer polypeptide) predicted by computer algorithm is indicated with ▭. ▨ indicates the presumed *HMSD-v* transcript region encoding a 53-mer polypeptide starting with an ATG codon and including the CTL-2A12 epitope. The location of the identified 2A12 epitope is shown below the *HMSD-v* cDNA. These 2 polypeptides have no homology because they are translated from different reading frames.

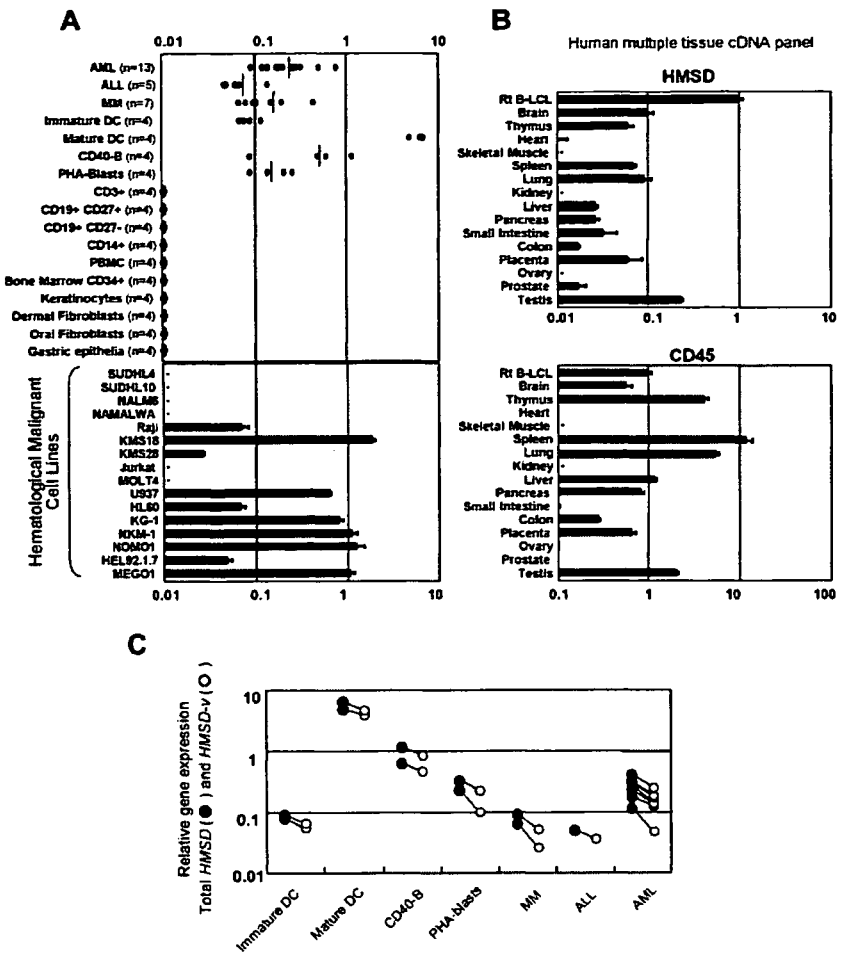

Figure 5. Selective mRNA expression of *HMSD* and *HMSD-v*. (A) Total *HMSD* expression was determined by real-time quantitative PCR in various normal tissues and malignant hematopoietic cell lines using a primer-probe set that detects the exon 3-4 boundary. Targeted mRNA expression in the recipient B-LCL is set as 1.0. In the top dotted plot graph, cDNAs prepared from $CD34^+$ subsets of primary leukemic cells and $CD138^+$ subsets of primary MM cells, freshly isolated hematopoietic cells, their subpopulations, immature and mature DCs, activated B and T cells, freshly isolated $CD34^-$ bone marrow cells, and primary cell cultures were similarly analyzed. Values in the parentheses indicate the number of the individuals tested. In the bottom and middle panels, cDNAs prepared from 16 hematologic malignant cell lines are shown. SUDHL4 and SUDHL10 are derived from B-cell non-Hodgkin lymphoma; NALM6 from acute B-lymphocyte leukemia; NAMALWA and Raji from Burkitt lymphoma; KMS18 and KMS28 from multiple myeloma (MM); Jurkat and MOLT4 from acute T-lymphocyte leukemia; U937 from histiocytic lymphoma; HL60, KG-1, NKM-1, NOMO1, and HEL92.1.7 from acute myeloid leukemia; and MEGO1 from chronic myeloid leukemia (blast crisis). (B) cDNAs of 15 normal tissue samples purchased from Clontech (MTC panels human I and II) were analyzed for total *HMSD* expression (top panel) and CD45 mRNA expression (bottom panel). Messenger RNA expression in the recipient B-LCL is set as 1.0. (C) *HMSD-v* expression levels (○) were compared with total *HMSD* expression levels (●) using a primer-probe set that detects the exon 1-3 boundary specific for *HMSD-v* mRNA. Among primary hematopoietic cells shown in the top of panel A, cells that were found to be heterozygous for ACC-6 allele were further selected and tested. Paired samples are linked.

CTL-2A12 (Figure 6A). The mRNA expression level of total *HMSD* in these AML cells was 47% (AML-1), 28% (AML-2), and 24% (AML-3) of that in the ACC-6-heterozygous recipient B-LCL, respectively.

Next, to determine whether the ACC-6 mHA recognized by CTL-2A12 is indeed expressed on LSCs and thus might have been involved in a GVL effect in AML patient UPN-027, we performed the LSC engraftment assay as previously reported[27] but substituted the significantly immunodeficient NOG mice because the absence of NK activity in NOG mice has been shown to facilitate the engraftment level of xenogenic human hematopoietic cells.[22] The $CD34^+$ fractions of primary AML cells that were lysed by CTL-2A12 (AML-2 in Figure 6A) were selected for this assay, since it was found to be negative for the HLA-B*4403-restricted mHA ACC-$2^{D,6}$ and not lysed by the ACC-2^D-specific clone CTL-3B5 (data not shown), which was used as an irrelevant control. These AML $CD34^+$ cells were incubated in vitro for 16 hours either alone or in the presence of CTL-2A12 or control CTL-3B5 at a T-cell/AML cell ratio of 5:1. Subsequently the mixtures were inoculated into NOG mice. After 5 weeks, flow cytometric analysis of BM and PBMCs was conducted to study the expression of human CD45, CD34, and CD8. Representative flow cytometric profiles are shown in Figure 6B. BM cells of control mice receiving AML-2 cells cultured in medium alone or with control CTL-3B5 before inoculation were found to contain 2.79% to 25.44% (mean, 20.29%) human $CD45^+$ $CD34^+$ cells, whereas PBMCs of the same 2 groups of mice contained 2.97% to 9.69% human cells. In contrast, human cells were not detectable in either BM or PBMCs of the mice inoculated with AML cells precultured with CTL-2A12. Percentage AML engraftment at 5 weeks after inoculation under these conditions is summarized in Figure 6C, indicating that CTL-2A12 eradicated AML stem cells with repopulating capacity ($P = .015$ for BM).

Follow-up of ACC-6–specific CTLs in peripheral blood from an AML patient (UPN-027)

To detect ACC-6–specific CTLs in peripheral blood from AML patient UPN-027 and from his donor, we performed real-time quantitative PCR (Figure 7A) using a set of primers and a fluorogenic probe specific for the unique CDR3 sequence of the CTL-2A12 TCR β chain at several time points. Although ACC-6-specific CTLs were not detected in blood samples from the donor and the patient before HCT, they became detectable in patient samples after HCT at frequencies of 0.11%, 0.23%, 0.83%, and 0.16% among $CD3^+$ cells at days 29, 91, 197, and 548, respectively (Figure 7B). During this period of time, there were no documented clinical manifestations of recurrent disease, and only grade 1 acute GVHD was noted.

Discussion

Antigenicity of the majority of previously identified human mHAs is generated by differences in amino-acid sequence between donor

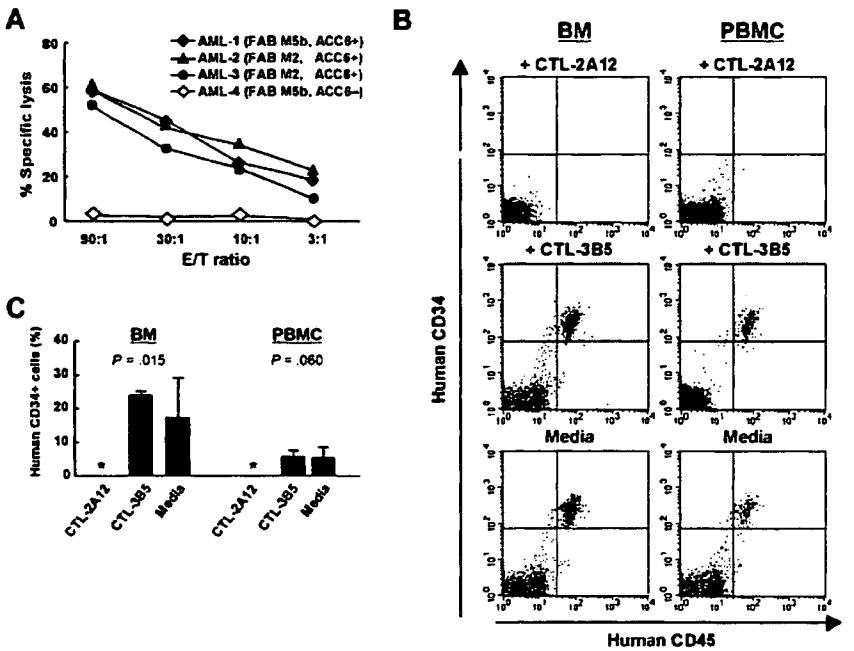

Figure 6. Inhibition of human AML stem cell engraftment in severely immunodeficient NOG mice by CTL-2A12. (A) Specific lysis by CTL-2A12 of primary leukemia cells. A standard 4-hour ^{51}Cr release assay was conducted at the indicated E/T ratios. The CD34$^+$ fraction of 3 primary AML cells positive for HLA-B*4403 and the ACC-6$^+$ allele by genotyping (AML-1, -2 and -3; the expression level of *HMSD* was 47%, 28%, and 24% of that in the recipient B-LCL, respectively) and 1 HLA-B*4403$^-$, ACC-6 allele-negative (AML-4) were tested. FAB denotes French-American-British classification. (B) Representative flow cytometric profiles of peripheral blood and BM cells from AML-inoculated NOG mice for the expression of human CD45 and CD34. Peripheral blood and BM cells were obtained 5 weeks after inoculation from mice receiving 7.0×10^6 AML-2 CD34$^+$ cells (negative for ACC-2^D mHA) that had been incubated with either CTL-2A12 (top), control CTL-3B5 (middle; HLA-B*4403-restricted, ACC-2^D mHA-specific CTL), or culture medium alone (bottom) at a T-cell/AML cell ratio of 5:1. (C) Summary of results from engraftment experiments. Mean (± SD) percentage of CD45 and CD34 double-positive cells of 3 mice in each group at 5 weeks after inoculation and the *P* values examined by 1-way ANOVA test are shown. Asterisk indicates that CD45 and CD34 double-positive cells were not detectable in NOG mice inoculated with AML-2 cells preincubated with CTL-2A12.

and recipient due to nonsynonymous SNPs. In this study, we identified a novel HLA-B44–restricted mHA epitope (ACC-6) encoded by an allelic splice variant of *HMSD* (*HMSD-v*) in which exclusion of exon 2 due to alternative splicing was completely controlled by an intronic SNP at IVS2+5. Indeed, by RT-PCR, the novel *HMSD-v* was not detected in cDNA samples from mHA$^-$ B-LCLs, whereas it was detectable in mHA$^+$ B-LCLs. An interesting question is why the splicing of exon 2 was completely controlled by the intronic SNP. In general, during intron splicing reactions, U1snRNA first binds the 5′ splice site of an intron, spliceosome assembly starts, lariat formation is made with several other factors, and thereafter the intron is spliced out (reviewed in Valadkhan[28]). Here U1snRNA is an important initiator of the cascade. It has been shown that aberrant splicing can result from mutations that either destroy or create splice-site consensus sequences at the 5′ splice site such that approximately half of the observed aberrant splicing is exon skipping while intron retention is rarely observed.[29] In this case, we speculate that the G-to-A substitution of the intronic SNP at nucleotide 5 in intron 2 (IVS2+5G>A, 5′-GUA*C*AU-3′), in addition to the presence of nonconsensus IVS2+4C (underlined), which is commonly observed in both mHA$^+$ and mHA$^-$ alleles and thus is likely to be permissive, completely disrupts the consensus alignment sequence critical for U1snRNA binding (5′-GUAAGU-3′) such that U1snRNA cannot stably bind the 5′ end of intron 2 in the precursor mRNA from the mHA$^+$ allele. A similar mutation (IVS3+5G>C, 5′-GUAACU-3′) and resultant exon 3 skipping was reported as a disease-causing mutation in the *NF1* gene.[30] Accordingly, intron 2 cannot be spliced out; a large lariat consisting of intron 1, exon 2, and intron 2 is formed; and then the large lariat is spliced out. In the latter case, 1 nucleotide (IVS1+4) does not match the U1snRNA sequence, but this mismatch is again likely to be permissive. Indeed, it has been shown that a mismatch at nucleotide 3, 4, or 6 of the 5′ splice site is not critical compared with others.[31,32] To our knowledge, this is the first demonstration of an mHA whose antigenicity is controlled by alternative splicing due to an intronic SNP, which may represent an important mechanism for the generation of mHAs.

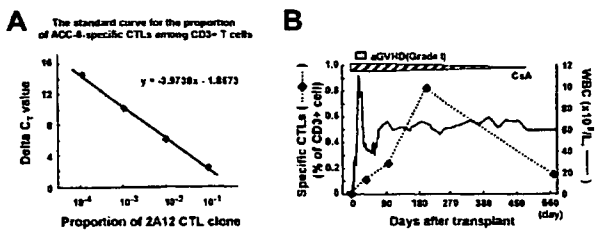

Figure 7. Detection of ACC-6–specific CTLs in peripheral blood from the AML patient (UPN-027) by real-time quantitative PCR using a set of primers and fluorogenic probe specific for the CTL-2A12 CDR3 sequence. (A) The standard curve for the proportion of ACC-6–specific CTL-2A12 serially diluted into CD3$^+$ cells from healthy donors using the comparative C_T (threshold cycle) method. The y-axis is delta C_T value. The x-axis is the log proportion of ACC-6–specific CTLs among CD3$^+$ T cells. (B) The frequency of T cells carrying the CDR3 sequence of CTL-2A12 over a period of 1.5 years after HCT. The percentages of such T cells among CD3$^+$ T cells (left y-axis) were estimated by using a standard curve in panel A and are indicated before HCT and after HCT at day 29, day 91, day 197, and day 548, respectively (diamonds with dotted line). Also noted are white blood cell (WBC) counts (right y-axis), acute GVHD (gray bar), and immunosuppressive therapy with cyclosporine A (CsA; hatched bar) during the same time period.

LSCs, which are present at very low frequencies, have a particularly strong capacity for proliferation, differentiation, and self-renewal[34] and likely play an important role in disease refractoriness or relapse after chemotherapy and transplantation. Thus, complete eradication of such stem cells is critical for cure in any treatment modalities. The LSC engraftment assay of AML cells in

immunodeficient mice has been shown to be a powerful method for testing the effect of treatment, here mHA-specific CTLs, on LSCs. In addition, preliminary analysis has shown that CTL-2A12 lysed the $CD34^+CD38^-$ fraction of AML cells (Figure S1, available on the *Blood* website; see the Supplemental Figures link at the top of the online article), which is considered to contain leukemic stem-like cells.[35] These data clearly demonstrate that ACC-6 mHA is expressed on such stem cells and may serve as target for cognate CTL-2A12 in vivo.

We performed quantitative RT-PCR analyses for *HMSD* transcripts in various tissues with great interest because cytotoxicity assays suggested its limited expression in hematopoietic cells. Notably, *HMSD* showed selective expression in several hematopoietic primary tumor cells (especially those of myeloid lineage), mature DCs, and activated B and T cells. Since high expression was observed in mature DCs as in the case of *HMHA1* encoding HA-1 mHA,[36] immune responses to *HMSD*-derived mHAs may induce not only a GVL effect[37] against hematopoietic tumor cells but also GVHD,[38] since recipient DCs are responsible for initiating GVHD after HCT. Collectively, our data suggest that this novel mHA, ACC-6, might be a good target for immunotherapy inducing GVL if potential GVHD induction can be managed until recipient DCs have been eliminated early after HCT. Finally, relatively high expression of *HMSD* in the $CD138^+$ fraction of MM cells and their susceptibility to 2A12-CTL (Figure S2) suggest that ACC-6 may serve as a potential target for immunotherapy of multiple myeloma.

It is of interest to correlate clinical outcomes with ACC-6-specific T-cell kinetics after HCT using reagents such as tetramers. The preparation of HLA-B44 tetramer, however, is known to be very difficult,[39] so we used real-time quantitative RT-PCR using CTL-2A12 CDR3 sequence-specific primers/probe, because Yee et al[40] have previously shown strong concordance between semiquantitative RT-PCR analysis of a clone-specific CDR3 region and tetramer analysis used to monitor the fate of adoptively infused CTL clones for the treatment of melanoma. The highest frequency of 0.83% among $CD3^+$ cells was obtained at day 197 after HCT, concordant with the fact that CTL-2A12 was generated from the PBMCs collected at that time. This magnitude is somewhat lower than that observed in the case of LRH-1-specific T cells (1.6% of $CD8^+$ T cells) at the peak level after donor lymphocyte infusion (DLI)[16] but similar to that observed in the case of HA-1-specific T cells (1000 to 6000 tetramer-positive cells per mL blood, corresponding to 0.2% to 1.0% among $CD3^+$ cells).[41] The possibility that the ACC-6 mHA might preferentially induce GVL is supported by the fact that ACC-6–specific CTLs were detectable in the recipient's peripheral blood at a relatively high level after resolution of mild acute GVHD and that LSCs could be eradicated as shown in the NOG mice model. Whether or not ACC-6 mismatching in donor-recipient pairs may be associated with an increased risk of GVHD or morbidity would need to be studied using a large cohort of patients.

The therapeutic applicability of particular mHAs, calculated from the disparity rate and restricting HLA allele frequency, is an issue of interest.[42] The observed frequency of this $ACC\text{-}6^+$ phenotype was approximately 35% (n = 48/135) in healthy Japanese donors (data not shown) and HLA-B*4403 is present in around 20% of Japanese populations, so that ACC-6 incompatibility is expected to occur in approximately 4.6% of HCT recipient-donor pairs. Because CTL-2A12 lysed $HLA\text{-}B^*4402^+$ B-LCLs possessing the $ACC\text{-}6^+$ phenotype derived from white individuals, this novel epitope peptide can also bind to HLA-B*4402, which is a relatively common allele (around 20%) in white populations. Actually, data from the HapMap Project[43] demonstrate that the genotype frequency of carrying at least one IVS2+5A ($ACC\text{-}6^+$) allele is 0.381 for individuals registered in the Centre d'Etude du Polymorphisme Humain (CEPH) cell bank,[44] thus this mHA should also be applicable to white patients. These results together suggest that *HMSD*-derived products could be attractive targets for immunotherapy and that given the possible role of intronic SNPs, a mechanism of alternative splicing should be also taken into consideration when searching for novel mHAs.

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Authorship

Contribution: T.K., Y.A., and T.T. designed research; T.K., Y.A., and H.T. performed research; T.K., Y.A., S.O., and S.M. analyzed data; A.O., M.M., A.T., K.M., H.I., Y.M., and Y.K. contributed vital reagents or analytical tools; and T.K., Y.A., K.T., K.K., and T.T. wrote the paper.

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Review Article

Minor histocompatibility antigens as targets for immunotherapy using allogeneic immune reactions

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Minor histocompatibility antigens (mHag) were originally identified as antigens causing graft rejection or graft-versus-host disease in human leukocyte antigen (HLA)-matched allogeneic transplantation. Molecular identification has revealed most to be major histocompatibility complex (MHC)-bound short peptide fragments encoded by genes which are polymorphic due to single nucleotide polymorphisms (SNP). Genotypic disparity of SNP between transplantation donors and recipients gives rise to mHag as non-self antigens for both the donor and the recipient. Subsequently, mHag have been explored as immunotherapeutic antigens for use against recurring hematological malignancies after allogeneic hematopoietic cell transplantation (HCT), because mHag expressed only on hematopoietic cells are considered to augment graft-versus-leukemia/lymphoma (GVL) effects without increasing the risk of life-threatening graft-versus-host disease (GVHD). Accumulating evidence suggests that T-cell responses to mHag aberrantly expressed on solid tumor cells are also involved in the eradication of sensitive tumors such as renal cell carcinomas following HCT. Over the past decade, the number of putative GVL-directed mHag has increased to a level that covers more than 30% of the Japanese patient population, so that clinical trials may now be executed in the setting of either vaccination or adoptive immunotherapy. As it is expected that immune responses to alloantigens are more powerful than to tumor antigens mostly derived from overexpressed self-proteins, mHag-based immunotherapy may lead to a new treatment modality for high-risk malignancies following allogeneic HCT. (*Cancer Sci* 2007; 98: 1139–1146)

Allogeneic hematopoietic cell transplantation (HCT) was initially introduced to clinics as the last treatment choice against otherwise non-curable leukemia to reconstitute severely damaged patient hematopoietic cells after high-dose chemoradiotherapy with normal hematopoietic stem cells from a healthy donor. It did not take long until hematologists realized that allogeneic HCT offered an ultimate immunotherapy using donor T-cell-mediated allo-immune responses against residual leukemia cells, that is graft-versus-leukemia (GVL) effects.[1,2] This was confirmed by observation of the powerful antileukemic effects of donor lymphocyte infusion (DLI), which results in cure of some but not all recurring leukemia after HCT, although it is often accompanied by severe graft-versus-host disease (GVHD) or neutropenia.[3] Despite the advent of new treatment modalities, including imatinib for BCR-ABL-positive leukemias and rituximab for B-cell tumors, allogeneic HCT not only retains its position as the sole treatment offering cure to patients with advanced hematological malignancies, but the eligible patient population is continuously expanding through the introduction of reduced-intensity HCT,[4] or therapy for patients with solid tumors like renal cell carcinomas.[5] Now the powerful antitumor effect, the so-called graft-versus-tumor (GVT) effect, is considered to be mediated by graft-originated donor T cells that are reactive mainly with recipient alloantigens.[5,6] In the case of major histocompatibility antigen (major histocompatibility complex [MHC], human leukocyte antigen [HLA] in human) compatible HCT, minor histocompatibility antigen complexes (mHag) originating from gene polymorphisms between the donor and recipient have been shown to be targets for alloreaction when they are presented as MHC-bound short peptides to cognate T cells.[1,2] Unfortunately, GVHD, a life-threatening complication frequently accompanying allogeneic HCT, also caused by immune reactions against mismatched mHag, still offsets the favorable GVT effect in a substantial number of patients. Therefore dissecting GVT effects from GVHD and their further augmentation are the main aims of researchers mining novel mHag.

Selective induction of GVL effects by targeting mHag restricted to hematopoietic cells

In mouse models, adoptive transfer of cytotoxic T cells (CTL) specific for a single mHag disparity between the donor and recipient has been shown to be sufficient to eradicate implanted melanoma or leukemia cell lines,[7,8] suggesting robust GVT can be obtained by targeting an immunodominant mHag epitope. In these models, GVHD was not observed even though the mHag were expressed ubiquitously, while GVHD was induced when mHag-specific CTL were co-infused with naïve T cells. Because naïve T cells contain precursor cells reactive to other disparate mHag,[9] they cause inflammatory destruction of GVHD-prone organs once activated by antigens leaked from the cells initially targeted by the infused CTL through 'epitope spreading'. Although these data provide clues to the dissection of GVT from GVHD, caution is necessary because humans are a crossbred population and multiple mHag disparities do exist even between HLA-identical siblings. Thus, more selective targeting to mHag expressed only in hematopoietic cells, including hematological malignant cells, has been considered when designing immunotherapy against recurring tumors following allogeneic HCT in human to avoid GVHD.[1,2,10] If an mHag epitope is encoded by a polymorphic region of a gene whose expression is limited to normal and malignant hematopoietic cells, donor-derived T cells sensitized to the polymorphic region will induce immune reactions only to such recipient hematopoietic cells and not to non-hematopoietic cells, due to the lack of target gene expression (Fig. 1).

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