Identification of human minor histocompatibility antigens based on genetic association with highly parallel genotyping of pooled DNA

Running head: Efficient minor antigen mapping by SNP arrays

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Abstract

Minor histocompatibility (H) antigens are the molecular targets of allo-immunity responsible both for the development of anti-tumor 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 (WGAS) 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 two loci: one previously characterized (*HMSD* encoding ACC-6), and one novel. The novel minor H antigen encoded by the *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.
Introduction

Currently, allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been established as one of the most effective therapeutic options for hematopoietic malignancies and also implicated as a promising approach for some solid cancers. Its major therapeutic benefits are obtained from allo-immunity directed against patients’ tumor cells (graft-versus-tumor effects, GVT). However, the same kind of allo-immune reactions can also be directed against normal host tissues resulting in graft-versus-host disease, or 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 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 anti-leukemic therapies, while minimizing unfavorable GVHD reactions, one of the most serious complications of allo-HSCT. Unfortunately, the total number of such useful minor H antigens that are currently molecularly characterized is still disappointingly small – including HA-1, HA-2, ACC-1Y and ACC-2, DRN-7, ACC-6, LB-ADIR-1F, HB-1.
LRH-1,\textsuperscript{12} and 7A7-PANE1\textsuperscript{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 post transplant patients. 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,\textsuperscript{14} and has been successfully applied to identify novel minor H epitopes encoded by the \textit{BCL2A1} and \textit{P2RX5} genes.\textsuperscript{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 Polymorphism Humain (CEPH) could only localize minor H loci within a range of 1.64 to 5.5 Mb which still contained 11-46 genes,\textsuperscript{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 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.\textsuperscript{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. Moreover, since the presence of a target minor H allele in individual target cells can be determined by ordinary immunological assays using minor H antigen-specific CTL, 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.

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 single nucleotide polymorphism (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*. 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.

Materials and methods

Cell isolation and cell cultures

This study was approved by the Institutional Review Board of Aichi Cancer Center and University of Tokyo according to the Declaration of Helsinki. All blood or tissue samples were collected after written informed consent. 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 (PBMC) obtained posttransplant by stimulation with irradiated (33 Gy) recipient PBMC 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. CTL-1B9 was isolated from PBMC harvested on day 30 posttransplant 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 (CRA)**

Target cells were labeled with 0.1 mCi of $^{51}$Cr for 2 h, and $1 \times 10^3$ target cells/well were mixed with CTL at the E:T ratio indicated in a standard 4-h cytotoxicity. All assays were performed at least in duplicate. Percent specific lysis was calculated as follows:

$$\frac{(\text{Experimental cpm} - \text{Spontaneous cpm})}{(\text{Maximum cpm} - \text{Spontaneous cpm})} \times 100.$$ 

**Immunophenotyping by enzyme-linked immunosorbent assay (ELISA)**

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 of supernatant was collected and released IFN-γ was measured by standard ELISA.

**Construction of pooled DNA and microarray experiments**

Genomic DNA was individually extracted from immunophenotyped B-LCL. 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® Inc., Tokyo, Japan) according to the manufacturer’s protocol,19,20 where two independent experiments were performed for each array type (For more detailed statistical analysis for generated microarray data, see Supplemental Methods).

**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 h 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 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 (ver. 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: pre-denature, 50°C for 2 minutes, 95°C for
10 minutes followed by 35 cycles of 92°C for 15 s and 60°C for 1 min 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. 51Cr-labeled CTX-
donor B-LCL were incubated with graded concentrations of the peptides and then used as
targets in standard cytotoxicity assays.

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
two leukemia patients who had received HLA-identical sibling HSCT. Each clone
demonstrated specific lysis against the B-LCL of the recipient but not against donor B-LCL,
indicating recognition of minor H antigen (Figure 1A, and Reference No. 9). The minor H
antigen for CTL-2A12 had been previously identified by expression cloning⁹; on the other hand, the target minor H antigen for the HLA-A24-restricted CTL-1B9 clone, which was apparently hematopoietic lineage-specific (Figures 1A) and present in ~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 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® 100K and 500K 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 (see Supplemental Methods). A SNP was considered as positive for association if its test statistic exceeded an empirically determined threshold that provided a “genome-wide” p value of 0.05 in duplicate experiments (Supplemental Methods, and Supplemental Figures 1 and 2, and Supplemental Table 1). Threshold values for different pool sizes are also provided in Supplemental Table 2 for further experiments. The positive SNPs eventually obtained for both CTLs are summarized in Table 1, where the ten SNPs showing the highest test statistics are listed for individual experiments.

Mapping of the minor H loci by WGAS

All the SNPs significantly associated with susceptibility to CTL-2A12 were correctly mapped within a single 115kb LD block at chromosome18q21 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. 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 eight 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 two independent loci: SNP rs1879894 at 15q25.1 in 250K NspI (Table 1, Figures 4A and 4B, and Supplemental Figure 5) and SNP rs1842353 at 8q12.3 in 50K HindIII (Table 1 and Supplemental Figure 3A). 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 Supplemental Figure 3), the adjacent SNPs (rs6495463 and rs2562756, solid arrows in Figure 3B) around rs187894 (an open arrow in Figure 3B) did not show large test statistic values, reflecting the fact that no marker SNPs on 100K and 500K arrays exist in high LD (dashed red lines encompassing 26kb in Figure 3B) 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- B-LCLs from another set of 128 B-LCLs, and performed 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 and 4D)
and Supplemental Table 3). 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 two 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-1Y. 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 three 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 two SNPs using BIMAS software,\textsuperscript{22} since most
reported HLA-A*2402 binding peptides contain nine amino acid residues.\textsuperscript{23} Among these, a
nonameric peptide, DYLQC\underline{VLQI} (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 DYLQC\underline{VLQI} was strongly recognized by CTL-1B9, whereas its allelic counterpart,
DYLQ\underline{YVLQI}, was not. Decameric peptide, QDYLQ\underline{C}VLQI, 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 DYLQC\underline{VLQI} and cysteinylated DYLQ\underline{C}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
DYLQC\underline{VLQI} defines the cognate HLA-A*2402–restricted CTL-1B9 epitope, now designated
ACC-1\textsuperscript{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.\textsuperscript{24} Finally, real-time quantitative PCR revealed that T-cells
carrying the complementarity-determining region 3 sequence identical to CTL-1B9 became detectable in patient’s blood at the frequencies of 0.22%, 0.91%, 1.07% and 0.01% among TCRαβ+ T cells at days 30, 102, 196 and 395, post-transplant, respectively, suggesting that ACC-1C minor H antigen is indeed immunogenic (Figure 5D).

Discussion

Recent reports have unequivocally demonstrated that WGAS can be successfully used to identify common variants involved in a wide variety of human diseases.25-27 Our report represents a novel application of WGAS to transplantation immunology, which provides a simple but robust method to fine map the genetic loci of minor H antigens whose expression is readily determined by standard immunophenotyping with CTL clones established from post-transplant patients.

The current WGA/CTL method has several desirable features that should contribute to the acceleration of minor H locus mapping. In comparing the method to those of linkage analysis and other non-genetic approaches, including direct peptide sequencing of chemically purified minor H antigens5,6,10,13 and conventional expression cloning,8,9,11 there are differences in terms of power, sensitivity, and specificity. Direct sequencing of minor H antigen peptide guarantees that the purified peptide is surely present on the cell surface as
antigen, but it requires highly specialized equipment and personnel. Expression screening of
cDNA libraries is also widely used and has become feasible with commercially available
systems. However, it depends highly on the quality of the cDNA library and expression levels
of the target genes. In addition, it often suffers from false positive results due to the forced
expression of cDNA clones under a strong promoter. The current method of WGA/CTL
genetically determines the relevant minor H antigen locus, not relying on highly technical
protein chemistry utilizing specialized equipment, or repetitive cell cloning procedures. It is
also not affected by the expression levels of the target antigens.

As a genetic approach, the current method based on genetic association has several
advantages over conventional linkage analysis: the mapping resolution has been greatly
improved from several Mb in the conventional linkage analysis to the average haplotype block
size of less than 100kb,\(^{17,25-27}\) usually containing a handful of candidate genes, compared to the
dozens as typically found in linkage analysis. This means that the effort needed for the
subsequent epitope mapping will be substantially reduced. In fact, the 115kb region identified
for CTL-2A12 contains four genes compared to 38 genes as revealed by the previous linkage
study (data not shown), and the candidate gene was uniquely identified within the 26kb region
for CTL-1B9, for which linkage analysis had failed due to very rare segregating pedigrees
among the CEPH panels with this trait (now ACC-1C) (data not shown). In addition, before moving on to epitope mapping, it would be possible to evaluate the clinical relevance of the minor H antigens by examining the tissue distribution of their expression, based on widely available gene expression databases such as GNF (Genomic Institute of the Novartis Research Foundation, http://symatlas.gnf.org/SymAtlas/).

Second, the required sample size is generally small, and should be typically no more than 100 B-LCLs for common minor H alleles. This is in marked contrast to the association studies for common diseases, in which frequently thousands of samples are required. In the current approach, sufficiently high test statistic values could be obtained for the relevant loci with a relatively small sample size, since the minor H allele is correctly segregated between the CTX+ and CTX- pools by the highly specific immunological assay. Combined with high accuracy in allelic measurements, this feature allows for the use of pooled DNAs in WGAS, which substantially saves cost and time, compared to the genotyping of individual samples. Unexpectedly, our method allows for a considerable degree of error in the immunophenotyping, indicating the robustness of the current method; in fact, the minor H locus for CTL-2A12 was successfully identified in spite of the presence of eight (~10%) immunophenotyping errors. When the minor H allele has an
extreme allele frequency (e.g., <5% or >95%), which could be predicted by preliminary immunophenotyping. WGAS/CTL may not be an efficient method of mapping, due to the impractically large numbers of B-LCLs that would need to be screened to obtain enough CTX+ or − B-LCLs. However, such minor H antigens would likely have limited clinical impact or applicability.

Sensitivity of the microarray analysis seems to be very high when the target SNP has good proxy SNPs on the array, because we were able to correctly identify the single SNP correlated with the target of CTL-1B9 from more than 500,000 SNP markers. On the other hand, genome coverage of the microarray is definitely important. In our experiments on CTL-2A12, the association was successfully identified by the marker SNPs showing r² values of ~0.74 with the target locus of ACC-6. Since the GeneChip® 500K array set captures ~65% of all the HapMap phase II SNPs with more than 0.74 of r² (Nanya, et al. in press) and higher coverage will be obtained with the SNP 6.0 arrays having more than 1,000K SNP markers, these arrays can be satisfactorily used as platforms for the WGA/CTL method.

As shown in the current study, the intrinsic sensitivity and specificity of the WGA/CTL method in detecting associated SNPs were excellent. In other words, as long as target SNPs are captured in high r² values with one or more marker SNPs within the Affymetrix
500K SNP set, there is a high likelihood of capturing the SNP with the current approach. To evaluate the probability of a given minor H antigen being captured in high r² with marker SNPs, we checked the maximum r² values of known minor H antigen SNPs with the Affymetrix 500K SNPs, according to empirical data from the HapMap project (http://hapmap.org). Among 13 known minor H antigens, seven have their entries (designated minor H SNP) in the HapMap Phase II SNP set (HA-3, HA-8, HB-1, ACC-1 and ACC-2, LB-ADIR-1F and 7A7-PANE1), and were used for this purpose (note that absence of their entries in the HapMap data set does not necessarily mean that they could not be captured by a particular marker SNP set). As shown in Supplementary Table 4, all seven minor H SNPs are captured by at least one flanking SNPs that are included in the Affymetrix 500K SNP set with r² values of more than 0.74 in at least one HapMap panel. The situation should be more favorable in the recently available SNP6.0 array set with 1,000K SNPs, indicating the genome coverage with currently available SNP arrays would be sufficient to capture typical minor H antigens with our approach.

Most patients who have received allo-HSCT could be a source of minor H antigen-specific CTL clones to be used for this assay, since the donor T-cells are in vivo-primed and many CTL clones could be established using currently available methods. In fact,
substantial numbers of CTL clones have been established worldwide and could serve as the probes to identify novel minor H antigens. Once constructed, a panel of B-LCLs, including those transduced with HLA cDNAs, could be commonly applied to immunophenotyping with different CTL clones, especially when CTLs are obtained from the same ethnic group. In addition, by adopting other immunophenotyping readouts such as production of IL-2 from CD4+ T cells, this method could be applied to identification of MHC class II-restricted minor H antigens which have crucial roles in controlling CTL functions upstream. This may open a new field in the study of allo-HSCT since MHC class II-restricted mHags have been technically difficult to identify by conventional methods.

Finally, the discovery of ACC-1C as a novel minor H antigen indicates that all the mismatched transplants at this locus could be eligible for allo-immune therapies, since we have previously demonstrated that the counter allele also encodes a minor H antigen, ACC-1Y, which is preferentially expressed and presented on blood components including leukemic cells and may serve as a target of allo-immunity. Indeed, CTL specific for ACC-2, an HLA-B44-restricted minor H antigen restricted by the third exonic SNP on BCL2A1, was independently isolated from the peripheral blood of a patient with recurrent leukemia re-entering complete remission after donor lymphocyte infusion. The number of eligible
allo-HSCT recipients has now been effectively doubled, accounting for 50% of transplants with HLA-A24 or 20% of all transplants performed in the Asian population. In conclusion, we have described a simple but powerful method for minor H mapping to efficiently accelerate the discovery of novel minor H antigens that will be needed to contribute to our understanding of the molecular mechanism of human allo-immunity.
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Authorship

T.K. performed most of immunological experiments, preparation of pooled DNA and quantitative PCR, analyzed data and wrote the manuscript; Y.N. performed the majority of genetic analyses and analyzed the data; H.T. performed T cell receptor analysis and designed q-PCR primers and probes; G.Y. contributed to the organization of software for linkage analysis and simulation; S.M. contributed the preparation of pooled DNA; M.O., K.M. Y.K, and Y.M. collected clinical data and specimens; T.T. and K.K. contributed to data analysis and interpretation, and writing of the article; Y.A. and S.O. supervised the entire project, designed and coordinated most of the experiments in this study, and contributed to manuscript preparation.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
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### Table 1. Positive SNPs from pooled DNA analysis

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Genome-wide p < 0.05 *, p < 0.01 **, p < 0.001 ***

Significant SNPs appeared on both experiments are underlined.
Table 2. Correlation of BCL2A1 sequence polymorphisms with susceptibility to CTL-1B9

<table>
<thead>
<tr>
<th>Detected SNP</th>
<th>Position</th>
<th>Cytolysis by CTL-1B9</th>
<th>HLA-A*2402-positive B-LCLs§</th>
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§ Rt, recipient; Do, donor; UR, unrelated
¶ Nucleotide positions are shown according to NM_004092.2 mRNA sequence available at http://www.ncbi.nlm.nih.gov as GEO accession GSE10044.
Figure legends

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 h 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 two experiments and data are mean ± s.d. 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 50K XbaI (A), 50K HindIII (B), 250K NspI (C), and 250K 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 (arrows).

**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 is overlayed with SNPs available from Affymetrix® GeneChip® SNP-genotyping microarrays (arrows) and 4 genes in the 115kb block. SNPs that emerged repeatedly in the two independent experiments are indicated in blue. The genome-wide $p$ values for positive SNPs are shown as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. 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 is 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. *** indicates SNP with genome-wide $p < 0.001$. Two SNPs adjacent to the 26kb LD block (rs2562756 and rs6495463) never gave a significant genome-wide $p$ value.

**Figure 4**

**Reproducible detection of association with the immunophenotypes determined by CTL-1B9 at the BCL2A1 locus.**

The maximum test statistic value was observed at a single SNP (rs1879894) within 15q25.1 in
duplicate experiments for the first pools consisting of 57 CTX+ and 38 CTX− B-LCLs (A, B and C). The peak association at the same SNP was reproduced in the experiments with the second pools consisting of 75 CTX+ and 34 CTX− LCLs (D). Test statistic values (∆RA∆RB) are plotted by blue lines together with their RA (red) and RB (green) values. The expected ∆RA∆RB values multiplied by r² correlation coefficients for the adjacent SNPs within 500kb from the SNP rs1879894 are overlaid by red lines (A and B).

**Figure 5**  
Identification of the CTL-1B9 minimal minor H epitope

(A) Interferon-γ production from CTL-1B9 against HLA-A*2402-transduced 293T cells transfected with plasmid encoding full-length BCL2A1 cDNA cloned from either the recipient (Rt) from whom CTL-1B9 was isolated or his donor (Do). Rt B-LCL and Do B-LCL were used as positive and negative controls, respectively. Secreted interferon-γ was measured by ELISA and is expressed in arbitrary units (AU) corresponding to optical density at 630 nm. Results are typical of two experiments and data are mean ± s.d. of triplicates.

(B) A peptide reconstitution assay was conducted to determine the minimal epitope for CTL-1B9. Nonameric peptide (DYLQCVLQI), two nonameric peptides shifted by one amino acid to N- or C-terminus, N- and C-terminal extended decameric peptides, and its allelic counterpart (DYLQYVLQI) were synthesized and tested by adding to antigen-negative donor B-LCL at 10nM in a standard ⁵¹Cr release assay. Results are typical of two experiments and data are mean ± s.d. of triplicates. (C) Titration of the candidate minor H peptide by epitope reconstitution assay. Chromium-labeled donor B-LCLs were distributed to wells of 96-well round-bottomed plates, pulsed with serial dilutions of the indicated peptides for 30 min at room
temperature, and then used as targets for CTL-1B9 in a standard $^{51}$Cr release assay. A
cysteinylated peptide (indicated by an asterisk) was included as an alternative form of the
potential epitope. Results are typical of two experiments. (D) Tracking of ACC-1C-specific
T-cells in recipient’s peripheral blood. In order to longitudinally analyze the kinetics of the
ACC-1C-specific CTLs in peripheral blood from the patient from whom CTL-1B9 was
established, a real-time quantitative PCR was conducted. Complementary DNAs of peripheral
blood mononuclear cells from the donor and patient before and after HSCT were prepared from
the patient. Real-time PCR analysis was performed using a TaqMan assay as described
previously. The primers and fluorogenic probe sequences spanning the CTL-1B9
complementarity-determining region 3 (CDR3) were used to detect T cells carrying the CDR3
sequences identical to that of CTL-1B9. The primers and fluorogenic probe sequences spanning
constant region of TCR beta chain (TCRBC) mRNA were used as internal control. Samples
were quantified with the comparative CT method. The delta CT value was determined by
subtracting the average CT value for TCRBC from the average CTL-1B9 CDR3 CT value. The
standard curve for the proportion of CTL-1B9 among TCR$\alpha$β$^+$ T cells was composed by
plotting mean delta CT values for each ratio, and the percentages of T cells carrying the CDR3
sequence identical to CTL-1B9 were calculated by using this standard curve. During this
period, quiescent chronic GVHD which required steroid treatment developed, however,
involvement of immune reaction to ACC-1C minor H antigen was unlikely since its frequency
increased even after resolution of most chronic GVHD symptoms. c-GVHD, chronic GVHD;
CSA, cyclosporine A; PSL, prednisolone; WBC, white blood cell count.
Figure 1

A

Rt B-LCL
Do B-LCL
Rt CD40-B cells
Do CD40-B cells
Rt dermal fibroblasts
Rt primary AML cells

% Specific lysis

B

Rt B-LCL (A24, A11, B39, B51, Cw7, Cw14)
No.1 (A24, A26, B44, B61, Cw10, Cw14)
No.2 (A24, B52, Cw12)
No.3 (A24, B61, B61, Cw6, Cw12)
No.4 (A24, A2 B35, B51, Cw1, Cw8)
No.5 (A33, B14, Cw6)
No.5 transduced with HLA-A*2402

% Specific lysis
Figure 2

- CTL-2A12 (50K XbaI)
- CTL-2A12 (50K HindI)
- CTL-2A12 (250K Nsp)
- CTL-2A12 (250K Sty)

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Figure 3

A

Chr. 18: 59,649,725—59,805,277

B

Chr. 15: 77,999,379—78,122,388
Figure 4

A

CTL-1B9 (250K Nspl) 1st pools, Exp 1

B

CTL-1B9 (250K Nspl) 1st pools, Exp 2

C

CTL-1B9 1st pools, Exp 1

D

CTL-1B9 2nd pools, Exp 1
Figure 5
Identification of human minor histocompatibility antigens based on genetic association with highly parallel genotyping of pooled DNA

Takakazu Kawase, Yasuhito Nanya, Hiroki Torikai, Go Yamamoto, Makoto Onizuka, Satoko Morishima, Kunio Tsujimura, Koichi Miyamura, Yoshihisa Kodera, Yasuo Morishima, Toshitada Takahashi, Kiyotaka Kuzushima, Seishi Ogawa and Yoshiki Akatsuka