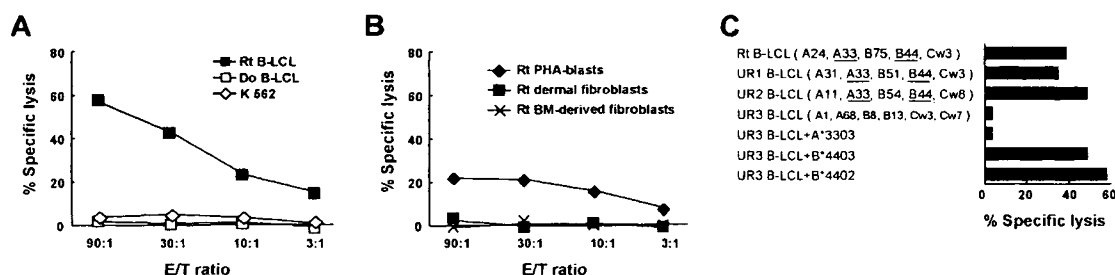


Figure 1. Specificity of the HLA-B44–restricted CTL clone 2A12. The cytolytic activity of CTL-2A12 was evaluated in a standard 4-hour ^{51}Cr release assay at the indicated E/T ratios. (A) CTL-2A12 recognition of target cells derived from recipient (Rt) but not donor (Do) B-LCLs. NK-sensitive K562 cells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

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

LSC engraftment assay of AML cells in immunodeficient NOG mice

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

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

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

Results

Characterization of a CTL clone

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

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

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

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

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

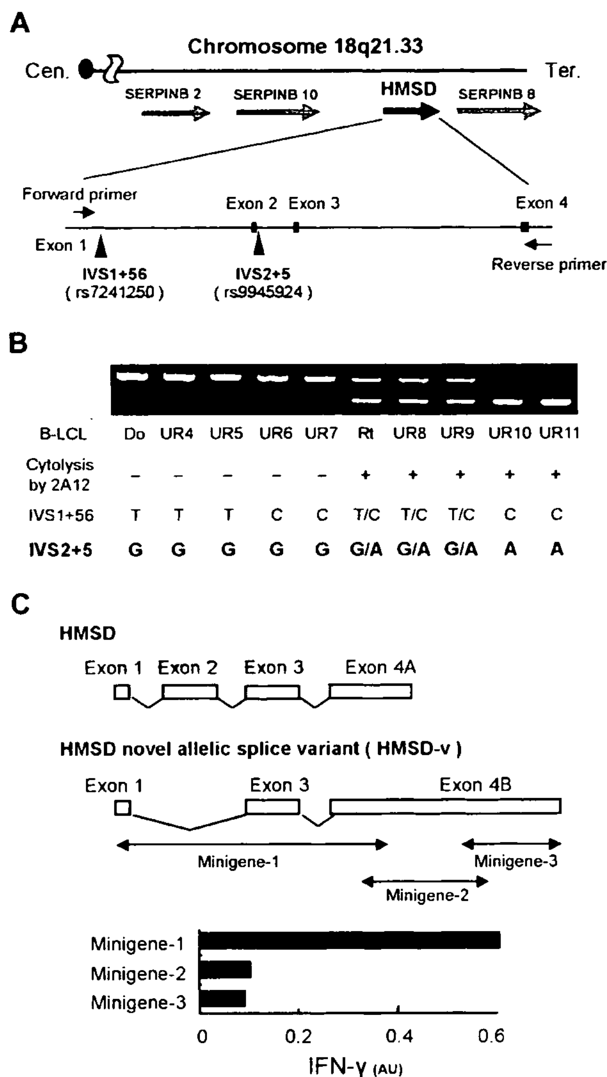


Figure 2. Identification of a novel splice variant transcript of *HMSD* encoding the mHA. (A) Summary of genome mapping around chromosome 18q21.33 showing relative positions of *HMSD*. Two identical cDNA clones were homologous to exons 1 and 3 plus exon 4 but lacked exon 2. This novel allelic splice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

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

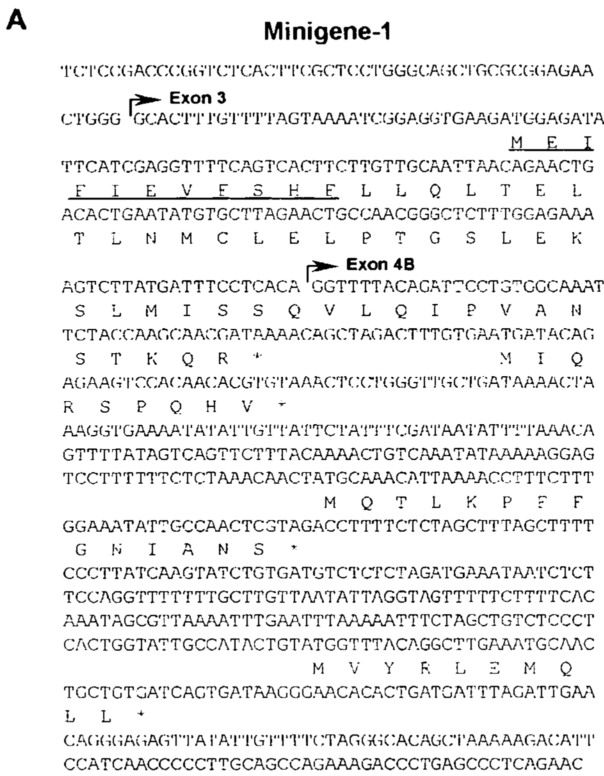
Identification of an HLA-B*4403-restricted epitope of *HMSD-v* and epitope reconstitution assay

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

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

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

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



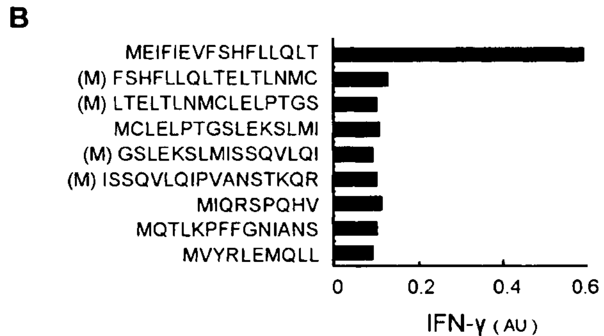


Figure 3. The nucleotide and deduced amino-acid sequences of minigene-1 encoding the CTL-2A12 mHA epitope. (A) Exon 2 encoding the original start codon in *HMSD* was deleted. After searching all frames, 2 reading frames in the *HMSD-v* transcript shown here were found to be able to encode polypeptides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

tissue cDNAs was unknown. High levels of expression were observed in primary AML and MM cells, mature DCs, CD40-B cells and PHA blasts (Figure 5A top panel), and malignant hematopoietic cell lines (especially those of myeloid lineage; Figure 5A bottom panel). In contrast, most normal tissues (Figure 5B top panel), including resting primary hematopoietic cells (Figure 5A top panel), showed lower or no expression, except for testis, which expressed a moderate amount of transcript. Weak expression observed in commercial cDNA from nonhematopoietic tissues including brain, lung, and placenta could be caused at least in part by contaminating hematopoietic cells or resident cells of hematopoietic origin such as pulmonary macrophages, because relatively high levels of *CD45* transcript were detected in those tissues (Figure 5B bottom panel).

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.

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

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

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

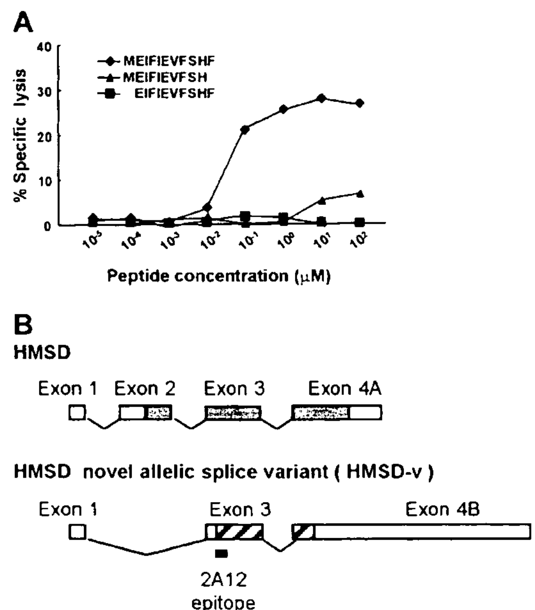


Figure 4. Identification of the CTL-2A12 minimal mHA epitope. (A) A peptide reconstitution assay was conducted to determine the concentration of peptides needed to stimulate CTL-2A12. Undecameric peptide (MEIFIEVFSHF), its C-terminal deleted decameric peptide (MEIFIEVFSH), and N-terminal deleted decameric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

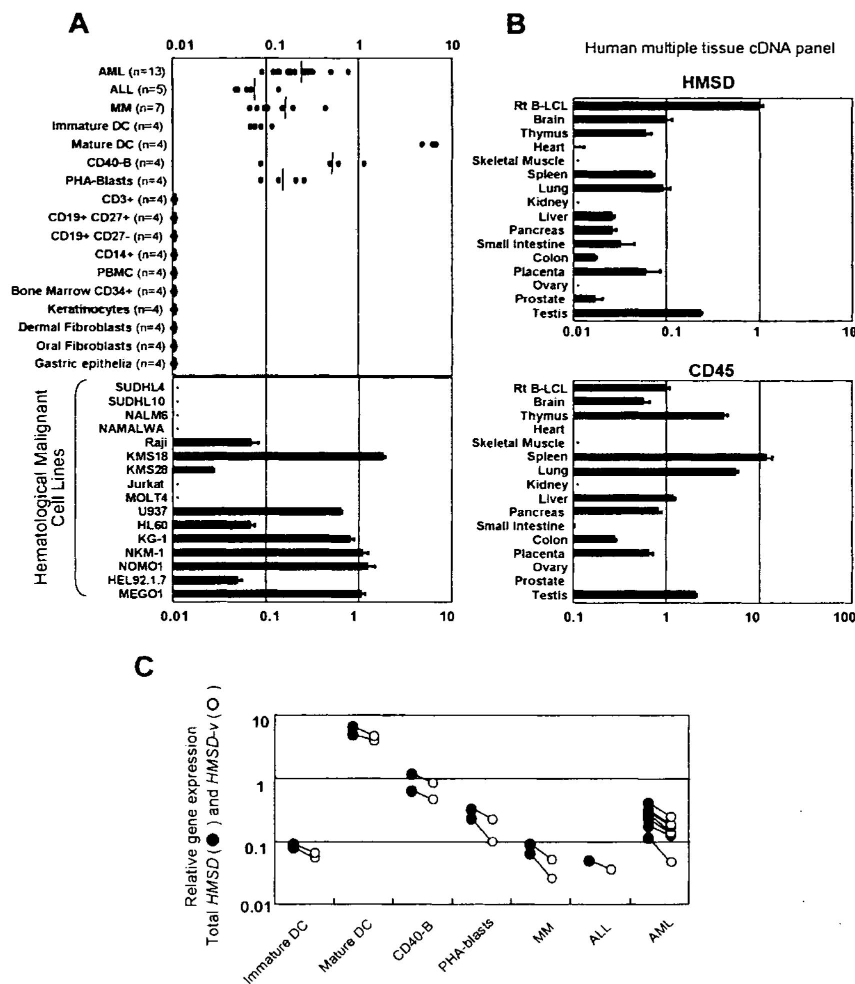


Figure 5. Selective mRNA expression of *HMSD* and *HMSD-v*. (A) Total *HMSD* expression was determined by real-time quantitative PCR in various normal tissues and malignant hematopoietic cell lines using a primer-probe set that detects the exon 3-4 boundary. Targeted mRNA expression in the recipient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

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

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

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

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

Discussion

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

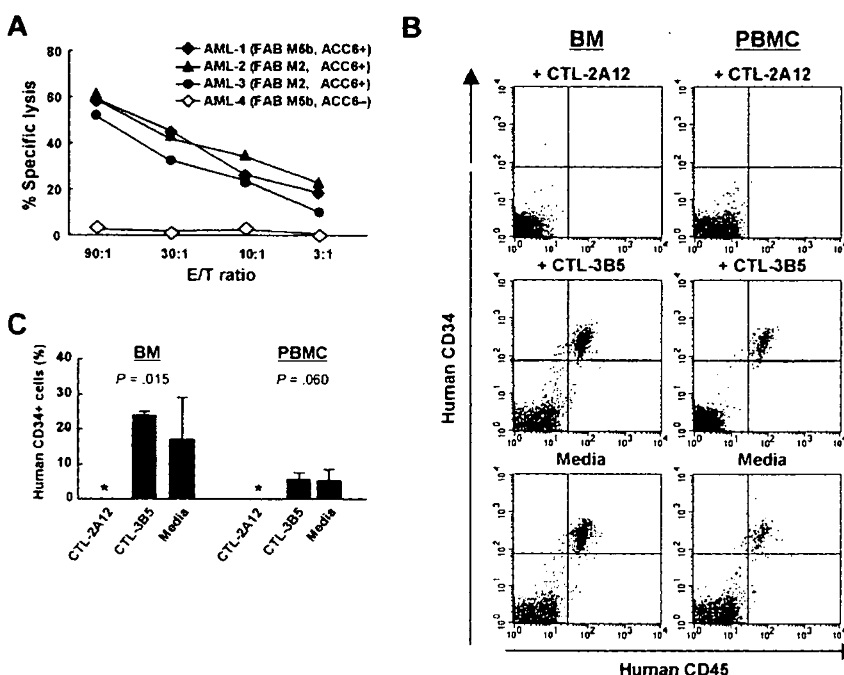


Figure 6. Inhibition of human AML stem cell engraftment in severely immunodeficient NOG mice by CTL-2A12. (A) Specific lysis by CTL-2A12 of primary leukemia cells. A standard 4-hour ^{51}Cr release assay was conducted at the indicated E/T ratios. The CD34$^+$ fraction of 3 primary AML cells positive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

and recipient due to nonsynonymous SNPs. In this study, we identified a novel HLA-B44–restricted mHA epitope (ACC-6) encoded by an allelic splice variant of *HMSD* (*HMSD-v*) in which exclusion of exon 2 due to alternative splicing was completely controlled by an intronic SNP at IVS2+5. Indeed, by RT-PCR, the novel *HMSD-v* was not detected in cDNA samples from mHA$^-$ B-LCLs, whereas it was detectable in mHA$^+$ B-LCLs. An interesting question is why the splicing of exon 2 was completely controlled by the intronic SNP. In general, during intron splicing reactions, U1snRNA first binds the 5′ splice site of an intron, spliceosome assembly starts, lariat formation is made with several other factors, and thereafter the intron is spliced out (reviewed in Valadkhan[28]). Here U1snRNA is an important initiator of the cascade. It has been shown that aberrant splicing can result from mutations that either destroy or create splice-site consensus sequences at the 5′ splice site such that approximately half of the observed aberrant splicing is exon skipping while intron retention is rarely observed.[29] In this case, we speculate that the G-to-A substitution of the intronic SNP at nucleotide 5 in intron 2

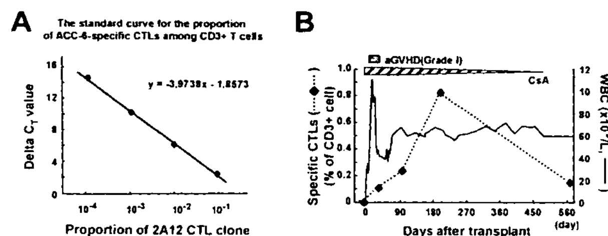


Figure 7. Detection of ACC-6–specific CTLs in peripheral blood from the AML patient (UPN-027) by real-time quantitative PCR using a set of primers and fluorogenic probe specific for the CTL-2A12 CDR3 sequence. (A) The standard curve for the proportion of ACC-6–specific CTL-2A12 serially diluted into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

(IVS2+5G>A, 5′-GUACAU-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.

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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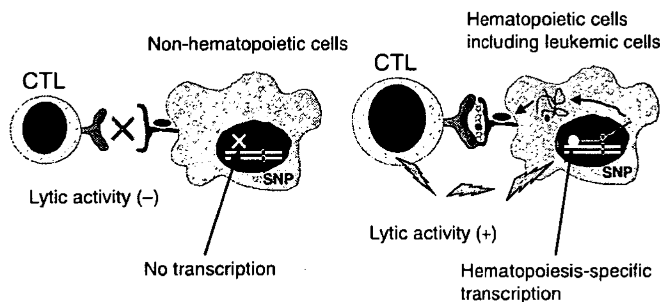

Fig. 1. Selective induction of graft-versus-leukemia/lymphoma (GVL) effects. Donor T cells sensitized to minor histocompatibility antigen complex (mHag) epitopes encoded by polymorphic genes, the expression of which is limited to hematopoietic cells, including hematological malignant cells, are responsible 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	RESEEESVSL	*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

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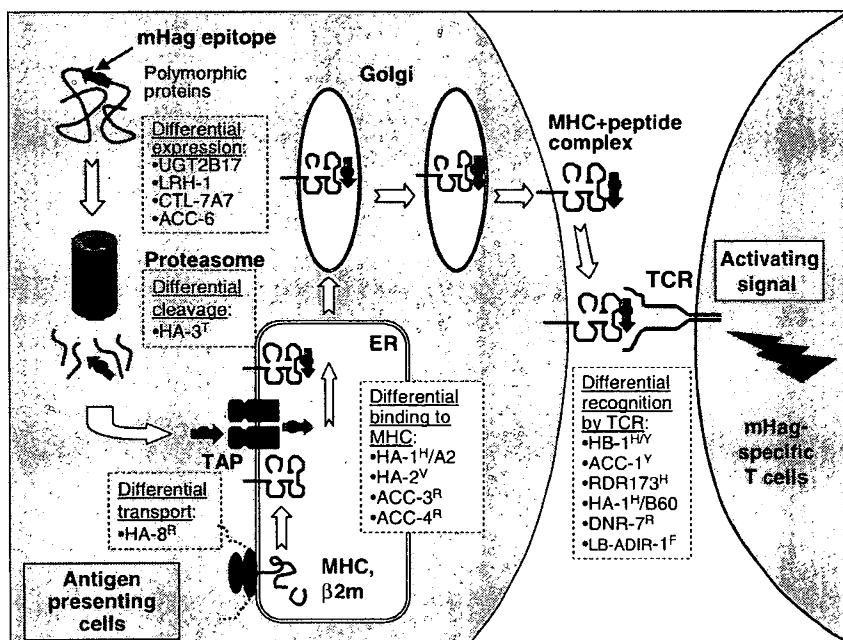

Fig. 2. Mechanisms involved in the generation of antigenicity of minor histocompatibility antigen complex (mHag) epitopes. In antigen-presenting cells, there is different machinery involved in the presentation of cellular proteins onto major histocompatibility complex (MHC) molecules on cell surfaces. 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

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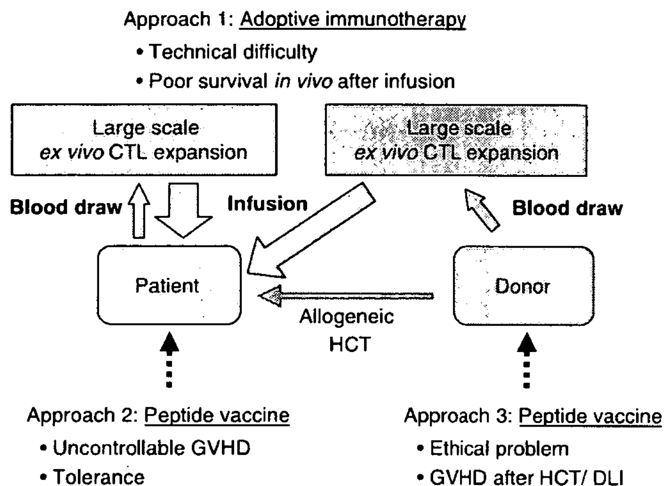

Fig. 3. Modalities of minor histocompatibility antigen complex (mHag)-based immunotherapy. Initially, adoptive immunotherapy with cytotoxic T cells (CTL) specific for mHag was developed largely due to the cell therapeutic nature of allogeneic cell transplantation (Approach 1). CTL are induced from either *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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Identification of human minor histocompatibility antigens based on genetic association with highly parallel genotyping of pooled DNA

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Minor histocompatibility (H) antigens are the molecular targets of allo-immunity responsible both for the development of antitumor effects and for graft-versus-host disease (GVHD) in allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, despite their potential clinical use, our knowledge of human minor H antigens is largely limited by the lack of efficient methods of their characterization. Here we report a robust and efficient method of minor H gene discovery that combines whole genome association scans (WGASs) with cytotoxic T-lymphocyte (CTL) assays, in which the genetic loci of minor H genes recognized by the CTL clones are precisely identified using pooled-DNA analysis of immortalized lymphoblastoid cell lines with/without susceptibility to those CTLs. Using this method, we have successfully mapped 2 loci: one previously characterized (*HMSD* encoding ACC-6), and one novel. The novel minor H antigen encoded by *BCL2A1* was identified within a 26 kb linkage disequilibrium block on chromosome 15q25, which had been directly mapped by WGAS. The pool size required to identify these regions was no more than 100 individuals. Thus, once CTL clones are generated, this method should substantially facilitate discovery of minor H antigens applicable to targeted allo-immune therapies and also contribute to our understanding of human allo-immunity. (Blood. 2008;111:3286-3294)

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Introduction

Currently, allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been established as one of the most effective therapeutic options for hematopoietic malignancies[1] and is also implicated as a promising approach for some solid cancers.[2] Its major therapeutic benefits are obtained from allo-immunity directed against patients' tumor cells (graft-versus-tumor [GVT] effects). However, the same kind of allo-immune reactions can also be directed against normal host tissues resulting in graft-versus-host disease (GVHD). In HLA-matched transplants, both GVT and GVHD are initiated by the recognition of HLA-bound polymorphic peptides, or minor histocompatibility (H) antigens, by donor T cells. Minor H antigens are typically encoded by dichotomous single nucleotide polymorphism (SNP) alleles, and may potentially be targeted by allo-immune reactions if the donor and recipient are mismatched at the minor H loci. Identification and characterization of minor H antigens that are specifically expressed in hematopoietic tissues, but not in other normal tissues, could contribute to the development of selective antileukemic therapies while minimizing unfavorable GVHD reactions, one of the most serious complications of allo-HSCT.[3,4] Unfortunately, the total number of such useful minor H antigens that are currently molecularly characterized is still disappointingly small, including HA-1,[5] HA-2,[6] ACC-1Y and ACC-2,[7] DRN-7,[8] ACC-6,[9] LB-ADIR-1F,[10] HB-1,[11] LRH-1,[12] and 7A7-PANE1,[13] limiting the number of patients eligible for such GVT-oriented immunotherapy.

Several techniques have been developed to identify novel minor H antigens targeted by CTLs generated from patients who have undergone transplantation. Among these, linkage analysis based on the cytotoxicity of the CTL clones against panels of lymphoblastoid cell lines (B-LCLs) from large pedigrees was proposed as a novel genetic approach,[14] and has been successfully applied to identify novel minor H epitopes encoded by the *BCL2A1* and *P2RX5* genes.[7,12] Nevertheless, the technology is still largely limited by its resolution, especially when large segregating families are not available. Linkage analysis using B-LCL panels from the Centre d'Etude du Polymorphisme Humain (CEPH) could only localize minor H loci within a range of 1.64 Mb to 5.5 Mb, which still contained 11 to 46 genes,[7,12,14] thus requiring additional selection procedures to identify the actual minor H genes.

On the other hand, clinically relevant minor H antigens might be associated with common polymorphisms within the human

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population, and therefore could be ideal targets of genetic association studies, considering recent advances of large-scale genotyping technologies and the assets of the International HapMap Project.[15,16] In this alternative genetic approach using the extensive linkage disequilibrium (LD) found within the human genome, target loci can be more efficiently localized within relatively small haplotype blocks without depending on limited numbers of recombination events, given the large number of genotyped genetic markers.[17] Moreover, since the presence of a target minor H allele in individual target cells can be determined by ordinary immunologic assays using minor H antigen-specific CTLs, the characterization of minor H antigens should be significantly more straightforward than identifying alleles associated with typical common complex diseases, for which typically weak-to-moderate genetic effects have been assumed.[18]

In this report, we describe a high-performance, cost-effective method for the identification of minor H antigens, in which whole genome association scans (WGASs) are performed based on SNP array analysis of pooled DNA samples constructed from cytotoxicity-positive (CTX^+) and cytotoxicity-negative (CTX^-) B-LCLs as determined by their susceptibility to CTL clones. Based on this method, termed WGA/CTL, we were able to map the previously characterized ACC-6 minor H locus to a 115-kb block containing only 4 genes, including *HMSD*.[9] Moreover, using the same approach, a novel minor H antigen encoded by the *BCL2A1* gene was identified within a 26-kb block containing only *BCL2A1* on chromosome 15q25. Surprisingly, the pool size required to identify these regions was no more than 100 individuals. Thus, this WGA/CTL method has significant potential to accelerate the discovery of minor H antigens that could be used in more selective, and thus more effective, allo-immune therapies in the near future.

Methods

Cell isolation and cell cultures

This study was approved by the institutional review board of the Aichi Cancer Center and the University of Tokyo. All blood or tissue samples were collected after written informed consent was obtained in accordance with the Declaration of Helsinki. B-LCLs were derived from allo-HSCT donors, recipients, and healthy volunteers. B-LCLs were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate.

Generation of CTL lines and clones

CTL lines were generated from peripheral blood mononuclear cells (PBMCs) obtained after transplantation by stimulation with irradiated (33 Gy) recipient PBMCs harvested before HSCT, thereafter stimulated weekly in RPMI 1640 supplemented with 10% pooled human serum and 2 mM L-glutamine. IL-2 was added on days 1 and 5 after the second and third stimulations. CTL clones were isolated by standard limiting dilution and expanded as previously described.[7] CTL-1B9 was isolated from PBMCs harvested on day 30 after transplantation from a patient receiving a marrow graft from his HLA-identical sibling (HLA A11, A24, B39, B51, Cw7, Cw14), and CTL-2A12 has been described recently.[9]

Chromium release assay

Target cells were labeled with 0.1 mCi (3.7 MBq) of ^{51}Cr for 2 hours, and 10^3 target cells/well were mixed with CTL at the effector-to-target (E/T) ratio indicated in a standard 4-hour cytotoxicity. All assays were performed at least in duplicate. Percent specific lysis was calculated as follows: ((Experimental cpm − Spontaneous cpm) / (Maximum cpm − Spontaneous cpm)) × 100.

Immunophenotyping by enzyme-linked immunosorbent assay

B-LCL cells (20 000 per well, which had been retrovirally transduced with restriction HLA cDNA for individual CTLs, if necessary) were plated in each well of 96-well round-bottomed plates, and corresponding CTL clones (10 000 per well) were added to each well. After overnight incubation at 37°C, 50 μL supernatant was collected and released IFN-γ was measured by standard enzyme-linked immunosorbent assay (ELISA).

Construction of pooled DNA and microarray experiments

Genomic DNA was individually extracted from immunophenotyped B-LCLs. After DNA concentrations were measured and adjusted to 50 μg/mL using the PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, OR), the DNA specimens from CTX^+ and CTX^- B-LCLs were separately combined to generate individual pools. DNA pools were analyzed in pairs using Affymetrix GeneChip SNP-genotyping microarrays (Affymetrix, Tokyo, Japan) according to the manufacturer's protocol,[19,20] where 2 independent experiments were performed for each array type (for more detailed statistical analysis for generated microarray data, see Document S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Estimation of LD blocks

LD structures of the candidate loci were evaluated based on empirical data from the International Hap Map Project (http://www.hapmap.org/).[15] LD data for the relevant HapMap panels were downloaded from the HapMap web site and further analyzed using Haploview software (http://www.broad.mit.edu/mpg/haploview/).[21]

Transfection of 293T cells and ELISA

Twenty thousand 293T cells retrovirally transduced with HLA-A*2402 were plated in each well of 96-well flat-bottomed plates, cultured overnight at 37°C, then transfected with 0.12 μg of plasmid containing full-length *BCL2A1* cDNA generated from either the patient or his donor using Trans IT-293 (Mirus, Madison, WI). B-LCLs of the recipient and his donor were used as positive and negative controls, respectively. Ten thousand CTL-1B9 cells were added to each well 20 hours after transfection. After overnight incubation at 37°C, 50 μL of supernatant was collected and IFN-γ was measured by ELISA.

SNP identification by direct sequencing

Complementary DNA prepared from B-LCLs was polymerase chain reaction (PCR) amplified for the coding region of *BCL2A1* using the following primers: sense: 5'-AGAAGATGACAGACTGTGAATTTGG-3'; antisense: 5'-TCAACAGTATTGCTTCAGGAGAG-3'.

PCR products were purified and directly sequenced with the same primer and BigDye Terminator kit (version 3.1) by using ABI PRISM 3100 (Applied Biosystems, Foster City, CA).

Confirmatory SNP genotyping

Genotyping was carried out using fluorogenic 3'-minor groove binding (MGB) probes in a PCR assay. PCR was conducted in 10-μL reactions containing both allelic probes, 500 nM each of the primers, 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), and 1 μL (100 ng) DNA. PCR cycling conditions were as follows: predenature, 50°C for 2 minutes, 95°C for 10 minutes, followed by 35 cycles of 92°C for 15 seconds and 60°C for 1 minute in a GeneAmp PCR System 9700 (Applied Biosystems). The PCR products were analyzed on an ABI 7900HT with the aid of SDS 2.2 software (Applied Biosystems).

Epitope reconstitution assay

The candidate *BCL2A1*-encoded minor H epitope and its allelic counterpart (DYLQYVLQI) peptides were synthesized by standard Fmoc chemistry. ^{51}Cr-labeled CTX^- donor B-LCLs were incubated with graded concentrations of the peptides and then used as targets in standard cytotoxicity assays.

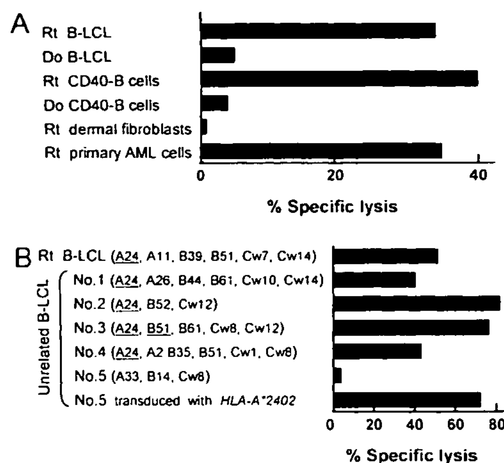

Figure 1. Specificity of CTL-1B9 against hematopoietic cells and its restriction HLA. (A) The cytolytic activity of CTL-1B9 was evaluated in a standard 4-hour ^{51}Cr release assay (E/T ratio, 20:1). Targets used were B-LCL, CD40-activated (CD40-B) B cells, dermal fibroblasts, and primary acute myeloid leukemia cells from the recipient (Rt), and B-LCL and CD40-B cells from his donor (Do). Rt dermal fibroblasts were pretreated with 500 U/mL IFN-γ and 10 ng/mL TNF-α for 48 hours before ^{51}Cr labeling. (B) Cytolytic activity of CTL-1B9 against a panel of B-LCLs derived from unrelated individuals, each of whom shared 1 or 2 class I MHC allele(s) with the recipient from whom the CTL-1B9 was generated. The shared HLA allele(s) with the recipient are underlined. B-LCLs (no. 5) which did not share any HLA alleles with the recipient, were retrovirally transduced with HLA-A*2402 cDNA and included to confirm HLA-A*2402 restriction by CTL-1B9. Results are typical of 2 experiments and data are the mean plus or minus the standard deviation (SD) of triplicates.

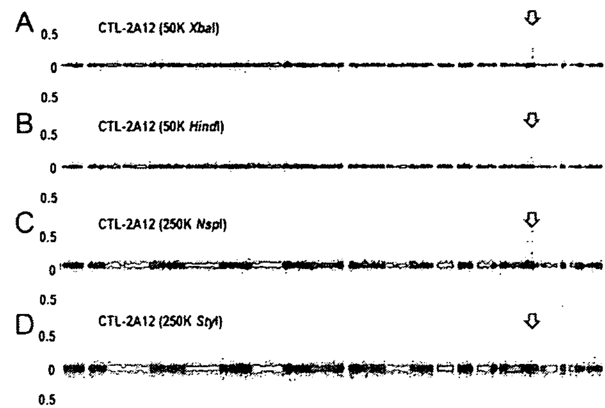

Figure 2. Whole genome association scans performed with pooled DNA generated based on immunophenotyping with CTL-2A12. Pooled DNAs generated from 44 CTX$^+$ and 44 CTX$^-$ B-LCLs were analyzed with 50 K *XbaI* (A), 50 K *HindIII* (B), 250 K *NspI* (C), and 250 K *StyI* (D) arrays. Test statistics were calculated for all SNPs and plotted in the chromosomal order. In all SNP array types, a common association peak is observed at 18q21, to which the minor H antigen for CTL-2A12, encoded by the *HMSD* gene, had been mapped based on expression cloning[9] (arrows).

Results

CTL-based typing and SNP array analysis of pooled DNA

CTL-2A12 and CTL-1B9 are CTL clones established from the peripheral blood of 2 patients with leukemia who had received HLA-identical sibling HSCTs. Each clone demonstrated specific lysis against the B-LCLs of the recipient but not against donor B-LCLs, indicating recognition of minor H antigen (Figure 1A and Kawase et al[9]). The minor H antigen for CTL-2A12 had been previously identified by expression cloning[9]; on the other hand, the target minor H antigen for the HLA-A24–restricted CTL-1B9 clone, which was apparently hematopoietic lineage–specific (Figure 1A) and present in approximately 80% of the Japanese population (data not shown), had not yet been determined. Using these CTL clones, a panel of B-LCLs expressing the restriction HLA (HLA-B44 for CTL-2A12 and HLA-A24 for CTL-1B9) endogenously or retrovirally transduced, were subjected to "immunophenotyping" for the presence or absence of the minor H antigen by ELISA and, if necessary, by standard chromium release assay (CRA). Based on the assay results, for CTL-2A12 we initially collected 44 cytotoxicity-positive (CTX$^+$) and 44 cytotoxicity-negative (CTX$^-$) B-LCLs after screening 132 B-LCLs, while 57 CTX$^+$ and 38 CTX$^-$ B-LCLs were obtained from 121 B-LCLs for CTL-1B9. From these sets of B-LCL panels, pools of DNA were generated and subjected to analysis on Affymetrix GeneChip 100 K and 500 K microarrays in duplicate.[19,20]

Detection of association between minor H phenotypes and marker SNPs

Genetic mapping of the minor H locus was performed by identifying marker SNPs that showed statistically significant deviations in allele-frequencies between CTX$^+$ and CTX$^-$ pools based on the observed allele-specific signals in the microarray experiments. For this purpose, we evaluated the deviations of observed allele ratios between CTX$^+$ and CTX$^-$ pools for each SNP on a given array (Document S1). An SNP was considered as positive for association if its test statistic exceeded an empirically determined threshold that provided a "genome-wide" P value of .05 in duplicate experiments (Document S1, Figures S1,S2, and Table S1). Threshold values for different pool sizes are also provided in Table S2 for further experiments. The positive SNPs eventually obtained for both CTLs are summarized in Table 1, where the 10 SNPs showing the highest test statistics are listed for individual experiments.

Mapping of the minor H loci by WGASs

All the SNPs significantly associated with susceptibility to CTL-2A12 were correctly mapped within a single 115 kb LD block at chromosome 18q21 containing the *HMSD* gene (Figures 2 and 3A), which had been previously shown to encode the ACC-6 minor H antigen recognized by CTL-2A12.[9] According to the above criteria, no false-positive SNPs were reported in any array types (Table 1). Confirmation genotyping of individual B-LCLs from both panels revealed none of the 44 that had been immunophenotyped as CTX$^-$ were misjudged, while 8 of the 44 CTX$^+$ B-LCLs were found to actually carry no minor H-positive allele for ACC-6, which was likely due to the inclusion of individual B-LCLs showing borderline cytotoxicity (data not shown).

On the other hand, positive association of the target minor H antigen with CTL-1B9 was detected in 2 independent loci: SNP rs1879894 at 15q25.1 in 250 K *NspI* (Table 1, Figure 4A-B, and Figure S5) and SNP rs1842353 at 8q12.3 in 50 K *HindIII* (Table 1 and Figure S3A). We eventually focused on rs1879894, as it showed a much more significant genome-wide P value than SNP rs1842353 (Table 1). In contrast to the CTL-2A12 case, where many mutually correlated SNPs around the most significant one created a broad peak in the statistic plots (Figure 2 arrows and Figure S3), the adjacent SNPs (rs6495463 and rs2562756; Figure 3B solid arrows) around rs187894 (Figure 3B open arrow) did not show large test statistic values, reflecting the fact that no marker SNPs on 100 K and 500 K arrays exist in high LD (Figure 3B dashed red lines encompassing 26 kb) with this SNP according to the HapMap data. To further confirm the association, we generated additional B-LCL pools consisting of 75 CTX$^+$ and 34 CTX$^-$

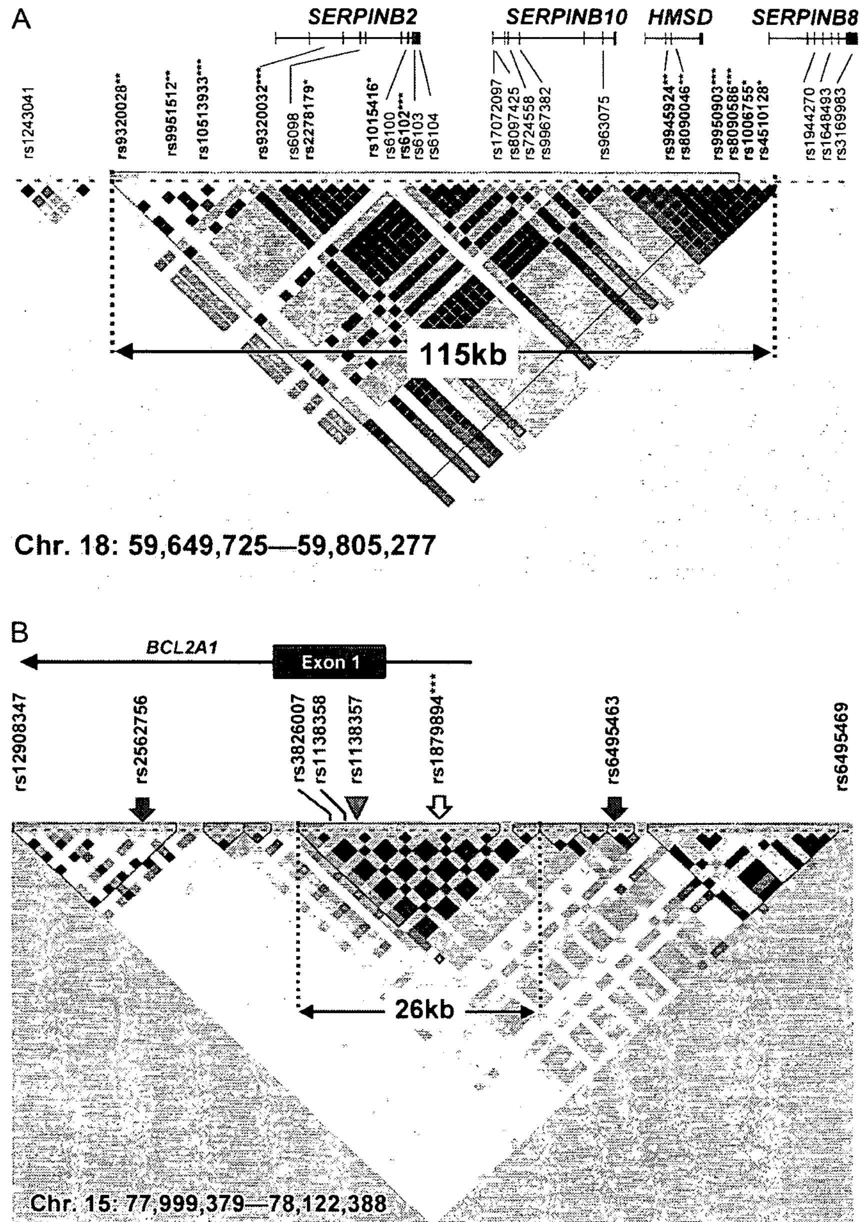

Figure 3. Linkage disequilibrium (LD) block mapped by CTL-2A12 and CTL-1B9. (A) An LD block map identified by pairwise r^2 plot from HapMap CEU data are overlayed with SNPs available from Affymetrix GeneChip SNP-genotyping microarrays (arrows) and 4 genes in the 115 kb block. SNPs that emerged repeatedly in the 2 independent experiments are indicated in blue. The genomewide *P* values for positive SNPs are shown as follows: *$P < .05$; **$P < .01$; ***$P < .001$. The intronic SNP (rs9945924) controlling the alternative splicing of *HMSD* transcripts and expression of encoded ACC-6 minor H antigen is indicated in red. (B) LD blocks identified by pairwise r^2 plot from HapMap JPT data are overlayed with SNPs available from Affymetrix GeneChip SNP-genotyping microarrays (arrows) and exon 1 of the *BCL2A1* gene. The only SNP showing a high association with CTL-1B9 immunophenotypes (rs1879894) is shown as an open arrow. The nonsynonymous SNP (rs1138357) controlling the expression of the minor H antigen recognized by CTL-1B9 is indicated by a red arrowhead. ***SNP with genomewide $P < .001$. The 2 SNPs adjacent to the 26 kb LD block (rs2562756 and rs6495463) never gave a significant genomewide *P* value.

B-LCLs from another set of 128 B-LCLs, and performed a WGAS. As expected, the WGAS of the second pools also identified the identical SNP with the highest test statistic value in duplicate experiments, unequivocally indicating that this SNP is truly associated with the minor H locus of interest (Figure 4C,D and Table S3). The association was also detected when the references in the first and second pools were swapped (data not shown).

Identification of the minor H epitope recognized by CTL-1B9

The LD block containing SNP rs1879894 that was singled out from more than 500 000 SNP markers with 2 sets of DNA pools only encodes exon 1 of *BCL2A1* (Figure 3B). To our surprise, this was the region to which we had previously mapped an HLA-A24–restricted minor H antigen, ACC-1^{Y}.[7] We first confirmed that full-length *BCL2A1* cDNA cloned only from the recipient but not his donor could stimulate interferon-γ secretion from CTL-1B9 when transduced into donor B-LCL (Figure 5A), indicating that *BCL2A1* is a bona fide gene encoding minor H antigen recognized by CTL-1B9. We next genotyped 3 nonsynonymous SNPs in the *BCL2A1* exon 1 sequence (Figure 3B) and comparison was made between the genotypes and the susceptibility to CTL-1B9 of 9 HLA-A*2402$^+$ B-LCLs, including ones generated from the recipient (from whom CTL-1B9 was established) and his donor. Susceptibility to CTL-1B9 correlated completely with the presence of guanine at SNP rs1138357 (nucleotide position 238, according to the mRNA sequence for NM_004049.2) and thymine at SNP rs1138358 (nucleotide position 299) (Table 2), suggesting that the expression of the minor H epitope recognized by CTL-1B9 is controlled by either of these SNPs. We searched for nonameric amino acid sequences spanning the 2 SNPs using BIMAS software,[22] since most reported HLA-A*2402 binding peptides contain 9 amino acid residues.[23] Among these, a nonameric peptide, DYLQCVLQI (the polymorphic residue being underlined), has a predicted binding score of 75 and was considered as a candidate minor H epitope. As shown in Figure 5B, the DYLQCVLQI was strongly recognized by CTL-1B9, whereas its allelic counterpart,

Table 1. Positive SNPs from pooled DNA analysis

CTL-2A12, Exp 1				CTL-2A12, Exp 2				CTL-1B9, Exp 1				CTL-1B9, Exp 2			
rsID	Chr	Position	$\Delta R_{A\Delta R_B}$	rsID	Chr	Position	$\Delta R_{A\Delta R_B}$	rsID	Chr	Position	$\Delta R_{A\Delta R_B}$	rsID	Chr	Position	$\Delta R_{A\Delta R_B}$
50K *X bal*															
rs10513933	18	59699669	0.366*	rs10513933	18	59699669	0.511†	rs1363258	5	103297593	0.239	rs10499174	6	131209689	0.352*
rs9320028	18	59668150	0.255‡	rs9320028	18	59668150	0.360*	rs726083	3	67093729	0.203	rs30058	5	122325602	0.240
rs6102	18	59721450	0.221	rs10485873	7	3503743	0.157	rs639243	5	31392931	0.198	rs150724	16	61960443	0.213
rs724533	23	116440574	0.137	rs219323	14	59510440	0.150	rs1936461	10	56519024	0.186	rs1993129	8	63618836	0.208
rs1341112	6	104919391	0.136	rs10506892	12	82478539	0.147	rs763876	12	94922502	0.186	rs356946	13	69066751	0.201
rs470490	18	61182216	0.136	rs10492269	12	97786333	0.144	rs958404	7	133054441	0.179	rs2869268	4	86421898	0.184
rs2826718	21	21471423	0.134	rs10483466	14	35986827	0.139	rs10486727	7	41672315	0.178	rs287002	12	40312537	0.183
rs10506697	12	73241741	0.128	rs5910124	23	116408616	0.137	rs2833488	21	32010112	0.176	rs1146808	13	67688608	0.182
rs10506891	12	82393029	0.127	rs10512545	17	66337079	0.134	rs379212	5	60977687	0.172	rs10501287	11	42446011	0.180
rs308995	14	59657919	0.125	rs295678	5	58186928	0.131	rs1954004	14	58627872	0.170	rs564993	5	31393476	0.177
50K *HindIII*															
rs9320032	18	59712191	0.486†	rs9320032	18	59712191	0.506†	rs1842353	8	63617543	0.244*	rs9300692	13	101216476	0.225‡
rs8090046	18	59773066	0.207‡	rs8090046	18	59773066	0.245*	rs10521202	17	12755289	0.201‡	rs1842353	8	63617543	0.210‡
rs1474220	2	108525317	0.193‡	rs10498752	6	41876488	0.210‡	rs7899961	10	59696431	0.198‡	rs10520983	5	31314700	0.195‡
rs10498752	6	41876488	0.178	rs1941538	18	37994337	0.176	rs9320974	6	124421441	0.197‡	rs1334375	13	80897038	0.173
rs2298578	21	21632551	0.167	rs7682770	4	152748018	0.174	rs10520983	5	31314700	0.179	rs10519164	15	75412758	0.163
rs7516032	1	91618962	0.165	rs1445862	5	3675257	0.169	rs1862446	5	147460749	0.170	rs9322063	6	146852196	0.152
rs5030938	10	70645922	0.164	rs4696976	4	21058616	0.167	rs1358778	20	13266796	0.169	rs8067384	17	37926265	0.150
rs1883041	21	44921845	0.158	rs5030938	10	70645922	0.165	rs1873790	4	83422480	0.166	rs10521202	17	12755289	0.147
rs3902916	4	189045176	0.155	rs3902916	4	189045176	0.165	rs1220724	4	70888705	0.162	rs7914904	10	62749969	0.141
rs1000551	20	58709208	0.154	rs1883041	21	44921845	0.164	rs9300692	13	101216476	0.157	rs1220724	4	70888705	0.141
250K *NspI*															
rs9950903	18	59781783	0.534†	rs9950903	18	59781783	1.036†	rs1879894	15	78055874	0.846†	rs1879894	15	78055874	1.072†
rs1463835	3	23539615	0.532†	rs8090586	18	59781864	0.518†	rs9646294	16	6110019	0.484†	rs6771859	3	190642054	0.387†
rs16975459	18	37802275	0.383*	rs6473170	8	80664840	0.338*	rs17734332	5	134945240	0.365†	rs10512261	9	98804394	0.299*
rs8090586	18	59781864	0.367*	rs4510128	18	59782312	0.310‡	rs566619	7	41381538	0.345*	rs12122772	1	60384564	0.287*
rs16872621	4	22081055	0.312‡	rs1006755	18	59782026	0.300‡	rs17737566	6	50345280	0.310*	rs2153155	4	26034162	0.248‡
rs870582	6	125097114	0.301‡	rs7039378	9	118735938	0.258	rs3849955	9	28350374	0.285*	rs17126896	14	53320494	0.246‡
rs1015416	18	59720363	0.270‡	rs1860563	16	6418899	0.258	rs4616156	13	86581518	0.273*	rs1328652	13	35607527	0.240
rs2155907	11	97599883	0.227	rs4699126	4	105709109	0.212	rs2484698	1	217474460	0.263*	rs7021551	9	27446645	0.237
rs2112948	5	50994294	0.222	rs10275055	7	156212079	0.204	rs17139603	11	79638632	0.262*	rs252817	5	106752487	0.237
rs2919747	2	129681506	0.217	rs1526411	7	124658309	0.201	rs2156737	4	100642529	0.246‡	rs10772587	12	12681356	0.235
250K *StyI*															
rs6102	18	59721450	0.597†	rs6102	18	59721450	0.495†	rs9383925	6	151975774	0.819†	rs201204	6	104842863	0.688†
rs9951512	18	59690885	0.374*	rs9945924	18	59771746	0.407*	rs6497397	16	19646258	0.311‡	rs12556155	23	108836419	0.442†
rs6496897	15	90493249	0.320‡	rs9951512	18	59690885	0.317‡	rs917252	7	22219990	0.289‡	rs4791422	17	10605304	0.435†
rs9945924	18	59771746	0.315‡	rs1983205	3	157782892	0.314‡	rs1019403	3	7823997	0.260‡	rs7749012	6	106459559	0.336*
rs12707805	8	107404746	0.303‡	rs950865	5	2720684	0.307‡	rs17053134	5	155373544	0.259‡	rs509951	5	31385483	0.308‡
rs10971778	9	33893184	0.296‡	rs2278179	18	59715512	0.292‡	rs11710880	3	72214965	0.246	rs16879024	8	32225711	0.256‡
rs6565076	16	81487818	0.294‡	rs10427722	22	36417752	0.289‡	rs17167866	7	13919264	0.237	rs2100054	15	75293482	0.252
rs2278179	18	59715512	0.291‡	rs17156659	7	82046820	0.271	rs10867062	9	137935241	0.237	rs11811023	1	143805934	0.240
rs7806238	7	29906442	0.290‡	rs4502324	18	4811261	0.262	rs5925800	23	23278707	0.235	rs17382798	15	75256074	0.231
rs965888	18	38062658	0.283‡	rs1348428	2	225927288	0.260	rs2255831	4	146614313	0.234	rs2030302	17	12526591	0.231

Significant SNPs that appeared on both experiments are underlined.

*Genomewide $P < .01$.

†Genomewide $P < .001$.

‡Genomewide $P < .05$.

DYLQYVLQI, was not. Decameric peptide, QDYLQCVLQI, on the other hand, appeared to be weakly recognized; however, it is likely that the nonameric form was actually being presented after N-terminal glutamine cleavage by aminopeptidase in the culture medium. Because it was possible that the cystine might be cysteinylated, recognition of synthetic peptides DYLQCVLQI and cysteinylated DYLQC*VLQI were assayed using CTL-1B9. Half-maximal lysis for the former was obtained at a concentration of 200 pM, whereas recognition of the latter was several-fold weaker (Figure 5C). Thus, we concluded that DYLQCVLQI defines the cognate HLA-A*2402–restricted CTL-1B9 epitope, now designated ACC-1^C. This incidentally provides a second example of products from both dichotomous SNP alleles being recognized as HLA-A*2402–restricted minor H antigens, the first example being the HB-1 minor H antigen.[24] Finally, real-time quantitative PCR revealed that T cells carrying the complementarity-determining region 3 sequence identical to CTL-1B9 became detectable in the patient's blood at the frequencies of 0.22%, 0.91%, 1.07% and 0.01% among TCR$\alpha\beta^+$ T cells at days 30, 102, 196, and 395 after transplantation, respectively, suggesting that ACC-1^C minor H antigen is indeed immunogenic (Figure 5D).

Discussion

Recent reports have unequivocally demonstrated that WGASs can be successfully used to identify common variants involved in a wide variety of human diseases.[25-27] Our report represents a novel