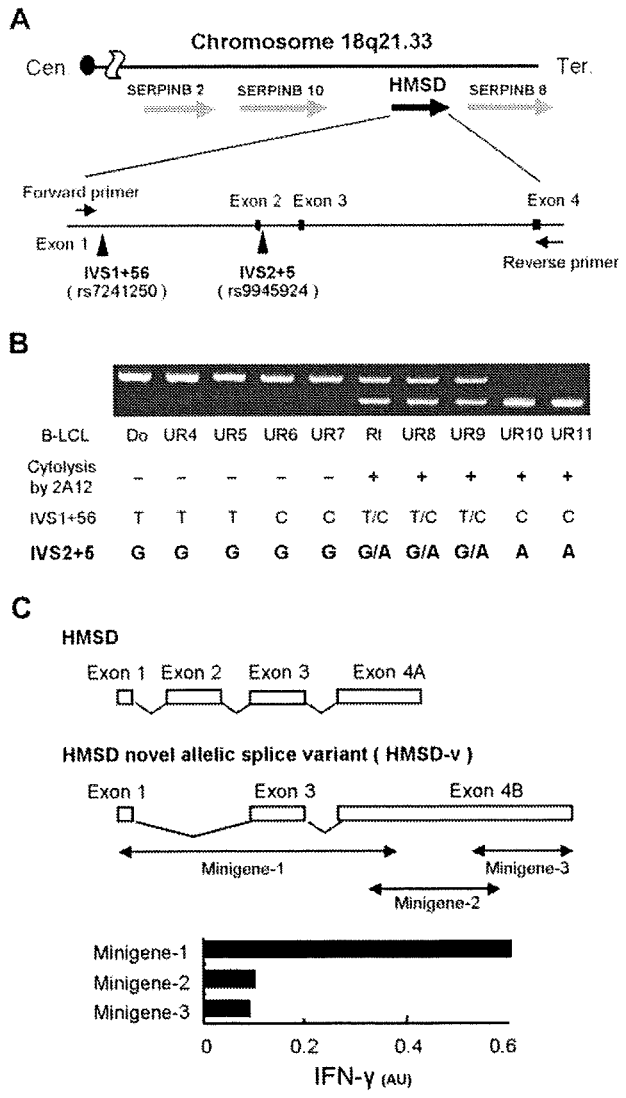

Figure 2. Identification of a novel splice variant transcript of *HMSD* encoding the mHA. (A) Summary of genome mapping around chromosome 18q21.33 showing relative positions of *HMSD*. Two identical cDNA clones were homologous to exons 1 and 3 plus exon 4 but lacked exon 2. This novel allelic splice 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

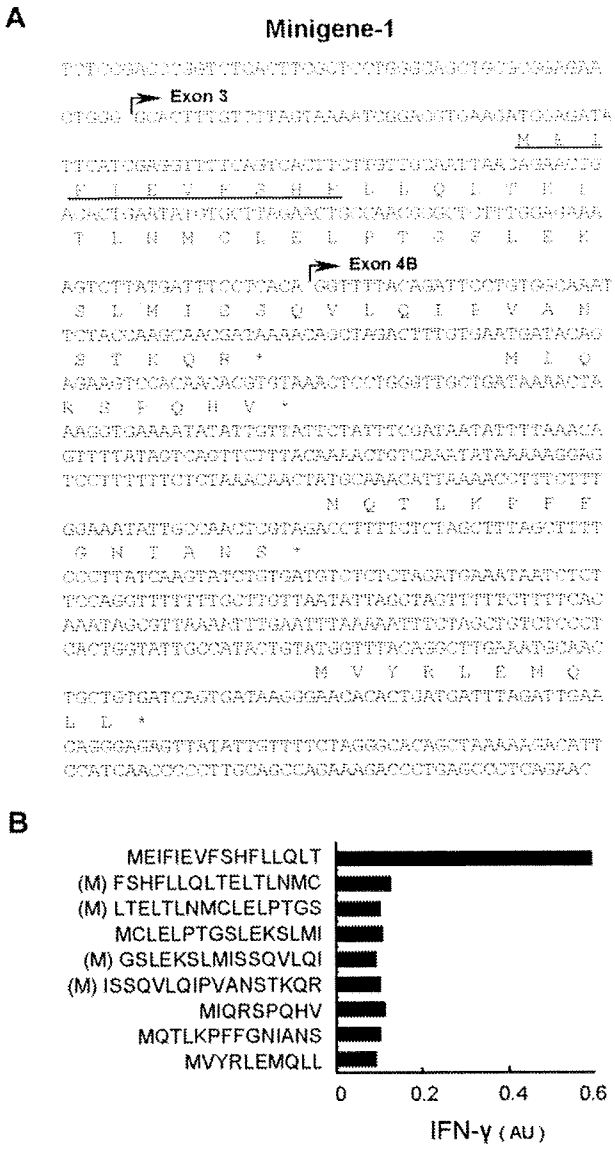

Figure 3. The nucleotide and deduced amino-acid sequences of minigene-1 encoding the CTL-2A12 mHA epitope. (A) Exon 2 encoding the original start codon in *HMSD* was deleted. After searching all frames, 2 reading frames in the *HMSD-v* transcript shown here were found to be able to encode polypeptides 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 $ACC\text{-}6^+$ allele (all heterozygous) by genotyping was efficiently lysed by

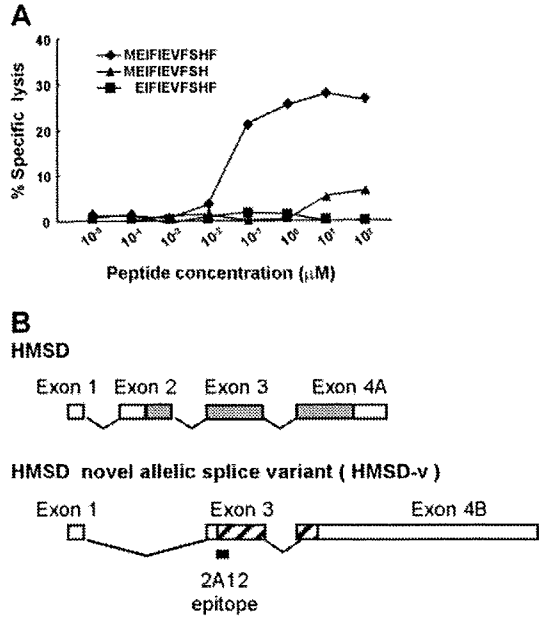

Figure 4. Identification of the CTL-2A12 minimal mHA epitope. (A) A peptide reconstitution assay was conducted to determine the concentration of peptides needed to stimulate CTL-2A12. Undecameric peptide (MEIFIEVFSHF), its C-terminal deleted decameric peptide (MEIFIEVFSH), and N-terminal deleted decameric 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.

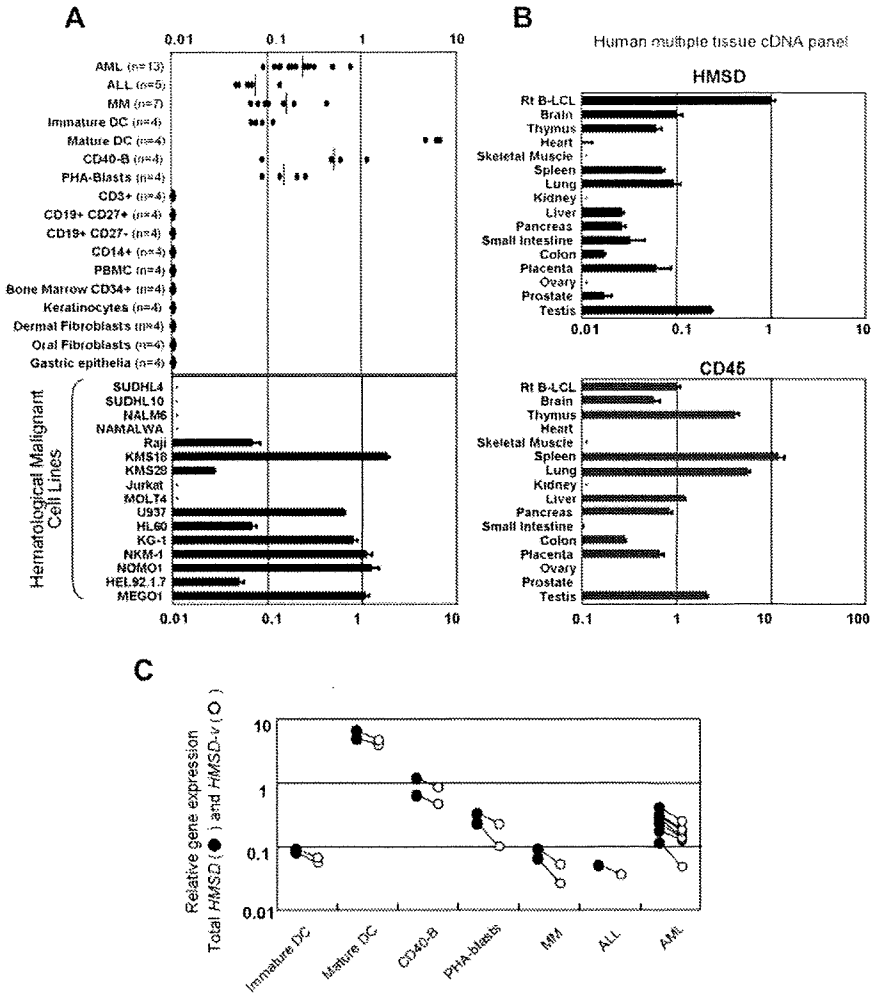

Figure 5. Selective mRNA expression of *HMSD* and *HMSD-v*. (A) Total *HMSD* expression was determined by real-time quantitative PCR in various normal tissues and malignant hematopoietic cell lines using a primer-probe set that detects the exon 3-4 boundary. Targeted mRNA expression in the recipient 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

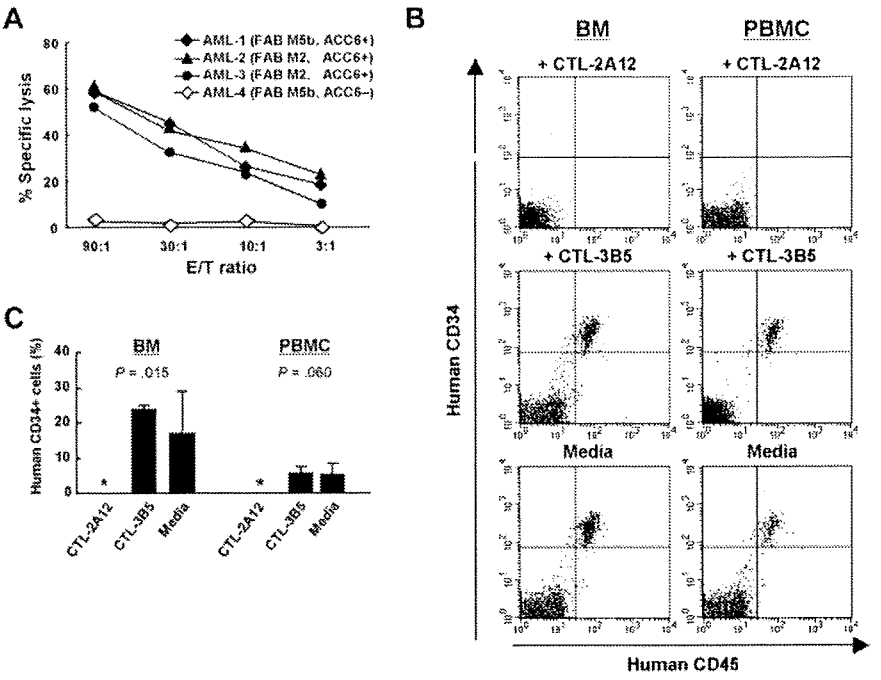

Figure 6. Inhibition of human AML stem cell engraftment in severely immunodeficient NOG mice by CTL-2A12. (A) Specific lysis by CTL-2A12 of primary leukemia cells. A standard 4-hour ^{51}Cr release assay was conducted at the indicated E/T ratios. The $CD34^+$ fraction of 3 primary AML cells positive for HLA-B*4403 and the $ACC\text{-}6^+$ 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 $HLA\text{-}B^*4403^+$, 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 $ACC\text{-}2^D$ mHA) that had been incubated with either CTL-2A12 (top), control CTL-3B5 (middle; HLA-B*4403-restricted, $ACC\text{-}2^D$ 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.

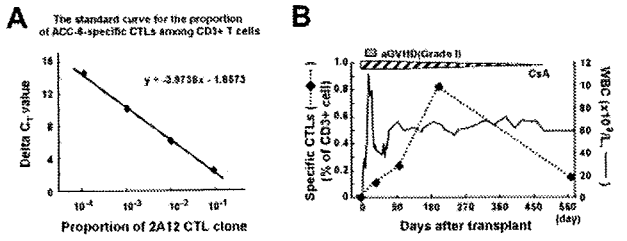

Figure 7. Detection of ACC-6–specific CTLs in peripheral blood from the AML patient (UPN-027) by real-time quantitative PCR using a set of primers and fluorogenic probe specific for the CTL-2A12 CDR3 sequence. (A) The standard curve for the proportion of ACC-6–specific CTL-2A12 serially diluted into $CD3^+$ 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.

The novel epitope was located on exon 3 and was transcribed from a reading frame different from the *HMSD* transcripts (Figure 4B). Although exon 3 is shared by *HMSD* and *HMSD-v*, it is speculated that polypeptide including the epitope was not being translated from *HMSD*, because donor B-LCL was not lysed by CTL-2A12. In general, ribosomes initiate translation from the first AUG start codon, but sometimes second or other AUG codons downstream can serve as start codons due to "leaky scanning."[33] However, it seems this is not the case for *HMSD* because the donor B-LCL homozygous for this allele was not lysed at all. This identification of an mHA unexpectedly generated from a previously unknown alternative transcript due to SNP has important implications for the identification of other new mHAs.

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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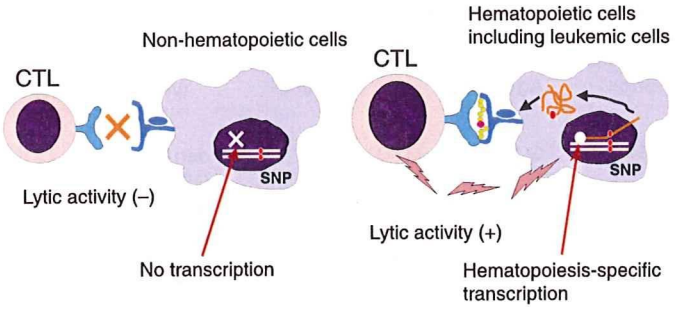

Fig. 1. Selective induction of graft-versus-leukemia/lymphoma (GVL) effects. Donor T cells sensitized to minor histocompatibility antigen complex (mHag) epitopes encoded by polymorphic genes, the expression of which is limited to hematopoietic cells, including hematological malignant cells, are responsible for selective GVL effects. (Left) Recipient non-hematopoietic cells are spared from attack by such donor-derived T cells because genes encoding mHag are not expressed due to the lack of hematopoiesis-specific transcription factors or other mechanisms. (Right) Recipient hematopoietic cells including residual or recurring hematological malignant cells after allogeneic hematopoietic cell transplantation (HCT) are eradicated by such donor-derived mHag epitope-specific T cells. Donor-derived normal hematopoietic cells are also spared because they are 'self' for donor-derived T cells and do not express mHag epitopes. CTL, cytotoxic T cells; SNP, single nucleotide polymorphism.

It was almost a decade ago when the first hematopoietic lineage-specific mHag, HA-1^H (the superscript indicates the polymorphic amino acid residue encoded by the antigenicity-positive allele), was identified at the molecular level.[11] The epitope determined biochemically from a pool of HLA-A*0201-binding peptides was found to be a nonameric amino acid encoded by the *KIAA0223* or *HMHA1* gene, the expression of which is restricted to normal and malignant hematopoietic cells,[11] and to some solid tumors due to 'aberrant expression'.[12,13] The HA-1^H most studied mHag thus has a long research history. CTL clones specific for HA-1^H were initially isolated from patient peripheral blood during acute GVHD following allogeneic HCT, and a first study by the same group at Leiden University showed a close association between donor-recipient HA-1 disparity and the development of severe acute GVHD.[14] Thus HA-1 mHag became regarded as responsible for GVHD induction. Subsequent studies, however, showed mixed results, ranging from no association to a significant association. Because of its restricted expression in hematopoietic cells and leukemic cells,[11] and the lack of cytotoxicity in a skin explant model,[12] the Leiden group moved to *in vitro* studies to induce HA-1^H-specific CTL lines for application in adoptive immunotherapy against recurring leukemia patients positive for both HLA-A*0201 and HA-1^H mHag following HCT.[15] Such HA-1^H-specific CTL lines were tested for antileukemic activity in an NOD/SCID mouse model in which mice implanted with leukemia cells 3 days prior to infusion of the CTL line showed a delayed outgrowth of leukemia compared with those receiving control CTL.[16] Although complete cure of leukemia was not achieved with a single CTL infusion in these mice, the data provided a rationale for use of mHag as effective targets for immunotherapy if *in vivo* maintenance of mHag-specific CTL activity could be further improved.

Mechanisms of mHag epitope generation

Accumulating evidence supporting the potential utility of mHag in the treatment of hematological malignancies prompted researchers to identify mHag of possible clinical use, and a series of mHag epitopes has now been reported (Table 1). As for the mechanisms of generation of mHag epitopes, not only coding single nucleotide polymorphisms (SNP) leading to amino acid substitution such as found in HA-1^H[11], but also non-coding SNP in both exons and introns appear to be involved.[17–19] Because millions of SNP have been reported throughout the human genome, it is possible that numerous mHag may exist, but, from the experimental results so far reported, less than 30 mHag have been identified to date, suggesting technical difficulty in their identification, as well as the presence of an immunological hierarchy in which immune responses tend to converge onto a limited number of immunogenic antigens. Technical problems may be commonly encountered in searching for novel epitopes, even with tumor or bacterial antigens, but the major difference is that mHag are only immunogenic if they are differentially expressed between the donor and recipient due to an SNP. This so-called 'immunodominance' is often the case because a CTL response to predetermined mHag is frequently and repeatedly detected in mHag-disparate recipients by means of HLA tetramer analyses.[17,20,21] Furthermore, mHag epitopes reported to date show a variety of mechanisms in their generation, that is, SNP can affect mRNA splicing,[19] translation (stop codon generation,[18] frameshift[17]), proteasomal digestion (destruction,[22] protein splicing[23]), transportation via transporter associated with antigen processing (TAP),[24] binding to MHC,[11,25–27] and interaction with T-cell receptor,[28–32] with one exception of gene deletion[33] (summarized in Fig. 2). These findings imply that a conventional reverse immunological approach to map epitopes around SNP regions based on HLA binding motifs may not be sufficient, which may make this research field less approachable or 'minor' even though the therapeutic potential of mHag seems to be 'major'.

Currently known mHag

To date, a total of 29 mHag epitopes have been identified at the molecular level, including 10 mHag epitopes mapped onto the Y chromosome (Table 1). Because the latter possesses several X-homolog genes, most of which are related to sex determination, the products of these genes contain many potential mHag that are of significant importance as GVHD targets exclusively in female to male HCT. These genes are, however, expressed broadly in most cases and thus are not discussed in this review but only listed in Table 1. Because there is no standard nomenclature system for mHag currently, the authors here employ both the originally proposed name and an alternative composed of the official gene symbol defined by the HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature/) with the polymorphic nucleotide encoding the mHag-positive allele as a superscript so as to be useful for genotyping/restriction HLA alleles. In addition to mHag identified at the molecular level, there are a number of examples defined at the cellular level, that is, 'CTL-defined' mHag. They are either waiting to be identified molecularly or are not being further explored because of limited use due to a broad tissue distribution (i.e. being potential GVHD targets) or too high or low allele frequencies resulting in only rare mismatching in clinical HCT.

The first report on mHag from Japan was made by Maruya *et al.*, who examined polymorphisms for 14 adhesion molecules and found significant associations of donor/recipient disparity in CD31, CD49b, and CD62L polymorphisms with acute GVHD development.[34] However, they did not confirm the mHag epitopes. Yazaki *et al.* successfully generated HLA-A31-restricted CTL that recognized an mHag by stimulating naïve but not *in vivo* primed HCT donor T cells with recipient leukemic cells, although the candidate gene was not identified.[35] It is noteworthy that only three autosomal mHag (HA-1^H, HA-2^V and HB-1^H) were identified at the molecular level by the end of the 20th century, but after the advent of publicly available on-line

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Table 1. mHag epitopes identified at the molecular level (arranged in principle in ascending order by year of publication)

mHag†	HLA restriction	Peptide sequence‡	mHag gene††	Chromosomal position	SNP Ag+/Ag–§	Proposed mHag name¶	Reporter (year; reference)
mHags encoded by genes on autosomal chromosomes							
HA-2^{V}	A*0201	YIGEVLVSV	***MYO1G***	7p13-p11.2	G/A	MYO1G^{G}/A2	den Haan (1995, 2001)[25,26]
HA-1^{H}	A*0201	VLHDDLLEA	***HMHA1***	19p13.3	A/G	HMHA1^{A}/A0201	den Haan (1998)[11]
HA-1^{H}	B60	KECVLHDDL	***HMHA1***	19p13.3	A/G	HMHA1^{A}/B60	Mommaas (2002)[32]
HA-1^{H}	A*0206	VLHDDLLEA	***HMHA1***	19p13.3	A/G	HMHA1^{A}/A0206	Torikai (2007)[36]
HB-1^{H}	B44	EEKRGSLHVW	***HMHB1***	5q31.3	C/T	HMHB1^{C}/B44	Dolstra (1999)[29]
HB-1^{Y}	B44	EEKRGSLYVW	***HNHB1***	5q31.3	T/C	HMHB1^{T}/B44	Dolstra (2002),[28]
HA-8^{R}	A*0201	RTLDKVLEV	*KIAA0020*	9p22.3	G/C	KIAA0020^{G}/A2	Brickner (2001)[24]
HA-3^{T}	A1	VTEPGTAQY	*AKAP13*	15q24-q25	C/T	AKAP13^{C}/A1	Spierings (2003)[22]
UGT2B17	A29	AELLNIPFLY	*UGT2B17*	4q13	Gene defect‡‡	UGT2B17/A29	Murata (2003)[33]
ACC1^{Y}	A24	DYLQYVLQI	***BCL2A1***	15q25.3	A/G	BCL2A1^{A}/A24	Akatsuka (2003)[30]
ACC2^{D}	B44	KEFEDDIINW			A/G	BCL2A1^{A}/B44	
LRH-1	B7	TPNQRQNVC	***P2RX5***	17p13.3	C/–§§	P2RX5^{+C}/B7	de Rijike (2005)[17]
CTL-7A7^{R}	A3	RVWDLPGVLK	***PANE1***	22q13.2	T/C	PANE1^{T}/A3	Brickner (2006)[18]
ACC-5^{R}	A*3101	ATLPLLCAR	*CTSH*	15q24-q25	A/G	CTSH^{A}/A3101	Torikai (2006)[27]
ACC-4^{R}	A*3303	WATLPLLCAR				CTSH^{A}/A3303	
RDR173^{H}	B7	RPHAIRRPLAL	***ECGF1***	22q13.33	A/G	ECGF1^{A}/B7	Slager (2006)[31]
DNR-7^{R}	A3	SLPRGTSTPK	***SP110***	2q37.1	A/G	SP110^{A}/A3	Warren (2006)[23]
LB-ADIR-1^{F}	A*0201	SVAPALALFPA	***TOR3A***	1q25.2	T/C	TOR3A^{T}/A0201	van Bergen (2007)[45]
ACC-6	B44	MEIFIEVFSHF	***HMSD***	18q21.33	A/G	HMSD^{A}/B44	Kawase (2007)[19]
mHags encoded by X-homolog genes on Y chromosomes					NA	JARID1D/B7	Wang (1995)[58]
SMCY	B7	SPSVDKARAEL	*JARID1D*	Yq11	NA	JARID1D/B7	Wang (1995)[58]
SMCY	A*0201	FIDSYICQV	*JARID1D*	Yq11	NA	JARID1D/A0201	Meadows (1997)[58]
DFFRY	A*0101	IVDCLTEMY	*USP9Y*	Yq11.2	NA	USP9Y/A0101	Pierce (1999)[59]
UTY	B8	LPHNHTDL	*UTY*	Yq11	NA	UTY/B8	Warren (2000)[60]
UTY	B60	RESEESVSL	*UTY*	Yq11	NA	UTY/B60	Vogt (2000)[61]
DBY	DQ5	HIENFSDIDMGE	*DDX3Y*	Yq11	NA	DDX3Y/DQ5	Vogt (2002)[62]
DBY	DRB1*1501	GSTASKGRYIPPHLRNREA	*DOX3Y*	Yq11	NA	DDX3Y/DRB1*1501	Zorn (2004)[63]
RPS4Y	DRB3*0301	VIKVNDTVQI	*RPS4Y1*	Yp11.3	NA	RPS4Y1/DRB3*0301	Spierings (2003)[64]
RPS4Y	B*5201	TIRYPDPVI	*RPS4Y1*	Yp11.3	NA	RPS4Y1/B5201	Ivanov (2005)[65]
ACC-3	A*3303	EVLLRPGLHFR	*TMSB4Y*	Yq11.221	NA	TMSB4Y/A3303	Torikai (2005)[66]

†Original mHag name, if applicable, with a single letter amino acid encoded antigenic allele as superscript. ‡Expressed in a single letter amino acid, if applicable, with the polymorphic amino acid underlined; in the case of Y-chromosome-associated mHag, amino acid difference from its X-homolog, if applicable, is underlined. §The polymorphic nucleotide determining antigenicity of the autosomal mHag is expressed for positive and negative alleles. Nucleotide deletion instead of SNP is expressed '–' and deleted nucleotide. ¶The authors' proposed mHag name consists of 'official gene symbol' defined by the HUGO Gene Nomenclature Committee with a nucleotide encoded by antigenic allele (expressed as superscript)/'restriction HLA allele'. ††Genes expressed mainly in hematopoietic cells, thus serving as potential targets for GVL effects, are shown in bold. ‡‡*UGT2B17* has a whole gene deletion type polymorphism. Transplants from donors lacking both *UGT2B17* alleles to recipient possessing at least one UGT2B17 allele are considered as graft-versus-host direction mismatches. §§This mHag is caused by differential protein expression in recipient and donor cells as a consequence of a homozygous frameshift due to deletion of polymorphism in the donor. One cytosine (C) nucleotide deletion in the nucleotide sequence of exon 3 of the *P2X5* gene in the donor results in early translation termination as well as a frameshift after the SNP portion. GVL, graft-versus-leukemia; HLA, human leukocyte antigen; mHag, minor histocompatibility complex antigen; SNP, single nucleotide polymorphism.

databases provided by the Human Genome Project and others, the number of novel mHag has grown at a much faster pace.

Hematopoietic cell-restricted mHag epitopes

HA-1^{H} (HMHA1^{A}/A*0201), HA-2^{V} (MYO1G^{G}/A2) and HB-1$^{H/Y}$ (HMHB1$^{C/T}$/B44). HA-1^{H} is the most well-known and extensively studied mHag, as described above and reviewed elsewhere,[1,10,11,14,15] partly due to its relatively high 'overall applicability' (>10%) considering both the donor/recipient mismatching rate (~25%) and the HLA-A*0201 frequency (~45% in Caucasian population).

HA-2^{V} is also an HLA-A*0201-restricted mHag and a potential GVL target,[10,25,26] but its clinical relevance is limited due to its low mismatching rate (~5%) compared with that of HA-1^{H} (Table 2). However, possible involvement in GVL reactions against recurring leukemia following DLI has been shown elegantly using HLA-A2 tetramers incorporating HA-1^{H} and HA-2^{V} epitope peptides.[20]

HB-1^{H} is an HLA-B44-restricted mHag encoded by the functionally unknown *HMHB1*, the expression of which is restricted to activated B cells and acute B-cell leukemia. This is the first mHag identified using cDNA expression cloning.[29] Interestingly, both alleles encoded by *HMHB1* (i.e. C encoding His and T encoding Tyr) are immunogenic reciprocally,[28] but their clinical applicability has yet to be fully explored.

HA-1^{H} (HMHA1^{A}/B60, HMHA1^{A}/A*0206). Originally HA-1^{H} was identified as an HLA-A*0201-restricted mHag as described above.[11] In searches for other epitopes restricted by different HLA alleles around the same SNP or other coding SNP on the

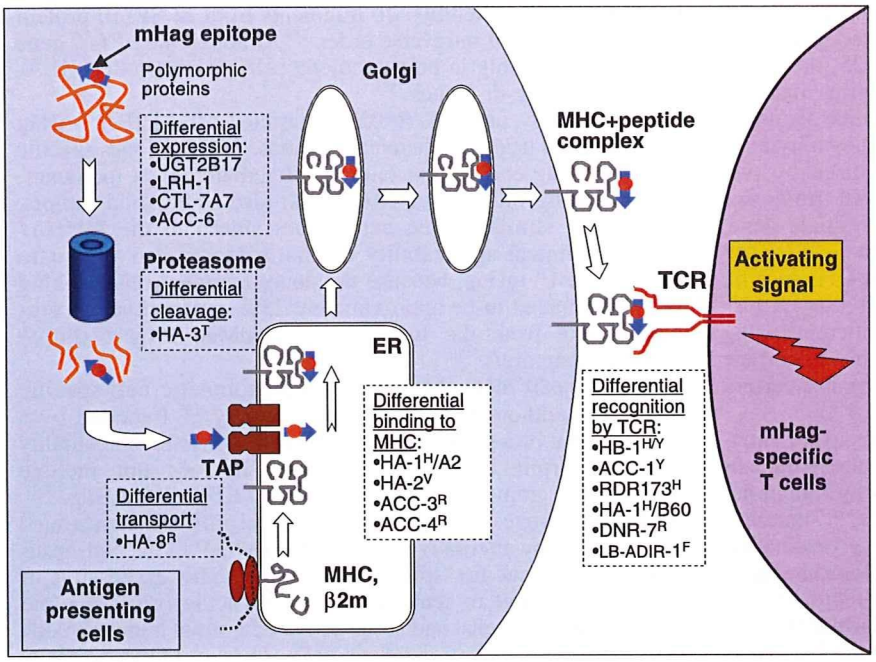

Fig. 2. Mechanisms involved in the generation of antigenicity of minor histocompatibility antigen complex (mHag) epitopes. In antigen-presenting cells, there is different machinery involved in the presentation of cellular proteins onto major histocompatibility complex (MHC) molecules on cell surfaces. The processing efficiency of each step (proteasome digestion, transporter associated with antigen processing [TAP] transport to endoplasmic reticulum (ER), peptide loading to MHC) can be affected by the amino acid sequence of individual peptides, so that an amino acid substitution due to a single nucleotide polymorphism (SNP) may give rise to differential expression of antigenic peptides. Besides these mechanisms, gene defects and alternative splicing/translation due to SNP may result in differential supply of antigenic polypeptides. Finally, even when polymorphic peptides are expressed on cell surface MHC at similar efficiencies, the repertoire or precursor frequency of corresponding T cells may differ, resulting in differential recognition by T-cell receptor (TCR).

Table 2. Potential therapeutic mHag epitopes as GVL targets applicable to hematological malignancies in the Japanese population restricted with HLA alleles, the frequency of which is more than 10%†

mHag	mHag* frequency (%)	Probability of mismatching (%)‡	Restriction HLA allele	HLA frequency (%)[37]	Applicability (%)§
ACC-1^{Y}	75¶	18.8	A*2402	59	11.1
ACC-2^{D}	43¶	24.5	B*4403/2	12.5	3.1
HA-1^{H}	62††	23.6	A*0201	22	5.1
			A*0206	16.6	3.9
ACC-6	18¶	14.8	B*4403/2	12.5	1.9
LRH-1	ND	NA	B*0702	11	NA
$RDR173^{H}$	ND	NA	B*0702	11	NA
LB-ADIR-1^{F}	54¶	24.8	A*0201	22	5.5
				Total	**30.6**

†Applicability for Caucasian populations was reported by Spierings *et al.*[55] ‡Calculated frequency of mismatching under the assumption of unrelated pairs. §Calculated by multiplying the probability of mismatching of a given mHag with the frequency of its restriction HLA. ¶Based on data from the International HapMap Project (http://www.hapmap.org/).[46] ††Kindly provided by Dr Saji (The HLA Laboratory). GVL, graft-versus-leukemia; HLA, human leukocyte antigen; mHag, minor histocompatibility complex antigen; NA, not applicable due to missing data; ND, not done in a Japanese population.

HMHA1 gene, a reverse immunological approach was employed by Goulmy's laboratory, and a single decameric peptide located three amino acid upstream of non-americ HA-1^{H}/A*0201 peptide was identified as a new epitope.[32]

The authors took a different approach, in which post-HCT peripheral blood T cells from patients receiving HA-1-disparate transplantation were stimulated with a 29-mer peptide spanning polymorphic Histidine in the middle, HA-1^{H}/A*0201 epitope, and successfully generated an HLA-A*0206-restricted CTL clone. Epitope analysis then revealed this to be identical to the HA-1^{H} peptide restricted by HLA-A*0201.[36] Presentation by HLA-A*0206 was unexpected from its preferred anchor motif, suggesting limitations of the reverse immunological approach. HLA-A*0206 is the second or third most common major HLA-A2 subtype in Asians,[37] thus the applicability of the HA-1^{H} mHag has almost become doubled (Table 2).

ACC-1^{Y} (BCL2A1^{A}/A24) and ACC-2^{D} (BCL2A1^{A}/B44). ACC-1^{Y} (restricted by HLA-A24) and ACC-2^{D} (HLA-B44) are mHag epitopes that were identified successfully for the first time using linkage analysis and subsequent *in silico* analysis in Aichi Cancer Center (ACC) in 2003.[30] CTL clones were generated from post-HCT peripheral blood of two independent patients with advanced leukemia using a CTL cloning method targeting HLA alleles of interest the authors developed.[38] To map gene(s) encoding mHag recognized by these CTL, a panel of B lymphoid cell lines (B-LCL) registered to the Center d'Etude du Polymorphisme Humain (CEPH) foundation was screened after transfecting restriction HLA alleles using an efficient retrovirus system.[39] The CEPH cell lines comprise B-LCL from large families, individuals of which have been genetically mapped. Thus, computational linkage analysis based on the cytotoxicity data of individual B-LCL by CTL clones, regarded as an inherited

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trait, and their genetic information facilitated mapping of the regions responsible for the generation of mHag recognized by the CTL clones. In the mapped region at 15q24-25, there was only one gene that fulfilled two critical criteria, namely, hematopoietic tissue-specific expression and a peptide sequence with the required HLA-binding motif and a non-synonymous coding SNP. It was surprising that mHag recognized by two CTL clones of different HLA restriction (derived from two different patients) were located in two SNP in a single gene, *BCL2A1*, but this may imply that mHag encoded by *BCL2A1* are particularly immunogenic. Indeed, a report that CTL specific for ACC-2^D were isolated from a Caucasian patient after DLI by random cloning[40] may support this idea. Similar attempts using linkage analysis have been conducted previously, but a shortage of genetic information before the Human Genome Project era hampered the approach.[41]

BCL2A1 protein, a homolog of BCL2, functions as an antiapoptotic molecule and is expressed only in normal and malignant hematopoietic cells.[30] Because it is up-regulated by inflammation or exposure to some chemotherapeutic agents,[42] immunotherapy with CTL specific for these mHag or active vaccination may be an ideal option against recurring hematological malignancies following HCT. At the same time, up-regulation of BCL2A1 by inflammatory cytokines can also pose a risk for GVHD,[43] but the authors' analysis using HLA-A24-positive, HLA genotypically matched unrelated marrow recipients through the Japan Marrow Donor Program did not indicate any potential association of ACC-1 disparity with GVHD development.[21] In addition, $CD8^+$ cells stained using HLA-A24/ACC-1^Y tetramers were detectable for up to 7 months in ACC-1-disparate HCT recipients,[21,44] suggesting the presence of immune surveillance by mHag-specific CTL. Furthermore, the authors have observed that CTL specific for ACC-1^Y can survive longer as memory T cells in marrow than in peripheral blood, as found for other memory T cells.[44]

LRH-1 (P2RX5^{+C}/B7), CTL-7A7^R (PANE1^C/A3), RDR173^H (ECGF1^A/B7), DRN-7^R (SP110^A/A3) and LB-ADIR-1^F (TOR3A^T/A*0201). LRH-1 mHag, termed 'lymphoid-restricted histocompatibility antigen-1', is generated by differential expression of the P2RX5 protein between the donor and recipient due to a frameshift resulting from a cytosine deletion polymorphism in the donor. It has also been identified using linkage analysis with some modifications.[17] Although P2RX5 mRNA is expressed highly in lymphoid cells and leukemic stem cells, clinical applicability is so far limited because the HLA restriction for LRH-1 is HLA-B7, the frequency of which in Japanese is less than 10%.[37] However, the *P2RX5* gene may encode more mHag epitopes, as both alleles encode different polypeptides due to a frameshift that is long enough to include binding motifs to HLA molecules of other alleles.[17]

The HLA-A3-restricted CTL-7A7^R mHag was identified using a biochemical method,[18] as with HA-1^H,[11] HA-2^V,[26] HA-3^T,[22] and HA-8^R.[24] The epitope is encoded by a PANE1 κ transcript that is only expressed in resting B cells and chronic B-cell leukemia cells. An individual homozygous for C→T SNP in the transcript resulting in the formation of stop codon (CGA→TGA) becomes mHag negative. Unlike the case of LRH-1 the differentially expressed region contains only 12 amino acids, so that its applicability is low in populations in which HLA-A3 is rare, such as Asians.[37]

The HLA-B7-restricted RDR173^H mHag was identified using expression cloning,[31] like HB-1^H,[29] ACC-4^R and ACC-5^R.[27] The *ECGF1* gene encoding this mHag is expressed in hematopoietic cells and some solid tumors, as expected from its gene name, angiogenic endothelial-cell growth factor-1.[31] Clinical applicability of this mHag in Asians is again not high because of its HLA-B7 restriction.[37]

The HLA-A3-restricted DRN-7^R mHag was found to be generated using a unique mechanism, 'protein' splicing mediated by proteasomes, whereby two fragments from an SP110 protein digest were ligated in reverse order.[23] Although the *SP110* gene is expressed mainly in hematopoietic cells, its restriction HLA-A3 allele is rare in Asians.[37]

Very recently, an HLA-A*0201-restricted LB-ADIR-1^F mHag was identified using biochemical methods.[45] CTL clones specific for this mHag epitope not only lysed hematological malignancies including multiple myelomas but also some solid tumors, implying a similar tissue expression pattern to the *HMHA1* gene.[11] Clinical applicability of this mHag is also similar to that of HA-1^H mHag, because the donor/recipient mismatching rate is estimated to be approximately 25% in Japanese, according to data from the International HapMap Project (http://www.hapmap.org/).[46]

ACC-6 (HMSD^A/B44). ACC-6 is a hematopoietic cell-specific mHag the authors have found just recently.[19] Encoded by a splice variant of a pseudogene termed *HMSD* (*H*istocompatibility (*M*inor) *S*erpin *D*omain containing), it does not include polymorphic amino acids, like LRH-1 and RDR173^H mHag,[17,31] as detailed above. This was the first novel mHag demonstrated to be formed by alternative splicing due to SNP in the consensus sequence critical for spliceome binding. The expression of *HMSD* is limited to leukemic cells, particularly of myeloid origin, myeloma cells, and some activated normal hematopoietic cells, including mature dendritic cells. *In vivo* immunogenicity was also proved by the presence of T cells specific for ACC-6 (0.8% of all $CD3^+$ cells at the peak time) using a quantitative polymerase chain reaction (PCR) method with clone-specific primers and a probe,[19] because HLA-B44 is notorious as a molecule unsuitable for tetramer construction. The observed magnitude of T-cell response appears comparable with those observed for HA-1^H-specific T-cell responses following DLI against recurrent leukemia.[17,20,31] In addition, as with LRH-1 mHag,[17] both alleles of *HMSD* can encode substantial polypeptides whose amino acid sequences are distinct due to a frameshift, so that it is possible that different HLA alleles may present other epitopes encoded by not only *HMSD-v* but also the original *HMSD* (encoding a 193-mer polypeptide). Furthermore, eradication of leukemic stem cells by the CTL was confirmed using severe immunodeficient common γ-chain knockout NOD/SCID (NOG) mice,[47] transplanted with CD34-selected acute myeloid leukemic cells with or without preincubation with either ACC-6-specific or irrelevant CTL clones.[19] These findings collectively suggest that ACC-6 is a promising target for immunotherapy at least against recurring myeloid leukemia post-HCT.

Clinical application of mHag

The involvement of mHag-directed immune responses in the eradication of recurrent leukemia following DLI has been shown by longitudinal association analysis between the emergence or increase of mHag-specific $CD8^+$ T cells detectable by tetramers and the decline or disappearance of leukemic cells.[17,20,31] GVL effects of human mHag-specific CTL against leukemia stem cells have also been shown in immunodeficient NOD/SCID mouse models.[16,48] By using congenic mice disparate for a limited number of mHag, adoptive transfer of CTL from mice primed with cells disparate for a single immunodominant mHag was much more effective than that from mice primed with cells possessing six tumor antigens, implying superior immunogenicity of mHag than tumor antigens.[8] Kircher *et al.* reported that HA-1 mHag-specific T cells were readily expanded *in vitro* when peripheral blood T cells of a patient with Ph^+ acute lymphocytic leukemia following DLI against relapsed disease were stimulated with HA-1 peptide, while no P190 BCR/ABL peptide-specific T cells were expanded.[49] In the authors' preliminary analyses using

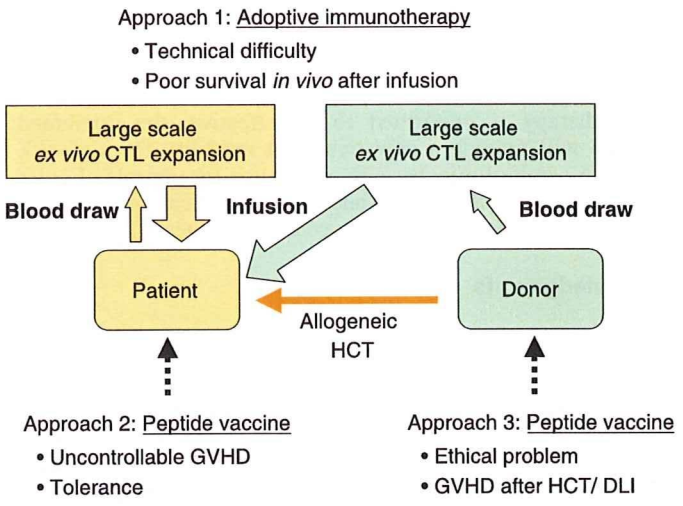

Fig. 3. Modalities of minor histocompatibility antigen complex (mHag)-based immunotherapy. Initially, adoptive immunotherapy with cytotoxic T cells (CTL) specific for mHag was developed largely due to the cell therapeutic nature of allogeneic cell transplantation (Approach 1). CTL are induced from either *in vivo*-sensitized T cells in the post-transplant recipient or naïve T cells of the donor, and then expanded *ex vivo* for infusion into the patient with recurrent disease. Recently, patient vaccination trials with predefined mHag peptides have been initiated (Approach 2). In the near future, donor vaccination prior to hematopoietic stem cell harvest or donor lymphocyte infusion (DLI) may undergo clinical trials with some modification such as enrichment of mHag-sensitized memory T cells so as to decrease graft-versus-host disease (GVHD; Approach 3). Potential limitations or current problems are listed under each step. HCT, hematopoietic cell transplantation.

post-HCT peripheral blood specimens, no WT-1-specific T cells were detected by tetramer (K. Kuzushima, unpublished observations, 2003), while mHag-specific T cells were frequently detected.[19,21,44] Therefore it is rational to use mHag as target antigens in the setting of allogeneic HCT. Indeed, allogeneic HCT is immunotherapy itself, taking advantage of allo-immune responses. The ultimate goal of defined hematopoiesis-specific mHag-based immunotherapy is to maximize GVL effects while minimizing unfavorable GVHD. In this context, it should be stressed that mHag encoded by hematopoiesis-specific genes such as *HMHA1*,[11] *HMHB1*,[29] *MYO1G*,[25] *BCL2A1*,[30] *P2RX5*,[17] *PANE1*,[18] *ECGF1*,[31] *SP110*,[23] *TOR3A*,[45] and *HMSD*,[19] are good candidates for immunotherapy for the majority or some fraction (e.g. B cells,[18,29] or myeloid cells,[31]) of hematological malignancies, depending on their preferential expression in hematopoietic cells.

Currently, the number of mHag applicable to a clinical purpose is limited, despite covering 30% of Japanese when including HA-1, ACC-1^Y, ACC-2^D, and ACC-6 that the authors have identified to date (in Table 2). The bottle-neck for mHag-based immunotherapy consists not only of the presence of restriction HLA alleles but also the necessity for an mHag disparity between the donor and patient. Further identification of mHag that are restricted by common HLA alleles in particular ethnic groups (e.g. HLA-A24 in Japanese,[37]) and that have an allelic frequency of 30–70% for greater chance of disparity is thus a high priority to allow expansion of the patient population eligible for mHag-targeted immunotherapy.

Historically, adoptive (passive) immunotherapy preceded active immunotherapies such as vaccination, probably because HCT itself is a kind of adoptive immunotherapy (Fig. 3). Problems associated with DLI, including GVHD or delayed response (usually 4 weeks), prompted mHag researchers to test the effects of adoptive immunotherapy with mHag-specific CTL clones against recurrent leukemia.[3] A protocol to generate HA-1^H and HA-2^V specific T-cell lines from mHag-negative donors was proposed for adoptive immunotherapy.[15] Another adoptive immunotherapy trial using CTL clones that lyse hematopoietic cells but not dermal fibroblasts has been performed in the Fred Hutchinson Cancer Research Center,[50] where a dramatic decline of cytomegalovirus (CMV)-related disease has already been demonstrated by adoptive transfer of CMV-specific CTL clones.[51] The authors also started a phase I/II study to test the toxicity and effectiveness of CTL clones specific for ACC-1^Y or ACC-2^D. The common problems associated with adoptive immunotherapy are an insufficient success rate in generation of CTL, laborious and time-consuming procedures to obtain therapeutic doses of T cells (10^9–10^{10}), and expensive facilities and running costs to meet the GMP standards. Indeed, one ACC-1-disparate patient was enrolled in the authors' study but dropped out due to failure in the expansion step.

A more feasible approach would be vaccination of post-HCT patients with defined mHag peptides synthesized and prepared under GMP conditions. The vaccination approach was tested extensively against solid tumors soon after the first identification of melanoma-associated tumor antigen.[52] Exploration of more effective adjuvants (e.g. dendritic cells, cytokines, Toll-like receptor stimulants), forms of antigens (e.g. peptide, protein, DNA) and routes of administration (e.g. injection into tumor, regional lymph-nodes, sub- or trans-cutaneous) has improved the antitumor effects, although both the tolerance barrier and autoimmunity still pose obstacles to the success of tumor (most of them are self) antigen-based immunotherapy (reviewed in Slingluff *et al.*[53]). In contrast, mHag epitope vaccines use alloantigens, so that there is less possibility of tolerance if GVHD induction is carefully avoided by administration at appropriate time points following HCT. The feasibility of inducing high avidity (i.e. CD8-independent) CTL specific for HA-1^H and HA-2^V has been shown in HLA-A2-transgenic mice using a DNA vaccine method.[54] A clinical peptide vaccination trial targeting HA-1^H and HA-2^V is reported to be underway in the USA,[55] although precise details are not available at present. Just recently, the authors also launched a phase I trial of mHag peptide vaccination targeting ACC-1^Y, ACC-2^D and HA-1^H against recurring hematological malignancies post-HCT to test the hypothesis of alloimmunity-based immunotherapy tailored to appropriate mHag for individual patients. If antitumor effects against recurrent tumors are observed in this trial, preventive vaccination after HCT will be initiated, because maximal effects would be expected in such a setting, as observed with other immunotherapeutics. The idea of donor immunization with mHag may be the ultimate goal to boost the GVL effects of HCT or DLI,[55] but it also poses a challenging ethical issue. Justification may be provided by the fact that normal postpartum mothers have T cells specific for mHag due to sensitization during pregnancy.[56]

Recently, allogeneic HCT for advanced solid tumors has been explored because of the development of reduced preconditioning regimens for patients of older age or in poor condition. Among the various tumor types tested, renal cell carcinomas responded fairly well,[5] suggesting a susceptibility to alloimmunity. Not only tumor antigens but also mHag have been shown to be involved in the GVT effect.[6] One possibility is that hematopoiesis-specific mHag that are aberrantly up-regulated in solid tumors may be targeted by cognate CTL. Indeed, the authors and others have provided evidence that the *HMHA1* gene is aberrantly expressed in some solid tumors, including lung cancers and renal cell carcinomas.[12,13] *ECGF1*-encoded mHag-specific CTL are also reported to be able to lyse melanoma cells.[31] In addition, the authors found just recently that *BCL2A1* is aberrantly expressed in melanoma cells (unpublished data, H. Torikai *et al.*, 2007). It is possible that

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mHag demonstrating tissue-specific expression may serve as target antigens, particularly when tumors originate in non-vital organs, although no such mHag have been identified to date. The available results do suggest that mHag-based immunotherapy may be applicable for at least a fraction of patients with solid tumors. Finally, mHag alleles are easy to determine, as most of them are controlled by a single SNP. Thus, it should be possible to predetermine patients eligible for mHag-based immunotherapy. Currently, one kit for genotyping mHag is commercially available at One Lambda, Inc., (Canoga Park, CA, USA) that has been used to establish a minor H database, named dbMinor (http://www.lumc.nl/dbminor).[57] In Japan, alternatively, The HLA Laboratory (Kyoto, Japan; http://www.hla.or.jp/) provides typing services.

Conclusions

It has already been a decade since the potential application of hematopoiesis-specific mHag was first proposed for HCT patients with recurring hematopoietic malignancies. The slow progression or translation toward clinical trials for testing mHag-based immunotherapy has been due at least in part to the limited number of potentially therapeutic mHag identified to date and also to the relatively small number of patients receiving HCT (~1700 allogeneic HCT per year in Japan). Thus, further efforts are needed to identify new mHag epitopes to allow immunotherapy coverage of most HCT patients with hematological malignancies when they unfortunately recur. Once the safety of mHag-based immunotherapy is confirmed, the ultimate aim will be preventive immunotherapy tailored for patients at high risk of relapse after HCT, providing a boost with either mHag vaccination or adoptive transfer of T-cell specific mHag at appropriate times after HCT. The authors believe that as HCT becomes safer through reduced-intensity conditioning and appropriate immune suppression, and as mHag-based immunotherapy is confirmed to be effective, the combined approach will provide a new treatment modality for high-risk patients. A significant increase in application for patients of older age and those with not only advanced hematological malignancies, but also solid tumors, may be expected in the near future.

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ORIGINAL ARTICLE

The HLA-A*0201-restricted minor histocompatibility antigen HA-1^H peptide can also be presented by another HLA-A2 subtype, A*0206

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HA-1^H is one of the most attractive minor histocompatibility antigens (mHA) as a target for immunotherapy of hematopoietic malignancies, but HLA-A*0201 and HLA-B60 molecules capable of presenting HA-1^H-derived peptides are less common in eastern Asian populations when compared with Caucasian populations. Therefore, an attempt was made to search for novel epitopes presented by HLA alleles other than those previously reported by generating CTL lines from patients undergoing HLA-identical, HA-1 disparate hematopoietic stem cell transplantation (hematopoietic SCT) by stimulation with a 29-mer HA-1^H peptide spanning a central polymorphic histidine (His). Two CTL clones established were found to be restricted by HLA-A*0206, which is the second or third most common HLA-A2 subtype worldwide. Epitope mapping revealed that the clones recognized the same nonameric peptide as A*0201-restricted HA-1^H, VLHDDLLEA. This epitope was unexpected, since it does not contain any preferred anchor motifs for HLA-A*0206. However, an HLA peptide binding assay revealed stronger binding of this peptide to A*0206 than to A*0201. Interestingly, HLA-A*0206-restricted CTL clones could lyse both HLA-A*0206$^+$ and HLA-A*0201$^+$ targets (including leukemic blasts) that express HA-1^H peptide endogenously, whereas an HLA-A*0201-restricted, HA-1^H-specific CTL clone failed to lyse HLA-A*0206$^+$ targets. This finding will expand the patient population who can benefit from HA-1^H-based immunotherapy.

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Introduction

Minor histocompatibility antigens (mHAs) are MHC-bound peptides derived from cellular proteins, which are immunogenic because of their differential expression between the donor and recipient, most often due to a single nucleotide polymorphism (SNP).[1,2] To date, significant efforts have been made to identify mHAs, particularly those specific for hematopoietic cells, since such mHAs have been speculated to contribute to the GVL effect following allogeneic stem cell transplantation (allogeneic SCT). Among these, HA-1, whose immunogenicity is controlled by a nonsynonymous SNP (dbSNP cluster ID: rs1801284) on the *HMHA1* gene (GeneID: 23526),[3] has been studied most extensively since it can be used for immunotherapy of recurring hematological malignancies after HA-1-mismatched SCT.[4] The originally reported HA-1 mHA is an HLA-A*0201-restricted nonameric peptide carrying His (HA-1^H) but not Arg (HA-1^R) at position 3.[3] In an effort to expand the patient population for HA-1-based immunotherapy, a decameric peptide containing His at position 6 from the same polymorphic region has been identified as an mHA presentable by the HLA-B60 molecule.[5] The only other mHAs with selective expression in hematopoietic cells described to date are HA-2,[6,7] BCL2A1 (ACC-1 and ACC-2),[8,9] HB-1[10,11] and PANE1,[12] the latter two of which are B cell lineage-specific. Thus, identification of novel hematopoietic system-specific mHAs is warranted to facilitate the development of effective immunotherapy to induce GVL reactions.

HLA-A*0201 is the most common allele in HLA-A2 subtypes and is distributed worldwide, especially among the general Caucasian populations (according to unpublished data from the 12th International Histocompatibility Workshop (IHW)). The next most common HLA-A2 subtypes appear to be HLA-A*0206 and/or -A*0207, which are found frequently in the eastern Asian populations, including Chinese and Japanese populations.[13,14] To date, it has not been examined whether an HLA-A*0201-restricted HA-1^H mHA epitope can be presented by HLA-A*0206, which possesses a single amino-acid substitution (9Phe-9Tyr) in the B-pocket compared with HLA-A*0201,[15] probably owing to the rarity of the HLA-A*0206 allele in

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Caucasian populations. While attempting to identify HLA alleles capable of presenting HA-1 mHAs other than the previously reported HLA-A*0201 and HLA-B60,[5] we unexpectedly found that HLA-A*0206 was able to present the nonameric peptide originally described as the A*0201-restricted HA-1^H mHA (VLHDDLLEA). These findings may be clinically beneficial for SCT recipients expressing HLA-A*0206, which accounts for 20–40% of HLA-A2 alleles in eastern Asia, by broadening the potential clinical relevance of an epitope that can be used for HA-1 mHA-based immunotherapy.

Materials and methods

Patients, cell culture and clones

This study was approved by the Institutional Review Board of Aichi Cancer Center according to the Declaration of Helsinki. All blood samples were collected after written informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll–Paque (Pharmacia, Uppsala, Sweden). CD4$^+$ cells were isolated from donor PBMCs using anti-human CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), expanded with 5 μg/ml PHA-L (Roche, Mannheim, Germany), 20 U/ml interleukin (IL)-2 (Chiron, Emeryville, CA, USA) and 20 ng/ml IL-7 (R&D Systems, Minneapolis, MN, USA) as previously reported,[16] and used as antigen presenting cells (APCs). CD3$^+$ cells were isolated from recipient PBMCs using anti-human CD3 microbeads (Miltenyi Biotec) and used as responder cells. CD34$^+$ leukemic cells were isolated from bone marrow specimens from acute myelocytic leukemia patients using anti-human CD34 microbeads (Miltenyi Biotec) and used as target cells. Epstein–Barr virus-transformed B lymphoblastoid cell lines (LCL) derived from donors, recipients and normal volunteers and the HLA class I-negative, LCL, 721.221 cell line[17] were maintained in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum (IBL, Takasaki, Japan). LCLs were transduced with retroviral vectors carrying individual HLA cDNAs by a method described previously.[18] HLA cDNA-transfected cell lines are referred to by their 'cell line name/HLA allele' designation (e.g. 721.221/A*0201). EH6-CTL is a CD8$^+$ clone previously generated from an HLA-A*0201$^+$ and HA-1^R healthy individual.[18]

Synthetic peptides and in vitro *CTL induction*

The peptide used for CTL induction consisted of 29 amino acids from the HA-1 polymorphic region with the histidine (underlined) in the middle (RFAEGLEKLKECVLHDDL-LEARRRRAHEC). All peptides were synthesized using standard Fmoc chemistry and dissolved in 100% DMSO.

Donor-derived PHA-activated CD4$^+$ cells were pulsed with the 29-mer peptide (50 μg/ml) for 2 h at room temperature in AIM-V medium (Invitrogen, Carlsbad, CA, USA). CTL lines were generated from post-SCT CD3$^+$ cells (5×10^4) by coculturing with irradiated (35 Gy) peptide-pulsed CD4$^+$ cells (5×10^4) in 0.2 ml RPMI 1640 supplemented with 6% pooled human serum, 2 mM L-glutamine and penicillin/streptomycin (referred to as CTL medium) in the presence of recombinant human IL-6 (20 ng/ml, R&D Systems) and IL-12 (20 ng/ml, R&D Systems) in a 96-well round-bottomed plate. IL-2 (10 U/ml) was added on day 3. Individual wells were similarly restimulated weekly with irradiated, peptide-pulsed (10 μg/ml) donor CD4$^+$ cells without cytokines except for IL-2 (10 U/ml) on day 1. After the fourth stimulation, T cells in culture wells showing lytic activity to both recipient LCL and 29-mer peptide-pulsed donor LCL but not unpulsed donor LCL were combined and cloned by limiting dilution. Putative CTL clones were expanded as previously described[19] and frozen until use. The cytotoxic activity of CTL lines and clones was evaluated by standard ^{51}Cr release assays. Percent specific lysis was calculated as follows: Experimental c.p.m.−spontaneous c.p.m./(maximum c.p.m.−spontaneous c.p.m. × 100. For tetramer blocking experiments, 0.25×10^6 CTL clones were incubated with 40 μg/ml of individual tetramers in PBS containing 2% fetal calf serum at room temperature for 15 min, then washed twice before coculturing with either 721.221/A*0201 or 721.221/A*0206 target cells.

Genotyping of HA-1 polymorphisms

Genomic DNA was isolated from each B-LCL with a DNA blood kit (QIAGEN, Valencia, CA, USA) and amplified by PCR. The primer sequences and amplification conditions used to amplify *HMHA1* gene encoding the HA-1 polymorphic region were (obtained from http://snp.ims.u-tokyo.ac.jp/cgi-bin/SnpInfo.cgi?SNP_ID = IMS-JST118551) as follows:

sense, 5′-CTGACCTCTGGCCTTTGACC-3′
antisense, 5′-ATCTGAGCCTCCCTCCCTTC-3′

PCR products were purified and directly sequenced with the same primer and a BigDye Terminator kit (ver. 3.1; PE Applied BioSystems, Foster City, CA, USA) using an ABI PRISM 3100 (PE Applied Biosystems).

Epitope reconstitution assay

^{51}Cr-labeled HA-1^R LCLs from either HLA-A*0201$^+$ or -A*0206$^+$ individuals were incubated for 30 min in medium containing 10-fold serial dilutions of test peptides and then used as target cells in standard 4 h cytotoxicity assays.

HLA peptide binding assay

A quantitative ELISA-based assay capable of measuring the affinity of the interaction between peptide and HLA was carried out as described previously,[20] with some modifications. In brief, purified recombinant HLA molecules in 8 M urea, 10 mM EDTA, 25 mM 2-(*N*-morpholino)-ethanesulfonicacid and 0.1 mM dithiothreitol were diluted to 4 μg/ml in refolding buffer containing 400 mM arginine, 100 mM Tris pH 8.0, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 0.2 mM phenylmethyl sulfonyl fluoride (all from Sigma-Aldrich) and 2 μM purified β2-microglobulin (β2m) on ice. Ten-fold dilutions of each peptide were made with 100% DMSO in 96-well round-bottomed polypropylene plates, then 1 μl individual aliquots were transferred into new plates and 99 μl of the above HLA-β2m mixture was added to each

well (i.e. 100-fold dilution for each peptide solution). The plates were incubated on a shaker at 4°C for 48–72 h. One day before ELISA analysis, 96-well ELISA plates (Costar, Cambridge, MA, USA) were coated with 50 μl/well W6/32 MoAb (10 μg/ml) in 50 mM carbonate–bicarbonate buffer, pH 9.6 (Sigma), and kept overnight at 4°C. After washing thrice with washing buffer containing 0.05% Tween 20 (Sigma-Aldrich) in PBS, the wells were blocked for 1 h. Just before the ELISA analysis, the reaction volume was diluted 10 times by PBS at 4°C, and 50 μl/well of aliquots were transferred in duplicate to the W6/32 MoAb-coated plates. The plates were incubated for 2 h at room temperature and then washed six times. To detect properly refolded complexes, plates were incubated for 2 h at room temperature with 100 μl/well of a horseradish peroxidase-conjugated anti-human β2m MoAb (1:1000 dilution; DAKO, Copenhagen, Denmark), followed by washing as above. Finally, color development was performed with 3,3′,5,5′-tetramethyl-benzidine as substrate (Sigma-Aldrich).

Tetramer construction and flow cytometric analysis

HLA-A*0201 or HLA-A*0206 tetramers incorporating the HA-1^H peptide (VLHDDLLEA) were produced as described previously.[21] For staining, cells were incubated with the tetramers at a concentration of 20 μg/ml at room temperature for 15 min followed by FITC-conjugated anti-CD3 (Becton-Dickinson, San Diego, CA, USA) and Tricolor anti-CD8 MoAb (Caltag, Burlingame, CA, USA) on ice for 15 min. Cells were analyzed with a FACSCalibur flow cytometer and CellQuest software (Becton-Dickinson).

Determination of TCR variable (V)-gene usage

TCR V-gene usage was assessed by RT-PCR using primers covering the entire families of functional TCR alpha (TCRAV) and beta (TCRBV) chains. Briefly, total RNA was extracted from individual CTL clones and cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen) and primer p(dT)$_{15}$ (Roche) and used for PCR. For TCRBV usage determination, each reaction was carried out with an optimal concentration of sense primers specific for four or five different BV families and a primer specific for the constant region of TCRB.[22] For TCRAV usage determination, each reaction was carried out with a single sense primer specific for individual alpha families and a primer specific for the constant region of TCRA.[23] The PCR products were size-fractionated on 2% agarose gels. Subsequently, the complementarity-determining region 3 (CDR3) of each positive PCR product was sequenced with corresponding antisense primer as above. TCR V-gene usage was determined by the international ImMuno GeneTics information system (IMGT) software, IMGT/V-QUEST (http://imgt.cines.fr).[24]

Results

Induction of CTL lines reactive to 29-mer HA-1^H peptide

A cohort of 28 patients were enrolled into our clinical study to generate mHA-specific T cells. Three of the 28 patients were found to have received an HA-1 disparate (namely, from HA-1$^{R/R}$ to HA-1$^{H/H}$ or HA-1$^{H/R}$) transplant. We chose the two patients negative for HLA-A*0201 since the presence of HLA-A*0201 was expected to induce T cells mostly specific for the immunodominant A*0201-restricted HA-1^H mHA. The two patients (designated by unique patient numbers (UPN)) and their corresponding HLA types were UPN012 (A*1101/2402, B*3901/5101, Cw*0702/1402, DRB1*0803/1302) and UPN022 (A*0206/3303, B*4006/4403, Cw*0801/−, DRB1*0901/1302). Both patients were typed as HA-1$^{H/R}$ and received HA-1$^{R/R}$ marrow from their donors. Peripheral blood CD3$^+$ cells obtained at days 102 and 196 from UPN012 and at days 28 and 99 from UPN022 were stimulated with peptide-pulsed, activated CD4$^+$ APCs prepared from their respective donor PBMC. Out of the four sets of cultures, two of 18 T-cell lines induced from day 99 CD3$^+$ cells of UPN022 preferentially lysed the recipient LCL and 29-mer HA-1^H peptide-pulsed donor LCL (Supplementary Figure 1). After the two lines were combined and cloned by limiting dilution, four putative CD8$^+$ CTL clones with the same specificity as the original T-cell lines were obtained (Table 1).

To test the HLA restriction of these putative clones, HLA class I-deficient 721.221 LCL were transduced with individual HLA class I cDNA of UPN022 and used as targets because this cell line was found to be positive for the HA-1^H allele. All four putative CTL clones demonstrated an identical pattern of HLA restriction, which was HLA-A*0206. Interestingly, these clones also lysed 721.221/A*0201 efficiently (Table 1). In contrast, EH6-CTL, a CD8$^+$ clone previously generated from an HLA-A*0201$^+$ and HA-1^R healthy individual,[18] was only able to lyse 721.221/A*0201 but not 721.221/A*0206, suggesting a higher degree of specificity. Out of the four putative clones from UPN022, 3B11-CTL and 4B1-CTL were found to be clonal by TCRBV usage analysis. Since the latter 4B1-CTL grew better, further experiments were carried out with it in comparison with EH6-CTL.

HLA restriction of 4B1-CTL was further tested using a panel of HLA-typed LCLs. As shown in Table 2, 4B1-CTL lytic activity was dependent on the presence of the HA-1^H allele and either the HLA-A*0206 or HLA-A*0201 allele. UR6 LCL carrying the HA-1^H allele and HLA-A*0207 was not lysed.

*Identification of the minimal epitope for the HLA-A*0206-restricted 4B1-CTL clone*

It has been shown that HLA-A*0206 has a single dominant anchor motif of Val at position 2 (P2), whereas A*0201 has two dominant anchor motifs, Leu at P2 and Val at P9.[25,26] Because HLA-A2 molecules incorporate nonameric peptides preferentially, we searched for such nonamers with Val at P2 in the 29-mer peptide used in the stimulation. One peptide, CVLHDDLLE, which had a Leu at P3 as a strong anchor motif for A*0206 as well, was the only candidate to fulfill the requirement. We conducted peptide reconstitution assays using 4B1-CTL and LCL from an HLA-A*0206$^+$, HA-1^R individual by pulsing the following peptides: CVLHDDLLE, its allelic variant CVLRDDLLE,

Table 1 HLA restriction element of putative CTL clones isolated from UPN022

Targets	*Putative CTL clones*				
	1E3	*2C3*	*3B11*	*4B1*	*EH6*[a]
% Specific lysis (E:T = 10:1)					
UPN022 Recipient LCL	73	68	70	66	−4
Donor LCL	3	2	−2	−1	−3
29-mer peptide[b] + donor LCL	72	73	71	71	3
721.221 LCL transfected with[c]					
HLA-A*0206	82	67	69	70	2
HLA-A*3303	2	−1	−1	−2	1
HLA-B*4406	5	0	−1	0	1
HLA-B*4403	5	−1	−1	1	1
HLA-Cw*0801	5	2	4	4	−1
HLA-A*0201	82	69	72	74	56
Clonality assessed by TCR usage[d]	No	No	Yes	Yes	Yes
Cell surface phenotype	CD8$^-$	CD8$^-$	CD8$^+$	CD8$^-$	CD8$^-$

Abbreviations: CTL = cytotoxic T lymphocyte; HLA = human leukocyte antigen; LCL = lymphoblastoid cell lines; TCR = T cell receptor for antigen.
[a]EH6, HLA-A*0201-restricted, HA-1^H-specific CTL clone.[18]
[b]Peptide used for induction (see Materials and methods).
[c]HLA class I-deficient LCL retrovirally transduced with individual HLA cDNA as reported previously.[18]
[d]The complementarity-determining region 3 of T-cell receptor β chain was sequenced by reverse transcription-PCR as previously reported.[22]

Table 2 Detailed restriction analysis of CTL clone 4B1 for HLA-A2 subtypes

Target[a]	*HLA-A*[b]	*HA-1 status*	*% Specific lysis*[c]
UPN022 recipient	0206, 3303	H/R	58
UPN022 donor	0206, 3303	R/R	0
UR1	0206, 2402	H/H	64
UR2	0206, 2402	R/R	2
UR3	0206, 0201	R/R	3
UR4	0201, 1101	H/R	57
UR5	0201, 2601	R/R	6
UR6	0207, 2402	H/R	4
UR7	2402, —	H/R	0
UR8	3101, —	H/R	0

Abbreviations: CTL = cytotoxic T lymphocyte; HA = histocompatibility antigen; HLA = human leukocyte antigen; LCL = lymphoblastoid cell lines.
[a]UR, LCL derived from unrelated individuals.
[b]HLA alleles shared with the UPN022 are underlined.
[c]A standard cytotoxicity assay was carried out at the E:T ratio of 30:1.

the original HA-1/A*0201 peptides (VLHDDLLEA and VLRDDLLEA) and decameric peptides of HA-1/A*0201 with a C-terminus extension of Arg (Figure 1a). Unexpectedly, 4B1-CTL recognized the HA-1^H/A*0201 peptide at half maximal lysis of ~1 nM and to lesser extent, its C-terminal extended decamer but could not recognize the predicted CVLHDDLLE nonamer at all.

To confirm that the HA-1^H/A*0201 peptide is indeed incorporated into the HLA-A*0206 molecule, an HLA peptide binding assay was carried out (Figure 1b). Surprisingly, the HA-1^H/A*0201 peptide was incorporated into HLA-A*0206 10-fold better than into HLA-A*0201 as assessed by means of quantifying peptide–MHC–β2m complexes refolded successfully.

Next, recognition of the HA-1^H/A*0201 peptide in the context of the two HLA-A2 subtypes was assessed by cytotoxicity assay. As expected from the results shown in Tables 1 and 2, 4B1-CTL recognized the HA-1^H/A*0201 peptide when pulsed onto either HLA-A*0206$^+$ or HLA-A*0201$^+$ LCL generated from HA-1^R individuals although recognition of HLA-A*0201-presented peptide was 10-fold lower (30 pM for HLA-A*0206$^+$ LCL versus 300 pM for HLA-A*0201$^+$ LCL by half maximal lysis; Figure 1c). In marked contrast, EH6-CTL recognized the HA-1^H/A*0201 peptide only when pulsed onto LCL generated from an HLA-A*0201$^+$ HA-1^R individual with a similar efficiency to that for 4B1-CTL. In addition, recognition of the HA-1^H/A*0201 peptide endogenously generated and presented by the two HLA-A2 subtypes was tested using the two CTL clones. As shown in Figure 1d, 4B1-CTL recognition of HA-1^H peptide presented by HLA-A*0206 was consistently better over the wide range of E:T ratio than both 4B1-CTL and EH6-CTL recognition of peptide presented by HLA-A*0201. These results indicate that the HA-1^H/A*0201 peptide, VLHDDLLEA, is presentable by both HLA-A2 subtypes and is sufficiently immunogenic. 4B1-CTL generated from an HLA-A*0206$^+$ SCT recipient showed less fine specificity in terms of scaffold molecules presenting VLHDDLLEA than EH6-CTL generated from an HLA-A*0201$^+$ individual.

Phenotypic and functional analyses using tetramers

We prepared HLA-A*0206 and HLA-A*0201 tetramers incorporating the HA-1^H/A*0201 peptide, VLHDDLLEA, and stained two CTL clones. As expected from the results shown in Table 2, 4B1-CTL was stained not only with A*0206 tetramer but also with A*0201 tetramer, although mean fluorescence intensity with the latter tetramer was nearly three-fold lower (67.5 versus 194.5; Figure 2, upper panels). In contrast, EH6-CTL was stained weakly with A*0206 tetramer (12.1%), while it was strongly stained with the cognate A*0201 tetramer (Figure 2, lower panels).

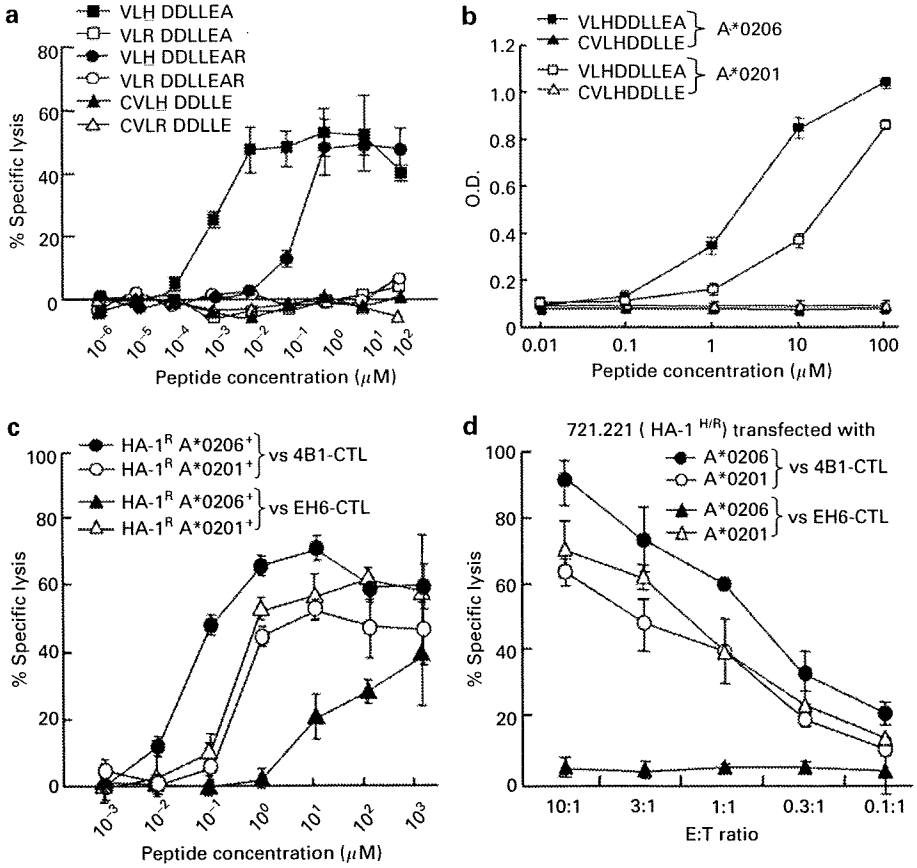

Figure 1 Identification of minimal epitope by epitope reconstitution and peptide-HLA binding assays. (**a**) UPN022 donor LCL were labeled with ^{51}Cr and distributed to wells of 96-well round-bottomed plates, pulsed with serial dilutions of the indicated peptides for 30 min at room temperature, and then used as targets for HLA-A*0206-restricted 4B1-CTL in a standard ^{51}Cr release assay (E:T ratio of 10:1). Data shown are representative of two independent experiments and the experiments were performed in duplicate. All values with error bars indicated in all figures applicable are shown as mean ± s.d. VLHDDLLEA was originally described as an HLA-A*0201-restricted *HMHA1*-derived mHA.[3] (**b**) Peptide-HLA binding assays were carried out as previously described[20] with some modifications (see Materials and methods). Purified HLA-A*0206 or HLA-A*0201 molecules were folded in folding buffer containing β2m and the serially diluted peptides indicated for 48–72 h. Amounts of properly folded HLA-A*0206 or HLA-A*0201 molecules were assessed by ELISA using plate-coated, conformation-dependent anti-HLA class I (clone W6/32) and horseradish peroxidase-tagged anti-β2m MoAbs. Folding efficiency is expressed in optical density (OD) at 630 nm. Data shown are representative of three independent experiments and the experiments were performed in duplicate. (**c**) Epitope reconstitution assay with VLHDDLLEA peptide for 4B1-CTL and HLA-A*0201-restricted, HA-1^H-specific EH6-CTL. HA-1^R-homozygous LCL positive for either HLA-A*0206 or HLA-A*0201 were pulsed with serial dilutions of VLHDDLLEA peptide for 30 min at room temperature and lytic activity by either 4B1-CTL or EH6-CTL was plotted. Data shown are representative of four independent experiments and the experiments were performed in duplicate. (**d**) Lytic activity of HLA-A*0206-restricted 4B1-CTL and HLA-A*0201-restricted EH6-CTL against HA-1^H-positive 721.221 LCLs transduced with either HLA-A*0206 or HLA-A*0201. A standard 4 h ^{51}Cr-release assay was carried out at the various E:T ratios indicated. Data shown are representative of two independent experiments and the experiments were performed in duplicate.

Next, we examined whether these tetramers could specifically block the recognition of target cells by the CTL clones (Figure 3a). The lysis of 721.221/A*0206 LCL by A*0206-restricted 4B1-CTL was efficiently inhibited by the cognate A*0206 tetramer, whereas inhibition with A*0201 tetramer was less significant. Similarly, the lysis of 721.221/A*0201 LCL by A*0201-restricted EH6-CTL was more efficiently blocked with the cognate A*0201 tetramer. Interestingly, A*0206 tetramer appeared to be able to block the recognition of EH6-CTL as well as A*0201 tetramer at the highest E:T ratio tested, suggesting that crossreactivity can be observed under certain conditions (consistent with the low degree of lysis by EH6-CTL of A*0206$^+$ LCL seen at high peptide concentrations in Figure 1c).

TCR variable region usage by HA-1^H-specific CTL clones

It has been shown previously that all HLA-A*0201-restricted clones specific for HA-1^H peptides from three individuals use the conserved TCRBV gene, TCRBV6S9,[27,28] corresponding to TCRBV7-9 by the IMGT classification.[24] We analyzed the variable and CDR3 usage of CTL clones, EH6, 4B1 and 3B11 (Table 3, and data not shown). Interestingly, not only HLA-A*0201-restricted EH6 but also the HLA-A*0206-restricted 4B1 and 3B11 CTL clones used the TCRBV7-9, although TRAV usage was different. The CTL clones 4B1 and 3B11 were found to have identical TCRAV, TCRBV and CDR3 sequences (data not shown). It is to be noted that HLA-A*0206-restricted 4B1-CTL had the Leu–Val amino-acid pair in the TCRB *N*-region (Table 3, underlined) which was

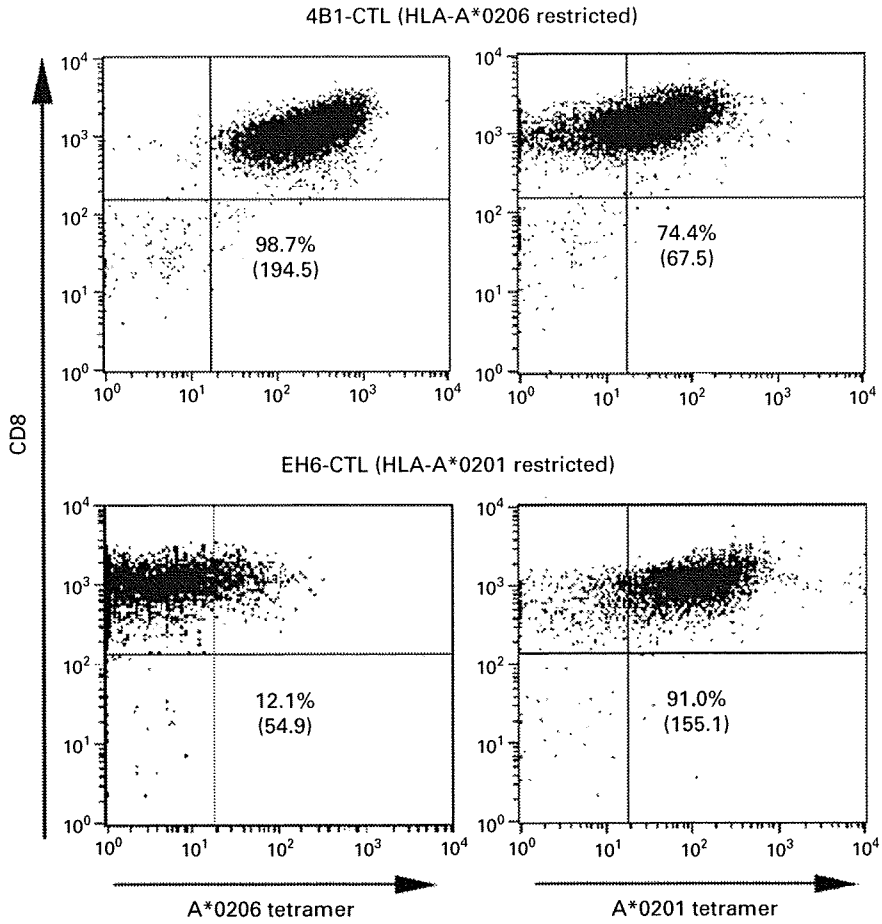

Figure 2 Representative staining profiles of CTL clones by tetramers. 4B1-CTL (upper panels) and EH6-CTL (lower panels) were stained with VLHDDLLEA peptide-incorporated HLA-A*0206 (left column) and HLA-A*0201 (right column) tetramers for 15 min at room temperature. Cells were washed and counterstained with CD3 and CD8 MoAbs, as described in Materials and methods. The percentage of T cells binding to the tetramer among all CD8$^+$ population is indicated in each panel and mean fluorescence intensity for tetramer$^+$ cells located in the upper right quadrant is shown in parentheses. Data shown are representative of three independent experiments.

also shown to be shared by HLA-A*0201-restricted HA-1^H-specific CTL clones.[27]

In vivo *relevance of the HLA-A*0206-restricted HA-1^H peptide-specific CTL*

Since the *HMHA1* gene is highly expressed in hematopoietic cells including hematopoietic malignancies,[3] we sought to examine whether 4B1-CTL could lyse leukemic CD34$^+$ cells carrying HA-1^H. As shown in Figure 3b, the CTL clone lysed not only HLA-A*0206$^+$ blasts but also HLA-A*0201$^+$ blasts as efficiently as UPN022 recipient-derived LCL. In contrast, the EH6-CTL recognized HLA-A*0201$^+$ but not HLA-A*0206$^+$ blasts (data not shown).

Finally, we examined the *in vivo* presence of HLA-A*0206$^+$-restricted, HA-1^H-specific T cells by means of tetramers. To this end, we stained PBMCs obtained at day 98 after SCT from a patient (UPN011) who was positive for both HLA-A*0201 and HLA-A*0206 and received HA-1 disparate marrow transplant from an unrelated, HLA-A, -B, -DR-matched donor (UPN022 PBMCs were no longer available). As shown in Figure 3c, minor populations of unstimulated CD8$^+$ cells were stained by HLA-A*0206 and HLA-A*0201 tetramers (0.06 and 0.18%, respectively) and with very low fluorescence intensity (compared to staining of the 4B1-CTL clone in Figure 2, upper left panel). To ascertain that these minor tetramer$^+$ populations were indeed HA-1^H specific, PBMCs were stimulated with peptide-pulsed APCs and assayed. After stimulation, individual tetramers detected around 15% tetramer$^+$ CD8$^+$ cells with subpopulations of cells (9.5 and 5.7%, boxed in Figure 3c) staining as brightly as the 4B1-CTL and EH6-CTL clones, respectively. It is speculated that these populations might represent HA-1^H-specific CD8$^+$ T cells restricted by HLA-A*0206 and HLA-A*0201 molecules, respectively.

Discussion

Among human mHAs identified to date, HA-1^H is one of the most promising mHAs involved in GVL and graft-versus-tumor effects due to its limited expression in hematopoietic cells[3] and some epithelial cancers.[29,30]