foreign tissue. In addition, Gubarev et al. (1996) reported the localization of a gene encoding mHa to chr 22. On the basis of these reports and in an attempt to improve efficiency by screening chromosomal regions of high gene density, chr 22 is a very attractive target for genome-wide association research of GVHD and other immune-related diseases.

As the first step to our genome-wide study, we retrospectively genotyped 155 microsatellite markers on chr 22 in 70 HLA-matched unrelated BMT recipient and donor pairs and associated at least eight significant allele frequency differences with aGVHD. In accordance with our previous study using microsatellite markers to identify mHa (Li et al. 2004), we performed individual DNA typing to investigate the association between statistically significant donor/recipient microsatellite marker mismatches.

Materials and methods

Recipient and donor pairs

A total of 70 unrelated donor/recipient pairs after BMT who were treated through the Japan Marrow Donor Program and completely allele-matched for the HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1 genes at the high resolution level were enrolled for this study after BMT (Sasazuki et al. 1998; Morishima et al. 2002). All 70 recipients underwent BMT from 1995 to 2000 for hematopoietic malignancy. None of the recipients received T-cell-depleted grafts. Patient, transplantation, and treatment information data are summarized in Table 1. All the donors and recipients provided informed consent for study, which was approved by the relevant institutional ethical committee.

Diagnosis and evaluation of the acute GVHD study group

Acute GVHD was diagnosed clinically and classified into four groups according to standard criteria (grades 0, I, II, III, and IV; Glucksberg et al. 1974; Thomas et al. 1975). The 30 recipients who experienced grades III and IV were designated in this study as the aGVHD group. The 40 recipients who had not developed aGVHD (grade 0) were designated as the aGVHD-free group. The recipients with GVHD grades I or II were excluded from this analysis to differentiate more efficiently between the aGVHD and aGVHD-free groups.

Microsatellite markers set

The association study was performed using 155 microsatellite markers spanning the long arm of chromosome 22.

Table 1 Numbers and ratios for the major clinical characteristics

Clinical characteristics	Number or Ratio
Recipient's age (median year, range)	27.5, 1–50
Donor's age (median year, range)	33.9, 21–52
Recipient's sex (M/F)	38:32
Sex combination (recipient/donor)	M/M 29
	M/F 19
	F/F 13
	F/M 9
Diagnosis	
Acute myeloid leukemia	28
Acute lymphoblastic leukemia	21
Chronic myeloid leukemia	21
Conditioning regimen	
CY+TBI	26
CY+CA+TBI	17
BU+CY+CA	2
BU+CY	7
CY+BU+TBI	3
CY+VP+TBI	4
BU+CY+TLI	1
LP+TBI	2
BU+VP+LP	1
CA+VP+TBI	1
CA+TBI	2
VP+TBI	1
BU+LP+TBI	1
CA+TBI	2
aGVHD frequency	
Grade 0	40
Grade III	20
Grade IV	10
GVHD prophylaxis	
CsA+MTX	64
FK+MTX	2
FK+PDR	1
CsA	1
CsA+MTX+PDR	1
CsA+MTX+FK	1

M Male, *F* female, *CY* cyclophosphamide, *TBI* total body irradiation, *CA* cytosine arabinoside, *BU* busulfan, *VP* etoposide, *TLI* total lymph node irradiation, *LP* melpharan, *CsA* cyclosporine A, *MTX* methotorexate, *FK* tacrolimus hydrate, *PDR* predonisolone

These markers were selected from Japan Biological Information Research Center (JBIRC) database (http://jbirc.jbic.or.jp/gdbs/). The markers covered the human genome from 15647099b (D22S0283i) to 49510061b (D22S0211i) on 22q with an average spacing of 200 kb.

Microsatellite genotyping

Genomic DNA was isolated from the peripheral blood lymphocytes of patients and donors. The PCR procedure was performed in 10 μl reactions using fluorescent-dye conjugated PCR primers that were unilaterally labeled at

the 5′-end with the fluorescent reagent, 6-FAM (Applied Biosystems Japan, Tokyo, Japan). The PCR reaction mixture contained 10 ng of genomic DNA, 1 μl of deoxyribonucleotide triphosphate (5 mM each), 1 μl of 10× buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), and 20 pmol of forward and reverse primers as well as 0.5 unit of Ampli Taq Gold DNA polymerase (Applied Biosystems Japan). After initial denaturation for 5 min at 96°C, amplification was carried out in an automated thermal cycler (Applied Biosystems Japan Co.) for 40 cycles of 1 min at 96°C, 45 s at 57°C, and 45 s at 72°C with a final extension of 7 min at 72°C. Each PCR product was diluted 1:40 with water. The samples containing 1 ul of the diluted PCR product, 10 μl Hi–Di formamide (Applied Biosystems Japan Co.) and 0.1 μl GeneScan-500LIZ size standard (Applied Biosystems Japan) were denatured for 3 min at 95°C, separated on capillary gels using an ABI PRISM 3730 automated sequencer, and the electrophoretic runs were analyzed with GeneMapper software (Applied Biosystems Japan).

Statistical analysis

Microsatellite allele frequency was calculated by direct counting. The strength of association was expressed by odds ratio (O.R), which was calculated from 2×2 contingency tables. Statistical significance was examined by the Fisher's double-sided exact test and the $m \times n$ contingency table. Univariate analysis was performed to determine the association between microsatellite mismatch and aGVHD incidence. The P value, except when comparing a mismatch, was corrected by multiplying the number of microsatellite alleles (corrected Pc value). The Pc value less than 0.05 was accepted as statistically significant, and the Pc value between 0.05 and 0.1 was indicative of a trend.

Definition of a microsatellite mismatch

Donor/recipient pairs were classified as matched or mismatched at each microsatellite marker locus. Pairs were defined as mismatched only when one or more recipient alleles are not shared by the corresponding donor (direction aGVHD).

Results

The overall genotyping results for paired transplantation donors and recipients

A total of 155 microsatellite markers spanning the long arm of chromosome 22 were used to genotype 70 pairs of transplantation patients and donors. Of the 70 transplantation recipients, 30 (42.8%) developed aGVHD with GVHD grade III in 20 patients and GVHD grade IV in 10 patients. The genotyping results obtained for the 70 transplantation recipients and the 70 transplantation donors were then analyzed and compared between the aGVHD-free group (grade 0 aGVHD) and the severe aGVHD group (group III to IV aGVHD).

Of the 155 markers, there were three markers with significant allele frequency differences between all donors and all recipients (D22S0052i-385; D22S0099i-412; D22S0115i-225; data not shown). As these three markers have different allele distribution between all donors and all recipients, they were considered to be inappropriate markers for the comparison between the aGVHD-free and the aGVHD group and were therefore excluded from further analysis. The remaining 152 markers were retained for further analysis in this study because they showed no significantly different allele distribution between all donors and recipients (data not shown).

Recipient age and GVHD prophylaxis

The recipient age was not significantly higher in the patients with aGVHD than the aGVHD-free group (P= 0.27 Student's t test). In regard to GVHD prophylaxis (Table 1), there was no significant association ($p>0.07$) of aGVHD factor risk between the patients in the total body irradiation (TBI) group and those in the non-TBI group.

Comparison of allele frequency differences between the aGVHD-free and the aGVHD group for the microsatellite polymorphisms in donors and recipients

The frequency differences for the microsatellite alleles between the aGVHD-free group and the aGVHD group were compared separately for the donors and recipients. The significant association ($P<0.05$) of markers with the occurrence of aGVHD was found for five markers (Table 2), with a significant difference (P and Pc<0.05) for two donor markers (D22S283 and D22S0141i) and for three recipient markers (D22S0021i, D22S0199i, D22S0222i). The comparison of individual allele frequencies of the microsatellite markers in the grade 0 (aGVHD-free) and grade III+IV (aGVHD) groups revealed the presence of possible risk (R) alleles (O.R>1) and protective (P) alleles (OR<1; Table 2). In the donors, the allele D22S0141i-431 was increased significantly (Pc=0.049) and the allele D22S283-132 was decreased significantly (Pc=0.008) in aGVHD when compared to the aGVHD-free group. Both of the marker loci were in position 22q12.3. In the recipients, the frequency of the allele D22S0021i-348 was significantly increased (Pc= 0.035) and three alleles (D22S0021i-357, D22S0199i-444,

Table 2 Statistically significant alleles associated with aGVHD grade in patients and donors, respectively

Marker	Position	No. of alleles	Significant allele	aGVHD grade (N=40)	aGVHD grade III+IV (N=30)	Odds ratio (95% confidence interval)	Protective (P) or at risk (R)	P value	Pc
Donor									
D22S283	22q12.3	12	132	35 (87.5%)	15 (50.0%)	0.14 (0.04–46)	P	0.0007	0.008
D22S0141i	22q12.3	7	431	4 (10.0%)	14 (46.7%)	7.87 (2.24–27.7)	R	0.007	0.049
Recipient									
D22S0021i	22q13.2	5	348	21 (52.5%)	25 (83.2%)	4.52 (1.44–14.2)	R	0.007	0.035
			357	38 (90.0%)	18 (67.7%)	0.16 (0.06–0.48)	P	0.004	0.020
D22S0199i	22q13.2	4	444	22 (55.0%)	7 (23.3%)	0.25 (0.09–0.72)	P	0.007	0.028
D22S0222i	22q13.3	7	258	17 (42.5%)	4 (13.3%)	0.21 (0.06–0.71)	P	0.007	0.049

and D22S0222i-258) were significantly decreased in aGVHD when compared to the aGVHD-free group.

In regard to the D22S0021i locus, we found both a risk allele (D22S0021i-348) and a protective allele (D22S0021i-357). On the basis of a genotype analysis, there was a significant association (P=0.001) between the D22S0021i genotype and aGVHD occurrence (Table 3).

Comparison of differences between the aGVHD-free and the aGVHD groups for microsatellite alleles that were matched or mismatched in donors and recipients

As a further comparison between the aGVHD-free and the aGVHD groups, we determined the significant differences between the number of alleles of the aGVHD-free and the aGVHD groups that were matched and mismatched for the donor and recipient pairs. We estimated that there were eight significant marker mismatches for an association with the occurrence of aGVHD (Table 4). Of these eight markers, three (D22S0267i, D22S0220i, and D22S683) were more often mismatched in the severe GVHD group (O.R>1), and therefore, these markers appear to be protective against the occurrence of severe aGVHD. As D22S0220i and D22S683 are located in a relatively close position to each other on 22q12.3 where they are 780 kb apart, we selected an additional five markers between D22S0220i and D22S683. As a result, three markers (Z67524, P=0.09, O.R=0.35; D22S0132i, P=0.07, O.R=2.54; D22S0075i, P=0.07, OR=0.03 in order from the centromere to telomere) showed a tendency of association with aGVHD (Fig. 1).

Table 3 Univariate analysis of D22S0021i genotype

Allele genotype	aGVHD grade 0 (N=40)	aGVHD grade III+IV (N=30)	P value
348/348	2	10	
348/357	19	15	
357/357	17	3	
			0.001

On the other hand, five markers (D22S0152i, P=0.0005; D22S0145i, P=0.017; Z66750, P=0.014; D22S0085i, P=0.035; D22S0197i, P=0.005) were more often mismatched in the aGVHD-free group (O.R<1), suggesting that they are significant susceptibility markers for aGVHD. Of these markers, D22S0152i and D22S0145i were located in a relatively close position to each other on 22q11.23 where they were 960 kb apart. We, therefore, genotyped an additional six markers (D22S0068i, D22S0186i, D22S0163i, D22S0169i D22S0184i, and D22S1174) but found that none of them were significantly associated with aGVHD (data not shown).

Candidate genes within the aGVHD susceptibility regions

Table 5 lists the candidate susceptibility genes that are located within or near to the genomic susceptibility region which was identified by microsatellite genotyping. These genes are in the close vicinity of the significant microsatellite markers that were found within intron 3 of CACNG2, intron 3 of PEX26, intron 4 of KIAA0376, intron 7 of LARGE, and intron 8 of TOM1. Other genes, such as MYH9, EP300, TCF20, ARSA, FLJ31568, EMID1, APOL3, and FLJ44385, are located within 10 kb to 172 kb of the significantly associated microsatellite markers.

Genomic map of the association of microsatellite polymorphisms on 22q12.3 with the occurrence of aGVHD

The P values for comparing the matching of microsatellite marker alleles between those of the aGVHD-free group and the aGVHD group were determined and plotted as a P value plot against the physical location of the microsatellite markers and the known genes on 22q12.3. Figure 1 shows a P value plot and the gene map of one of the aGVHD susceptibility regions determined by the association analysis using the microsatellite markers from D22S0220i to D22S683 and beyond the border of 22q12.3 and 22q13.1. The figure shows that the genes TOM1, HMOX1, and

Table 4 Correlation between matched mismatch donor–recipient pairs and aGVHD grade for each of the significant microsatellite markers on chromosome 22

Marker	Position	aGVHD grade 0		aGVHD grade III+IV		Odds ratio (95% CI)	Protective (P) or at risk (R)	*P* value
		Matched	Mismatched	Matched	Mismatched			
D22S0267i	22q11.21	38	2	23	7	5.78 (1.10–30.24)	P	0.028
D22S0152i	22q11.23	25	15	29	1	0.05 (0.01–0.41)	R	0.0005
D22S0145i	22q11.23	8	32	14	16	0.29 (0.10–0.82)	R	0.017
Z66750	22q12.1	28	12	28	2	0.17 (0.03–0.81)	R	0.014
D22S0085i	22q12.3	6	34	11	19	0.30 (0.10–0.96)	R	0.035
D22S0220i	22q12.3	17	23	4	26	4.80 (1.41–16.35)	P	0.008
D22S683	22q12.3	6	34	0	30	11.86 (0.64–219.35)	P	0.027
D22S0197i	22q13.33	16	24	22	8	0.24 (0.087–0.67)	R	0.005

MCM5 are in the region of the most significant *P* values and in close vicinity to the protective microsatellite marker D22S0220i.

Discussion

Of the 155 markers analyzed for differences between the aGVHD-free group and the aGVHD group and separately for the recipients and donors, only five markers on chr 22 (Table 2) were found to be significantly associated with aGVHD (Pc<0.05). Interestingly, of these five positive markers, the donor positive marker D22S283 was previously reported to be associated with schizophrenia (DeLisi et al. 2002), Sorsby's fundus dystrophy (Assink et al. 2000), and CDAGS (Mendoza-Londono et al. 2005). Although the susceptibility genes on chr 22 for those diseases are still unknown, the positive microsatellite marker D22S283 is located within the SC2D4 schizophrenia susceptibility locus (NCBI GeneID 6379) and could be associated with neuropsychological impairment that may evolve with aGVHD (Sostak et al. 2003).

The other four positive markers, D22S0141i, D22S0021i, D22S0199i, and D22S0222i, which were associated with aGVHD (Table 2) had not been previously associated with any human disease. However, these markers are located in a region of human chr 22 that was previously associated with the presence of strong mucosal and T-cell immune response against HIV-1 (Kanari et al. 2005) and, therefore, that could also affect the aGVHD. The p300 gene, which is a transcriptional factor located 10 kb from D22S0021i, is believed to participate in the activities of hundreds of

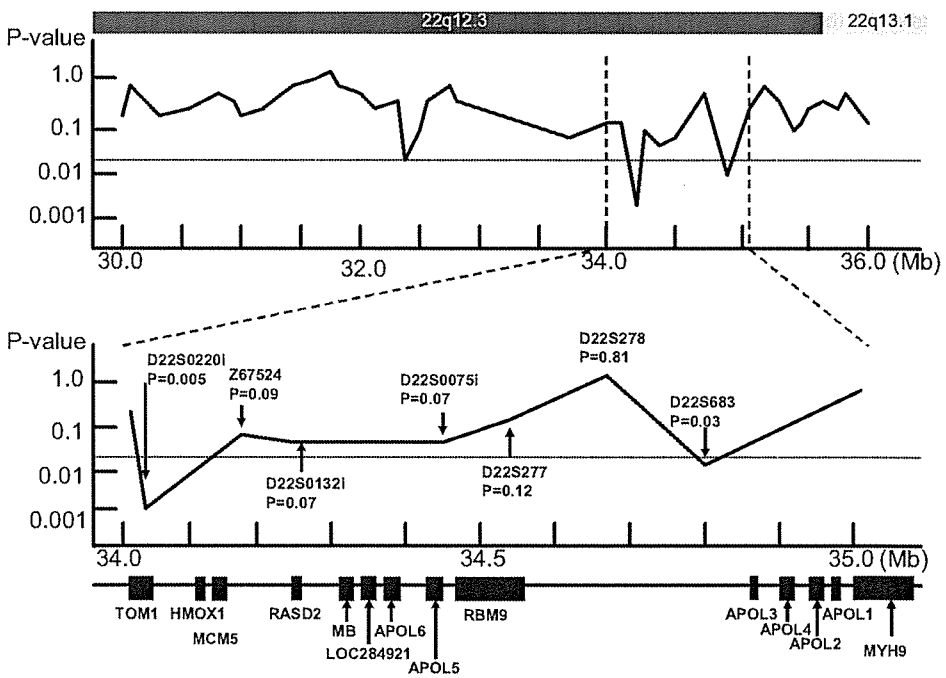

Fig. 1 aGVHD susceptibility gene mapping by association analysis using microsatellite markers on 22q12.3. *P* value (*y-axis*) was plotted against physical location of the microsatellite markers on 22q12.3 (*x-axis*), their distance (in Mb) in order from the centromere to the telomere. All markers were plotted according to their genetic map position taken from JBIRC database (http://jbirc.jbic.or.jp/gdbs/). The gene map at the *bottom* of the figure shows the representative genes that are indicated by *black boxes* on the locus near the two positive markers D22S0220i and D22S683 on 22q12.3. The *dotted horizontal line* shows the threshold for 5% significance

Table 5 Candidate genes close to the positive microsatellite markers

Marker	Position	Distance	Neighboring gene	Description	Function
Donor allele					
D22S283	22q12.3	172 kb	MYH9	Myosin, heavy chain 9, nonmuscle	Cytoskelton
D22S0141i	22q12.3	Intron 3	CACNG2	Calcium cannel, voltage dependent, gamma-2 subunit	Cell signaling
Recipient allele					
D22S0021i	22q13.2	10 kb	EP300	E1A binding protein, 300 KD	Transcription
D22S0199i	22q13.2	40 kb	TCF20	Transcription factor 20	Transcription
D22S0222i	22q13.33	45 kb	ARSA	Arylsulfatase A	Enzyme
Mismatching allele					
D22S0267i	22q11.21	Intron 3	PEX26	Peroxisome biogenesis factor 26	Protein degradation
D22S0152i	22q11.23	20 kb	FLJ31568	Hypothetical protein	Unknown
D22S0145i	22q11.23	Intron 4	KIAA0376	Hypothetical protein	Unknown
Z66750	22q12.1	10 kb	EMID1	BMI domain containing protein 1	Unknown
D22S0085i	22q12.3	Intron 7	LARGE	Acetyl glucosaminyltransferase-like protein	Unknown
D22S0220i	22q12.3	Intron 8	TOM1	Target of myb 1	Immuno regulation
D22S683	22q12.3	20 kb	APOL3	Apolipoprotien L-III	Lipid metabolism
D22S0197i	22q13.33	10 kb	FLJ44385	Hypothetical protein	Unknown

different genes (Vo and Goodman 2001). The p300 protein, together with the adenovirus serotype 5 E1A, has been reported to regulate the NKG2D ligand, NK cell lysis, and tumor rejection (Routes et al. 2005). In addition, p300-binding domains are known to interact with STAT1, 2, and 3, which play an important role in cytokine signal transduction (Pfitzner et al. 2004). Therefore, the p300 gene might be associated haplotypically with the D22S0021i marker, which has both a protective and risk allele for aGVHD (Table 3). These facts, together with our association results, strongly suggest that the loci at position 22q12–13 could affect the development of aGVHD. Whereas three of these positive markers are located 10 kb to 45 kb from any of the known genes, the positive microsatellite marker D2250141i is located within intron 3 of the CACNG2 gene that encodes the calcium channel, voltage dependent, gamma-2 subunit (Table 5). This protein appears to interact with neural proteins (Black and Lennon 1999; Chen et al. 2000), and it might have a role in neurological complications arising from aGVHD (Sostak et al. 2003).

We found eight microsatellite markers that were significantly different between the aGVHD-free group and the aGVHD group when matched or mismatched between the recipient and donor groups. Three of the eight markers, D22S0267i, D22S0220i, and D22S683, are considered to be protective because they were more often mismatched in the severe aGVHD group (OR>1), suggesting the existence of one or more protective candidate genes in close vicinity. Two of these markers, D22S0220i and D22S683, were approximately 780 kb apart (D22S0085i and D22S0220i) with another three markers (Z67524, D22S0132i, and D22S0075i) located between them that showed a positive trend ($P<0.1$) of association (Fig. 1). Interestingly, Gubarev et al. (1996) reported the localization of a gene encoding mHa on 22q12.3 in close vicinity to our significant markers by using T-cell clone and linkage-analysis. This report, which used different methods from our genome-wide approach, therefore strongly supports our results.

The highly significant protective microsatellite marker D22S0220i is located within intron 8 of the gene TOM1. The specific function of this gene has not yet been determined, but Tom1 may be a negative regulator of interleukin-1 and tumor necrosis factor-induced signaling pathways (Yamakami and Yokosawa 2004), and, therefore, affect aGVHD. D22S0220i is also located near to the HMOX1 gene (NCBI Gene ID 3162) that encodes the heme oxygenase (decycling) 1 protein. This association is biologically significant because HMOX1 (alias HO-1) is known to be a protective protein with anti-inflammatory and antiapoptotic properties (Willis et al. 1996; Brouard et al. 2002). Moreover, induction of HMOX1 in recipient mice of a BMT model resulted in a reduction in aGVHD and improved survival (Gerbitz et al. 2004). Therefore, HMOX1 is an excellent protective candidate gene for further aGVHD association studies specifically at the level of gene SNP analysis.

Another potential protective microsatellite marker D22S683 is located ~172.2 kb from the MYH9 gene (MIM 160775) and the Epstein syndrome locus (MIM 153650). The MYH9 mutations are known to result in the autosomal dominant giant-platelet disorders such as the May–Hegglin anomaly, the Fechtner syndrome, and the Sebastian syndrome (Seri et al. 2000). In addition, the MYH9 or the motor protein non-muscle heavy chain II A has been associated with the chemokine receptor CXCR4 in the T cell (Rey et al. 2002) and with the modulation of T cell motility (Jacobelli et al.

2004). Considering that one of the alleles of the microsatellite marker D22S283 is located within 172.2 kb of the MYH9 gene of the transplantation donors that were positively associated with aGVHD, then it can be envisaged that a neighboring SNP may affect the donor T cell behavior in a protective role against the occurrence and/or maintenance of aGVHD.

The five 'disease-negative' markers shown in Table 4 were associated with a risk of aGVHD because they were more often mismatched in aGVHD grade 0 group (OR<1) than the aGVHD group. This result seems to be paradoxical when considering the concept of a minor antigen mismatch, but it suggests that some gene products might need to be mismatched to prevent the development of disease. For example, it has been reported that the killer cell immunoglobulin-like receptor ligand (KIR-ligand), when mismatched between the donor and recipient, is associated with improved survival after stem cell transplantation for acute myeloid leukemia (Ruggeri et al. 2002). In this regard, the product of an unknown gene located near the 'disease-negative' microsatellite markers, when mismatched between donor and recipient, might help to prevent the development of aGVHD in a way that is analogous to the unique KIR-ligand mismatch involved with the NK-KIR biological system in response to transplantation (Malmberg et al. 2005).

To identify the candidate genes that are located within close vicinity to the significant microsatellite markers, we searched the human genome sequence deposited at NCBI for locations and annotations of genes in both directions of the microsatellite markers (Table 5). Interestingly, many of the genes that we identified near the associated markers, such as MYH9, CACNG2, EMID1, LARGE, and TCF20, have proximal STAT1- and STAT2-binding sites. Many DNA binding sites for STAT1 and STAT2 have been identified distributed across chr 22 in interferon-treated cells (Hartman et al. 2005). The STAT family proteins mediate transcriptional responses to many cytokines and are a useful system for studying inducible gene regulation. In addition, APOL3, EMID1, and LARGE exhibit IFN-sensitive expression changes. Considering the complex roles of cytokines, such as IFN, in the aGVHD occurring phase after BMT, the cytokine inducible candidate genes may play an important role in aGVHD.

The results of our study are largely dependant upon the hypothesis that microsatellite markers in LD will reveal an association between polymorphisms and the functional risk conferred by the variants or relevant genes so that certain marker alleles will be over represented in the aGVHD donors or patients compared with the GVHD-free donors or recipients (Ohashi and Tokunaga 2003; Zapata et al. 2001). In this study, we used 155 microsatellite markers whose spacing average was about 200 kb on the basis of the knowledge accumulated from a large number of recent data that the average length of LD between disease susceptible SNPs and nearby microsatellite alleles is ≥100 kb (Abecasis et al. 2001; Keicho et al. 2000; Oka et al. 1999; Ota et al. 1999). Although the LD pattern is variable between different regions of human genome depending on several factors such as allele frequency, mutation and recombination, and ethnic population, the 200 kb interval between markers is likely to be of sufficient distance for LD coverage of chr 22 in this study.

The multiple testing issues and the restricted sample size of our study limit the statistical power to find conclusive evidence of association particularly in the case of susceptibility genes with minor effects. It is statistically possible that at a probability level of less than 0.05 that 1 in 20 of our markers will represent false positives. We have analyzed 155 different microsatellite markers for association with aGVHD, and therefore, we could expect about eight false positive markers distributed randomly across the 40 Mb of the long arm of chr 22. Of the 13 microsatellite markers that were significantly different between the GVHD-free group and the GVHD severe group, the location of three of the markers, D22S0220i, D22S683, and D22S283, were relatively close to each other, which increases the probability that they represent a true association. Moreover, this GVHD susceptibility locus, from D22S0220i and D22S283, spans approximately 1 MB of genomic sequence and contains at least 14 candidate genes, including TOM1 and HMOX1 and MYH9, near the APOL1 to APOL6 gene cluster (Fig. 1).

In conclusion, we used 155 microsatellite markers distributed across the long arm of chr 22 and the 'genome-wide approach' in this genetic association study of aGVHD to identify and map potential aGVHD susceptibility and resistant regions on the basis of a small number of significant markers. It now remains to use the "candidate gene approach" and investigate the SNPs and haplotypes of the candidate genes, such as TOM1, HMOX, MCM5, and MYH9, which are located closely to the most significant microsatellite markers.

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ORIGINAL ARTICLE

Significance of additional high-dose cytarabine in combination with cyclophosphamide plus total body irradiation regimen for allogeneic stem cell transplantation

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The combination of cyclophosphamide (CY) and total body irradiation (TBI) has been used as a standard conditioning regimen for allogeneic transplantation. Several studies showed an advantage of adding high-dose cytarabine (HDCA) to this regimen. To clarify the significance of additional HDCA, we conducted a retrospective multicenter study and compared the clinical results of these two regimens. From June 1985 to March 2003, 219 patients with hematological malignancies underwent allogeneic transplantation after conditioning with CY + TBI 12Gy ($n = 73$) or CA + CY + TBI 12Gy ($n = 146$). Engraftment, overall survival, transplant-related mortality (TRM), relapse rate and incidence of graft-versus-host disease (GVHD) were compared according to risks and donors. Addition of HDCA had no impact on the relapse rate in all subgroups, and it was associated with lower TRM among standard-risk patients after related transplantation, and with higher TRM and worse survival among standard-risk patients after unrelated transplantation. The incidence of acute GVHD was not significantly different between the two regimens, and HDCA resulted in a higher incidence of chronic GVHD among standard-risk patients after related transplantation. In summary, addition of HDCA is not beneficial for high-risk patients, and is not recommended for standard-risk patients receiving unrelated transplantation.

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Introduction

For allogeneic stem cell transplantation, the conditioning regimen is one of the most important factors. The combination of cyclophosphamide (CY) and total body irradiation (TBI) has been used as a standard conditioning regimen for myeloablative hematopoietic stem cell transplantation.[1 4] Intensification of the conditioning regimen using high-dose cytarabine (HDCA) has been investigated as possibly reducing disease relapse in hematological malignancies. Some studies are encouraging additional HDCA,[5 11] whereas others are reporting more toxicity using HDCA particularly on the heart and lung.[12 16] Our previous preliminary report did not show any significant differences between CY + TBI and CA + CY + TBI in a small cohort.[17]

To clarify the significance of additional HDCA, we conducted a retrospective multicenter study of 219 patients, and compared the clinical results of these two regimens. We confirmed that addition of HDCA neither did improve overall survival, nor reduce the relapse rate.

Patients and methods

Patients, conditioning regimen and GVHD prophylaxis
From June 1985 to March 2003, a total of 219 patients with various hematological malignancies from 13 institutes

underwent allogeneic stem cell transplantation after conditioning with either CY + TBI ($n = 73$) or CA + CY + TBI ($n = 146$). CY was given at a dose of 60 mg/kg once daily intravenously (i.v.) on days −5 and −4 (total dose 120 mg/kg), CA at a dose of 2 g/m^2 twice daily i.v. over 3 h on day −6 and 2 g/m^2 once daily i.v. over 3 h on days −5 and −4 (total dose 8 g/m^2) and TBI at a dose of 300 cGy fractions twice daily on days −2 and −1 (total dose 12 Gy). Seven institutions used only one regimen, either CY + TBI or CA + CY + TBI. The other six institutions used both regimens at the same time. There were no consistent indications for either regimen in any institution. Donors were HLA-fully-matched related donors or HLA-fully-matched unrelated donors. GVHD prophylaxis consisted of either cyclosporine (CsA) and short-term methotrexate (sMTX) or taclorimus (FK) and sMTX.

Statistical analysis

Engraftment, overall survival, transplant-related mortality (TRM), relapse rate and incidence of graft-versus-host disease (GVHD) were compared between the two regimens in each subgroup, which was defined according to risk (standard or high) and donor (related or unrelated). TRM was defined as mortality owing to any cause other than relapse or disease progression. Standard-risk patients are defined as those with acute myeloblastic leukemia (AML) or acute lymphoblastic leukemia (ALL) in first complete remission, chronic myelogenous leukemia (CML) in first chronic phase, or myelodysplastic syndromes (MDS) as refractory anemia (RA) or refractory anemia with ringed sideroblasts (RARS). High-risk patients were those with AML or ALL in subsequent complete remission, in relapse or of induction failure, Philadelphia-chromosome-positive ALL, CML in subsequent chronic phase, accelerated phase or blastic phase or MDS as RAEB or overt leukemia with MDS. The χ^2 test and Fisher's exact test were used for comparison of the two groups. Overall survival was calculated using the Kaplan–Meier method and *P*-values were calculated using the log-rank test. Cumulative incidence curves for TRM and relapse, with or without death, were constructed, reflecting time to relapse and time to TRM as competing risks. *P*-values were calculated at the fixed point in time as reported by Klein *et al.*[18] Univariate and multivariate analyses were performed using the Cox proportional hazard regression model, and variables were selected using stepwise method. A two-sided *P*-value of less than 0.05 was considered significant. Data were analyzed as of March 2003.

Results

Patient characteristics

Patient characteristics of each subgroup are summarized in Table 1. One hundred and twenty-seven patients received transplantation from a related donor whereas 92 received from an unrelated donor. GVHD prophylaxis consisted of CsA + sMTX in 182 patients and FK + sMTX in 37 patients. FK was used in one patient after related transplantation in 1999, and in 36 patients after unrelated

Table 1 Patient characteristics

Risk	*Standard*				*High*			
Donor	*Related*		*Unrelated*		*Related*		*Unrelated*	
Conditioning	*CY + TBI(19)*	*CA + CY + TBI(71)*	*CY + TBI(24)*	*CA + CY + TBI(40)*	*CY + TBI(14)*	*CA + CY + TBI(23)*	*CY + TBI(16)*	*CA + CY + TBI(12)*
Median age (range)	29 (20–50)	33 (16–53)	33 (18–54)	31 (17–50)	39 (24–51)	9 (16–44)	27 (15–48)	31 (16–50)
Sex, F/M	6/13	25/46	8/16	15/25	3/11	8/15	3/13	6/6
Diagnosis								
AML	5	26	2	4	5	6	3	3
ALL	8	18	4	16	3	10	9	4
CML	4	26	15	19	4	5	1	5
MDS	2	1	3	1	2	2	3	0
P-value[a]		0.25		0.09		0.31		0.41
GVHD prophylaxis								
CsA + sMTX	18	71	10	26	14	23	11	9
FK + sMTX	1	0	14	14	0	0	5	3
P-value		0.48		0.12		—		1.0

Abbreviations: ALL = acute lymphoblastic leukemia; AML = acute myeloblastic leukemia; CA = cytarabine; CML = chronic myelogenous leukemia; CY = cyclophosphamide; CsA = cyclosporine; FK = taclorimus; MDS = myelodysplastic syndromes; sMTX = short-term methotrexate; TBI = total body irradiation.
[a]Myeloid malignancy vs lymphoid malignancy.

transplantation since 1996. All stem cell sources were from bone marrow except for three patients who received peripheral blood stem cell transplantation from a related donor. Diagnosis and GVHD prophylaxis did not differ significantly between conditioning regimens in each subgroup. The median follow-up period of survivors was 979 days (range 31–4704 days).

Engraftment

All evaluable patients achieved sustained engraftment (an absolute neutrophil count of $>0.5 \times 10^9$/l for three consecutive days) in both regimens.

Overall survival

Overall survival did not differ significantly in any patient between the two regimens (58 vs 56% at 3 years, $P=0.90$) (Figure 1a). Addition of HDCA resulted in significantly worse survival among standard-risk patients after unrelated transplantation (45 vs 81% at 3 years, $P=0.02$) (Figure 1b), whereas it resulted in comparable survival among standard-risk patients after related transplantation (80 vs 60% at 3 years, $P=0.27$).

No significant differences were observed among high-risk patients (40 vs 40% at 3 years, $P=0.48$ among patients after related transplantation; and 11 vs 28% at 3 years, $P=0.93$ among patients after unrelated transplantation).

TRM and hazard analysis for TRM

TRM did not differ significantly in any patient between the two regimens (28 vs 32% at 3 years, $P=0.56$). Addition of HDCA was associated with significantly lower TRM among standard-risk patients after related transplantation (7.8 vs 35% at 3 years, $P=0.027$) (Figure 2a), whereas it resulted in higher TRM among standard-risk patients after unrelated transplantation (51 vs 19% at 3 years, $P=0.0082$) (Figure 2b).

No significant differences were observed among high-risk patients (22 vs 16% at 3 years, $P=0.65$ among patients after related transplantation; and 69 vs 58% at 3 years, $P=0.64$ among patients after unrelated transplantation).

Univariate analysis among standard-risk patients after related transplantation showed that addition of HDCA, female patients, age over 40 and GVHD prophylaxis with CsA + sMTX were significant factors affecting TRM. Addition of HDCA remained a significant factor on multivariate analysis (relative risk = 0.18; confidence interval, 0.052–0.63) (Table 2a). Univariate analysis among standard-risk patients, after unrelated transplantation, showed that addition of HDCA and GVHD prophylaxis

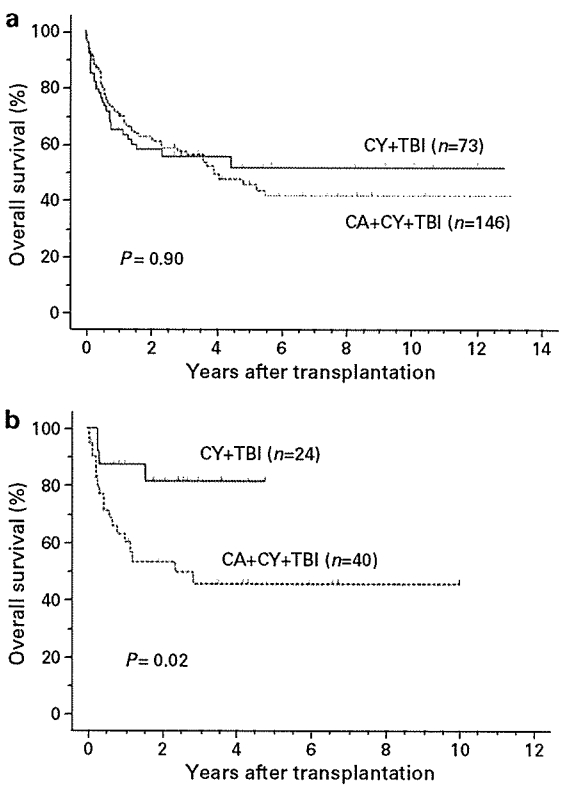

Figure 1 Overall survival. (a) No significant differences were observed between CA + CY + TBI and CY + TBI ($P=0.90$) in all patients. (b) CA + CY + TBI resulted in significantly worse survival than CY + TBI among patients who received transplantation from unrelated donors ($P=0.02$).

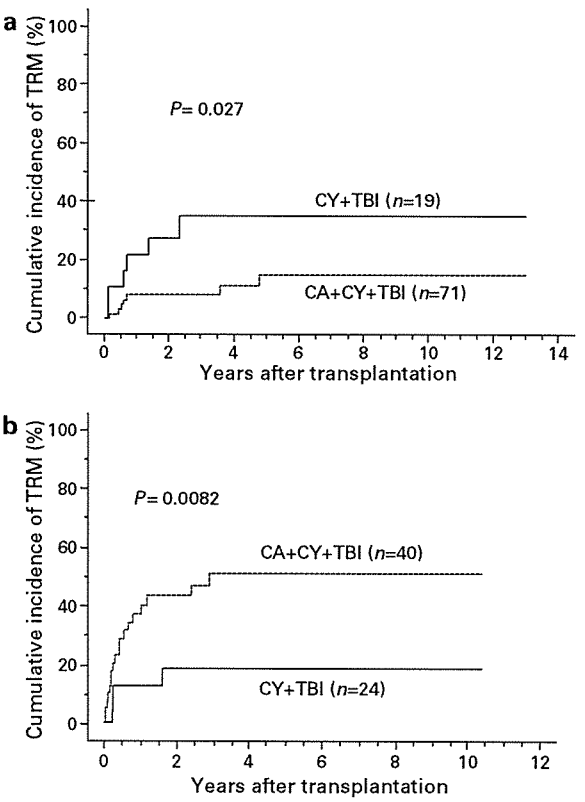

Figure 2 Cumulative incidence of TRM in patients with standard-risk disease. (a) CA + CY + TBI resulted in significantly lower TRM than CY + TBI among patients who received transplantation from related donors ($P=0.027$). (b) CA + CY + TBI resulted in significantly higher TRM than CY + TBI among patients who received transplantation from unrelated donors ($P=0.0082$).

Table 2 Prognostic factors affecting TRM

Variables	*Unfavorable factors*	*Univariate*		*Multivariate*[a]	
		Hazard ratio (CI)	P-*value*	*Hazard ratio (CI)*	P-*value*
(a) Related standard risk					
Conditioning	CA + CY	0.32 (0.11–0.94)	0.038	0.18 (0.052–0.63)	0.0070
Sex	Female	3.3 (1.1–10)	0.039	7.0 (2.0–25)	0.0030
Female to male	Yes	0.95 (0.29–3.1)	0.94		
Disease	Other than CML (CP)	1.4 (0.44–4.7)	0.55		
Age	>40	3.8 (1.2–12)	0.020	8.4 (2.4–30)	0.0010
GVHD prophylaxis	CsA + sMTX	0.12 (0.016–0.98)	0.047	0.53 (0.051–5.5)	0.59
Transplant year	~1996	1.9 (0.50–7.0)	0.35		
(b) Unrelated standard risk					
Conditioning	CA + CY	3.2 (1.1–9.3)	0.038	2.7 (0.90–8.1)	0.078
Sex	Female	0.70 (0.27–1.8)	0.45		
Female to male	Yes	0.74 (0.22–2.5)	0.63		
Disease	Other than CML (CP)	1.1 (0.46–2.5)	0.88		
Age	>40	1.1 (0.41–2.7)	0.93		
GVHD prophylaxis	CsA + sMTX	2.6 (1.0–6.6)	0.048	2.2 (0.84–5.6)	0.11
Transplant year	~1996	0.88 (0.29–2.6)	0.82		

Abbreviations: CA = cytarabine; CI = confidence interval; CML = chronic myelogenous leukemia; CsA = cyclosporine; CY = cyclophosphamide; sMTX = methotrexate.
[a]Final model.

of CsA + sMTX were significant factors influencing TRM. On multivariate analysis, addition of HDCA was associated with a trend for increased TRM (relative risk = 2.7; CI, 0.90–8.1) (Table 2b).

Relapse rate

Relapse rate did not differ between the two regimens (20 vs 13% at 3 years, $P = 0.23$). Addition of HDCA was not associated with any significant differences as to relapse rate in any subgroups (18 vs 5.6% at 3 years, $P = 0.085$ among standard-risk patients after related transplantation; 2.8 vs 0% at 3 years, $P = 0.31$ among standard-risk patients after unrelated transplantation; 51 vs 47% at 3 years, $P = 0.81$ among high-risk patients after related transplantation; and 17 vs 13% at 3 years, $P = 0.81$ among high-risk patients after unrelated transplantation).

Graft-versus-host disease

Results are summarized in Table 3. The incidence of grade II–IV acute GVHD did not differ between the two regimens in any subgroup. Addition of HDCA was associated with a significantly higher incidence of chronic limited and extensive GVHD among standard-risk patients after related transplantation (40/69 vs 5/19, $P = 0.029$).

Table 3 Incidence of acute and chronic GVHD

Risk	*Standard*		*High*	
Donor	*Related*	*Unrelated*	*Related*	*Unrelated*
Acute GVHD (II–IV)				
CY + TBI	6/19	6/24	3/11	8/14
CA + CY + TBI	9/71	11/40	6/22	6/11
P-value	0.11	1.0	1.0	0.78
Chronic GVHD				
CY + TBI	5/19	11/21	7/10	3/6
CA + CY + TBI	40/69	15/34	7/19	6/8
P-value	0.029	0.75	0.13	0.58

Abbreviations: CA = cytarabine; CY = cyclophosphamide; GVHD = graft-versus-host disease; TBI = total body irradiation.

Discussion

We examined a total of 219 patients, which is the largest series in the literature. Aurer and Gale[19] reviewed modified conditioning regimens in 1991, and failed to detect any major improvements in the overall survival with any of the new regimen. Although intensification of the conditioning regimen with HDCA is one of the approaches designed to improve outcome, particularly for high-risk hematological malignancies,[20 24] our retrospective analysis did not show any improvement in overall survival in any subgroups. In addition, no significant reduction in relapse rate was observed in any subgroups, suggesting that anti-leukemic activity may not be intensified by HDCA.

Many of the previous studies reported the superior anti-leukemic activity of HDCA for high-risk disease. Champlin *et al.*,[9] for example, showed that HDCA had good anti-leukemic activity before transplantation. Riddell *et al.*[21] reported a low relapse rate of 14% with the higher dose of CA (36 g/m^2), but an accurate relapse rate could not be fully evaluated because the day 100 TRM was as high as 50%. Mineishi *et al.*[22] reported a lower relapse rate of 11% after related transplantation compared to the 51% in our study. However, of 55 patients, 18 patients with AML/ALL with cytogenetic abnormalities in first remission were classified as high risk in their study. The difference in the definition of high-risk patients may be one reason for the lower relapse rate. In addition, the higher dose of CA (18 g/m^2) in their study may explain the lower relapse rate. Jillella *et al.*[10] also reported a similar outcome, but almost three-quarters of the patients had standard-risk disease. Woods

et al.[6] and Minami *et al.*[17] demonstrated a high relapse rate of 50–75% even with HDCA after related transplantation for high-risk disease. The dose effect of HDCA on anti-leukemic activity should be explored, but it may be offset by the increased toxicity reported in many earlier studies.

Interestingly, however, addition of HDCA was associated with lower TRM among standard-risk patients after related transplantation, and with higher TRM among standard-risk patients after unrelated transplantation. Thus, we performed multivariate analyses to clarify the factors affecting TRM, and confirmed that addition of HDCA still remained as a prognostic factor. Although the effects of the differences in unevaluable factors, such as supportive care, in each institute cannot be fully excluded, additional HDCA may play a role in the reduction of TRM after related transplantation. In contrast, a trend for increased TRM with HDCA after unrelated transplantation is reasonable. TRM is reported to be higher after unrelated than after related transplantation,[25,26] and intensification of the conditioning regimen increases TRM after unrelated transplantation.[27]

Intensity of conditioning is reported to modify the incidence of both acute and chronic GVHD,[28] but its effect on chronic GVHD is controversial.[29] Addition of HDCA was further associated with a significant increase in chronic GVHD among patients with standard-risk disease after related transplantation, but it was not associated with acute GVHD. Thus, other factors such as management of immunosuppresion may also have affected the incidence of chronic GVHD in our series.

In summary, addition of HDCA is not beneficial for patients with high-risk disease. It is not recommended for patients with standard-risk disease who will receive transplantation from unrelated donors because of increased TRM and decreased survival. It may be beneficial for patients with standard-risk disease who will receive transplantation from a related donor. Although the number of patients in this subgroup is somewhat small, such differences could not have emerged without underlying facts. Therefore, further studies are warranted to verify our results in this subgroup.

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European Journal of Immunology Immunity to infection

Epstein-Barr virus (EBV) latent membrane protein-1-specific cytotoxic T lymphocytes targeting EBV-carrying natural killer cell malignancies

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Epstein-Barr virus (EBV)-encoded latent membrane protein (LMP) 1 is a potential target for immunotherapy of some proportion of Hodgkin's disease cases, nasopharyngeal carcinomas, EBV-associated natural killer (NK)/T lymphomas, and chronic active EBV infection (CAEBV). Since it is unknown whether EBV-infected NK/T cells are susceptible to lysis by LMP1-specific cytotoxic T lymphohcytes (CTL), we here tested the ability of mRNA-transduced antigen-presenting cells (APC) to stimulate rare LMP1-specific CTL. A 43-amino acid N-terminal deletion mutant LMP1 (ΔLMP1) could be efficiently expressed in dendritic cells and CD40-activated B cells upon mRNA electroporation. ΔLMP1-expressing APC were found to stimulate LMP1-specific CTL from a healthy donor and a CTL clone recognized a peptide, IIIILIIFI, presented by HLA-A*0206 molecules. Processing and presentation of the antigenic peptide proved dependent on expression of an immunoproteasome subunit, low-molecular-weight protein-7, as confirmed by RNA interference gene silencing. Furthermore, an EBV-infected NK cell line derived from a patient with CAEBV, and another from an NK lymphoma with enforced HLA-A*0206 expression, were specifically lysed by the CTL. Overall, these data suggest that immunotherapy targeting LMP1 in EBV-associated NK lymphomas and CAEBV might serve as an alternative treatment modality.

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Introduction

EBV is involved in development of many malignancies, including Burkitt's lymphoma, Hodgkin's disease (HD), and nasopharyngeal carcinoma (NPC), as well as post-transplant lymphoproliferative disorder [1]. Viral protein expression is limited in latent infections and the patterns in these diseases have been classified into three types [2]. Only EBV nuclear Ag (EBNA) 1 is expressed in most Burkitt's lymphomas, referred to as latency I. Recently, a subset of Burkitt's lymphomas not displaying the typical latency I form of infection was identified [3, 4], the tumor cells instead expressing five nuclear Ag, namely EBNA1, EBNA3A, EBNA3B, EBNA3C, and a truncated (W1 W2 repeat domain only) EBNA leader protein, in the absence of EBNA2 and of latent membrane protein (LMP) 1 and LMP2. This was associated with transcription exclusively from Wp and was hence termed "Wp-restricted latency". Some cases of HD and NPC express LMP in addition to EBNA1 (latency II). In the post-transplant lymphoproliferative

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Abbreviations: **CAEBV:** chronic active EBV infection · **CD40-B:** CD40-activated B · **EBNA:** EBV nuclear antigen · **EGFP:** enhanced GFP · **ELISPOT:** enzyme-linked immunospot · **HD:** Hodgkin's disease · **ip-LMP:** low-molecular-weight protein of immunoproteasome subunit · **LGL: lymphoblastoid cell line** · **LMP:** EBV-encoded latent membrane protein · **ΔLMP1:** 43-amino acid N-terminal deletion mutant **LMP1** · **NPC:** nasopharyngeal carcinoma · **shRNA:** short hairpin RNA · **siRNA:** small interfering RNA

disorder, all EBV latent proteins, EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, leader protein, and LMP1 and LMP2 are expressed (latency III).

One distinct category is EBV infection in NK/T cells [1], including EBV-associated NK/T lymphomas. Chronic active EBV infection (CAEBV) is another disorder whereby EBV infects mainly NK/T cells to cause life-threatening lymphoproliferative disease [5]. EBV-infected NK cells express LMP1 [5–7], a transmembrane oncoprotein that enhances cell survival through up-regulation of anti-apoptotic genes [2]. Expression of LMP1 is essential for growth transformation of human B lymphocytes and is necessary for the proliferation of human monocytes under EBV-infected conditions [2]. LMP1 has also been found to induce tumorigenic transformation of the murine cell line BALB/c 3T3 and to generate B cell lymphomas in transgenic mice *in vivo* [2]. Moreover, LMP1 expression might be responsible for the proliferative capacity of EBV-positive NK cells [8].

There is increasing interest in immunotherapy for EBV-associated malignancies and adoptive transfer of *in vitro* activated EBV-specific CTL has proven effective for prevention and treatment of EBV-associated lymphoproliferative diseases after stem cell and organ transplants [9–13]. Extension of a similar strategy to other EBV-associated malignancies, such as HD [14] and NPC [15], has been reported to be efficacious in some patients. However, the majority of lymphoblastoid cell line (LCL)-activated CTL used in the reported studies were directed to immunodominant EBNA3A, EBNA3B, and EBNA3C Ag, which are not expressed in the malignant cells of HD and NPC cases.

A subdominant portion of LCL-activated CTL may recognize peptides derived from LMP2 [14, 15], which would contribute to immunotherapeutic effects in treated patients. However, T cells directing LMP1 peptides are rare [15], reflecting a low CTL precursor frequency [16]. To selectively activate the T cell repertoire specific to subdominant EBV Ag, Lin *et al.* [17] used monocyte-derived DC pulsed with LMP2 peptides to immunize NPC patients. For activation of CTL specific to LMP1, various modalities have been reported. Khanna *et al.* [16] first described HLA-A2-restricted LMP1 epitopes and induction of CTL using peptide-pulsed APC. They also showed the utility of a replication-incompetent adenovirus and a recombinant vaccinia virus encoding multiple LMP1 epitopes, successfully immunizing HLA-A2-transgenic mice and achieving inhibition of the growth of LMP1-transduced cells [18, 19]. Gottschalk *et al.* [20] reported effective induction of polyclonal LMP1-specific CTL using DC infected with a recombinant adenovirus expressing an N-terminally truncated, nontoxic LMP1 mutant. EBV-positive NK/T cell malignancies express EBNA1 and LMP1 as potential CTL targets [6, 7], but it has not been demonstrated that such NK/T cells can process LMP1 and generate HLA-restricted epitopes, so that they are susceptible to CTL-mediated cytolysis.

Induction of CTL with low precursor frequencies is a challenge for immunologists as well as clinicians wishing for immunotherapy targeting tumor-associated Ag. There is accumulating evidence that APC transduced with *in vitro* transcribed mRNA encoding certain Ag are potent inducers of CTL specific to tumor-associated Ag [21–24], even overcoming immunological tolerance to self Ag [25]. The advantages seem to derive from (1) complete deletion of antigenicity of vector backbone sequences; (2) highly reproducible yields with *in vitro* transcription; (3) high efficiency of transduction using electroporation. We infer that mRNA-transduced APC might be suitable for induction of LMP1-specific CTL. In the present study, we applied a 43-amino acid N-terminal deletion mutant (ΔLMP1) as an Ag to reduce LMP1 cytotoxicity [26] and its potential to induce IL-10 production [20].

We here document successful establishment of an LMP1-specific CTL clone, using mRNA-transduced APC, which recognizes a novel epitope presented by HLA-A*0206 molecules. Included is an analysis of the mechanisms involved in the generation of this epitope with its unusual hydrophobic primary structure. More importantly, we provide evidence that the CTL clone can kill EBV-infected NK cells derived from patients with NK lymphomas and CAEBV, suggesting potential application for immunotherapy against these tumors.

Results

Induction of LMP1-specific CTL using mRNA-transduced APC

DC and CD40-activated B (CD40-B) cells generated from PBMC of donors were electroporated with *in vitro* transcribed ΔLMP1 mRNA and analyzed for ΔLMP1 expression by FCM. More than 70% of both the DC and CD40-B cells were positive for ΔLMP1 (Fig. 1A). The viable populations exceeded 80% at 36–48 h post-electroporation (data not shown). These cells were used as APC to generate LMP1-specific T cells from five EBV-seropositive donors. After three rounds of stimulation, enzyme-linked immunospot (ELISPOT) assays were performed to test the specificity of the T cell lines. Polyclonal T cells from one out of five donors specifically secreted IFN-γ in response to ΔLMP1 mRNA-transduced autologous CD40-B cells, but not to non-transduced CD40-B cells (Fig. 1B).

We established a T cell clone, designated as H7, by limiting-dilution culture of the bulk CTL line. H7 was

found to lyse autologous ΔLMP1 mRNA-transduced CD40-B cells but not enhanced GFP (EGFP) mRNA-transduced CD40-B cells (data not shown). HLA genotyping revealed HLA-A*0206, HLA-A*2402, HLA-B*0702, HLA-B*4801, HLA-Cw*0304, and HLA-Cw*0702 for the blood donor. To identify the HLA molecule presenting the CTL epitope, fully HLA-mismatched LCL transduced with each HLA gene using retrovirus vectors were employed. H7 produced IFN-γ spots when incubated with LCL transduced with HLA-A*0206, demonstrating this to be the presenting molecule (Fig. 1C).

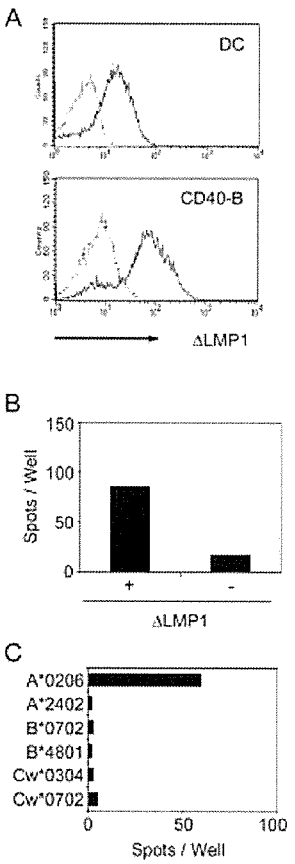

Figure 1. mRNA-transduced DC and CD40-B cells express ΔLMP1 and induce specific T cells. (A) DC and CD40-B cells were transduced with ΔLMP1 mRNA and analyzed for expression of ΔLMP1 by FCM at 36 h post-transduction. The dotted lines show non-transduced, and the solid lines ΔLMP1-transduced cells. (B) Peripheral $CD8^+$ T cells were stimulated with irradiated autologous ΔLMP1 mRNA-transduced APC three times and assayed by ELISPOT using ΔLMP1 mRNA-transduced or non-transduced CD40-B cells as APC. Data are numbers of spots per 500 $CD8^+$ T cells. (C) A CTL clone, H7, was stimulated with fully HLA-mismatched LCL transfected with each HLA gene and IFN-γ production was tested by ELISPOT assay (1000 H7 cells/well).

Identification of the LMP1 epitope

There have been no reports of HLA-A*0206-restricted LMP1-derived epitopes with the exception of the peptide YLLEMLWRL [16], which is HLA-A2 supertype-restricted. Since H7 did not produce IFN-γ with the peptide (data not shown), we decided to explore the epitope recognized by H7. For this purpose, we electroporated autologous CD40-B cells with truncated forms of the ΔLMP1 mRNA, and tested recognition by the H7 in ELISPOT assays. As demonstrated in Fig. 2A, antigenicity was lost upon C-terminal truncation

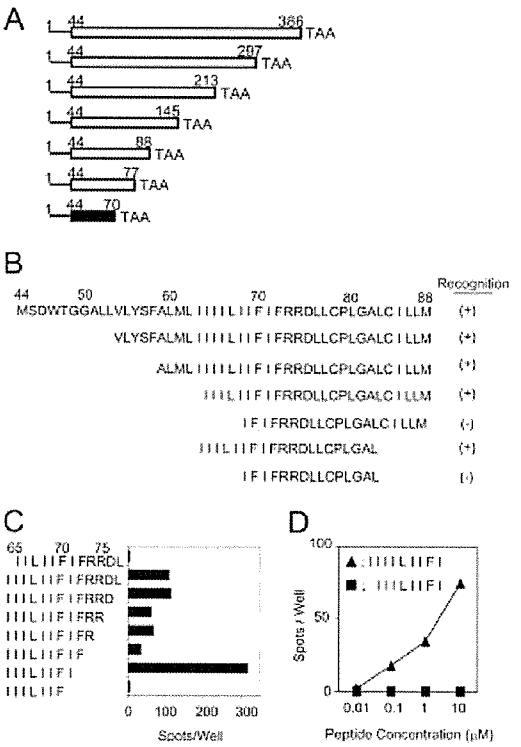

Figure 2. Identification of the LMP1 epitope peptide recognized by H7 CTL clone. (A) A series of C-terminally truncated ΔLMP1 mRNA were generated by *in vitro* transcription. A methionine at amino acid position 44 was used as the initiation codon for all constructs. CD40-B cells transduced with each truncated ΔLMP1 mRNA were used as stimulators in the ELISPOT assay. The constructs shown as open boxes were recognized by H7, while that shown as filled box was not. (B) A series of truncated fragments were amplified by PCR and cloned into the pcDNA3.1(+) vector. The predicted amino acid sequences are shown. H7 recognition of A0206–293T cells transfected with each plasmid was determined by ELISPOT assay (1000 H7 cells/well) and categorized as follows depending on IFN-γ spot production: (+), more than 50 spots; (–), less than 10 spots. (C) IFN-γ spot production of H7 stimulated with A0206–293T cells transfected with each minigene construct is shown. Each bar represents the number of spots per 1000 H7 cells. (D) ELISPOT assays were performed using A0206–293T cells pulsed with serial concentrations of synthetic peptides. Data are numbers of spots per 500 H7 cells.

between amino acid residues 70 and 77, indicating the C terminus of the epitope to be located between amino acid residues 71 and 77. Here we shifted to A0206–293T cells transfected with plasmids encoding truncated ΔLMP1 genes because they are more feasibly prepared than CD40-B cells and mRNA. With the A0206–293T cells as APC, antigenicity was lost when C-terminal truncation was between amino acid residues 77 and 88 (data not shown). The reason for the discrepancy with the data obtained using CD40-B cells is unclear. Here we used LMP1 truncated with the C terminus at position 88.

A series of plasmids with more deletions on the N-terminal side were prepared and analyzed (Fig. 2B). The shortest stimulatory fragment was identified as residues 64–83. To precisely define the N- and C-terminal ends, further truncation was performed within the region. As demonstrated in Fig. 2C, a plasmid encoding amino acid residues 64–71 (IIILIIFI) exhibited the strongest antigenicity, while deletion of either residue 64 or 71 completely abolished the antigenicity. Although amino acid residues 64–71 may constitute the minimal epitope for H7, it is possible that the N terminus methionine encoded by the start codon of the expression vector should substitute for isoleucine at position 63 to meet structural requirements for MHC binding and H7 recognition. For elucidation, a synthetic 8-mer peptide (residues 64–71, IIILIIFI), and a 9-mer peptide (residues 63–71, IIIILIIFI) were pulsed on the A0206–293T cells and the H7 reactivity was tested in ELISPOT assays. As demonstrated in Fig. 2D, only the 9-mer was recognized by H7, indicating the minimal epitope to start from isoleucine at position 63.

Requirement of the immunoproteasome subunit ip-LMP7 for generation of the LMP1 63–71 epitope

Regarding Ag processing in LMP1-transfected cells, we observed two discrepancies in the ELISPOT assays: First, H7 recognized CD40-B cells transfected with full-length ΔLMP1 mRNA (Fig. 2A) but not A0206–293T cells transfected with the plasmid expressing the same structure, although the expression of the LMP1 in A0206–293T cells following transfection was confirmed by Western blotting (data not shown); and second, H7 recognized CD40-B cells transfected with truncated ΔLMP1 mRNA encoding amino acid residues 44–77 (Fig. 2A) but not A0206–293T cells transfected with the plasmid expressing the same amino acid residues (data not shown). Here, we hypothesized that different machinery for Ag processing resulted in these discrepancies in generation of the LMP1 epitope. A0206–293T cells predominantly express standard proteasomes while in CD40-B cells and LCL immunoproteasomes are dominant [27, 28]. Standard proteasomes play a critical role in the Ag processing pathway, and exposure of cells to IFN-γ during immune responses alters the proteasome activity qualitatively and quantitatively by induction of newly synthesized immunoproteasome β subunits, such as low-molecular-weight protein (ip-LMP) 2 and ip-LMP7 [29], assembling immunoproterasomes.

To determine whether the effects of immunoproteasomes are critical for epitope processing, we used LCL in which expression of the immunoproteasome subunit was inhibited. The following two small interfering RNA (siRNA) targets, ip-LMP2 and ip-LMP7, were selected in this experiment because these are known to be crucial molecules in the generation of epitopes from transmembrane Ag such as EBV LMP2 [30] and MAGE-3 [31]. As shown in Fig. 3A, expression of either ip-LMP2 or ip-LMP7 was significantly reduced in LCL transfected with the corresponding short hairpin RNA (shRNA) vector. The effect of gene silencing on the LMP1 epitope generation was then assessed using ELISPOT assays. Interestingly, production of IFN-γ spots by the H7 clone was significantly reduced when stimulated with ip-LMP7-silenced LCL, whereas silencing of ip-LMP2 had negligible effects (Fig. 3B). These data indicate that ip-LMP7 is essential for processing and presentation of the LMP1 epitope.

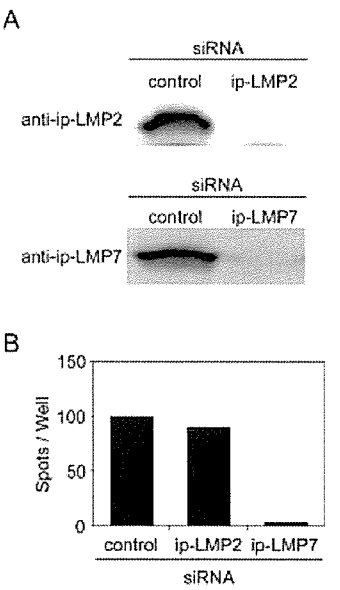

Figure 3. ip-LMP7 is essential for the LMP1 epitope processing. (A) Control siRNA, ip-LMP2 siRNA, or ip-LMP7 siRNA were retrovirally transduced into autologous LCL and the cells were selected for 14 days with puromycin, followed by Western blot analysis of ip-LMP2 (upper panel) and ip-LMP7 (lower panel). (B) IFN-γ spot production of H7 was estimated by ELISPOT assay using ip-LMP2- or ip-LMP7-silenced autologous LCL. Each bar represents the number of spots per 5000 H7 cells.

Cytotoxic activity of the LMP1-specific CTL clone against LCL

Next we explored functional activities of H7 on LCL. Standard CTL assays revealed that H7 could not efficiently lyse HLA-A*0206-positive LCL within a 4-h incubation (data not shown) but lysed autologous and HLA-A*0206-positive allogenic LCL after 16 h (Fig. 4A), suggesting insufficient LMP1 expression in the LCL for H7-mediated cell lysis in the 4-h CTL assay. The inability of CTL to kill LCL within 4 h has been reported previously for clones targeting other EBV Ag [32].We then examined LCL with forced expression of ΔLMP1 as target cells as shown in Fig. 4B. H7 specifically lysed exogenous ΔLMP1-expressing, but not EGFP-expressing LCL in the 4-h CTL assay

Cytotoxic activities of the LMP1-specific CTL clone against EBV-infected NK cell lines

EBV LMP1 is expressed in LCL with other proteins as latency III and also in NK/T cell malignancies as latency II [7]. In a final set of experiments, we tested the lytic activity of H7 against EBV-carrying NK cell lines as representative of EBV latency II malignancies and retaining characteristics of the original tumors, such as identical EBV clonality [6, 7, 33]. Among the three

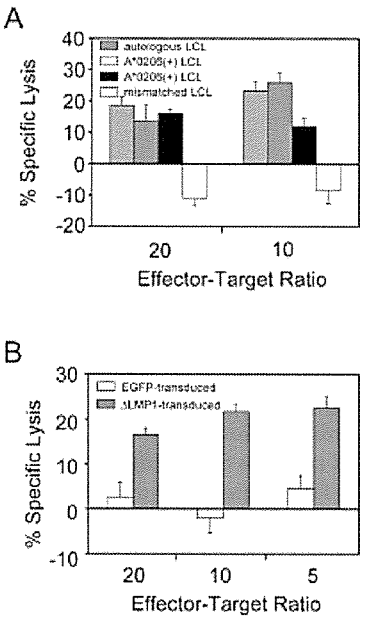

Figure 4. Cytotoxic activity of the LMP1-specific CTL clone H7. (A) Sixteen-hour CTL assays were performed using autologous, HLA-A*0206-shared, and fully HLA-mismatched LCL as target cells. (B) Four-hour CTL assays were performed using ΔLMP1- or EGFP-transfected LCL as target cells. Each bar represents the mean percentage cytolysis with standard deviations in triplicate wells.

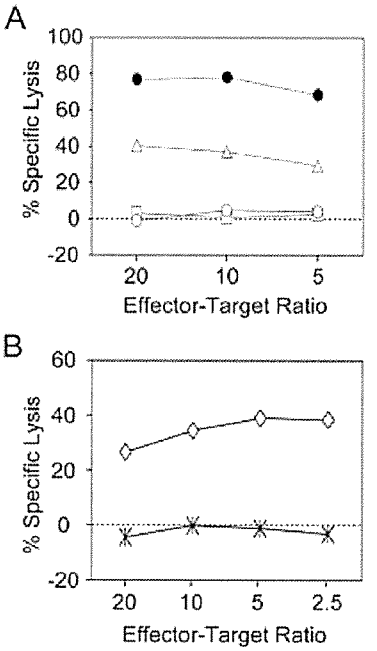

Figure 5. CTL specifically lyse EBV-infected NK cells. (A) Cytolytic activity of the CTL clone H7 was assessed against EBV-carrying NK cells in 16-h CTL assays. Data for two HLA-A*0206-positive NK cell lines (SNK-6 and SNK-10) and one HLA-A*0206-negative NK cell line (HANK-1) are shown with open circles, open triangles, and open squares, respectively. Lytic activity was measured in 4-h CTL assays using SNK-6 loaded with 100 nM cognate peptide (solid circles). (B) Sixteen-hour CTL assays were performed using HLA-A*0206- (diamonds) or HLA-A*2402-transduced (asterisks) HANK-1 cells.

LMP1-expressing NK cell lines examined, two were positive for HLA-A*0206. As shown in Fig. 5A, H7 lysed one of the HLA-A*0206-positive lines (SNK-10) but neither the other (SNK-6) nor HLA-A*0206-negative HANK-1 cells. HLA-A*0206-transfected HANK-1 cells were specifically lysed by H7 (Fig. 5B). Since the epitope peptide-pulsed SNK-6 cells were efficiently lysed by H7 (Fig. 5A), SNK-6 might have a mutation in the LMP1 epitope. Thus we sequenced genomic DNA flanking the LMP1 epitope. All three EBV-carrying NK cell lines demonstrated the same synonymous mutations, not affecting the amino acid sequence from position 55 to 80 (data not shown).

Discussion

For immunotherapy of EBV latency II malignancies such as HD, NPC and NK/T lymphoma, one focus is on EBNA1, LMP1 and LMP2 as target Ag. Of these, EBNA1 may not be seen by CTL because it is believed that the glycine-alanine repeat domain within the molecule prevents proteasomal cleavage [34]. Although there is

evidence that some CTL epitopes are produced and presented through the classical class I Ag presenting pathway [35–37], the significance of EBNA1-specific CTL for EBV-associated malignancies remains unclear. LMP1 has drawn particular attention as a target, but considering its oncogenic potential, it is unlikely that a vaccine or immunotherapeutic strategy based on full-length LMP1 could be used. We here applied an efficient approach to load APC with N-terminally truncated LMP1 using *in vitro* transcribed mRNA introduced *via* electroporation. Such mRNA-loaded DC have been proven to be able to stimulate the immune system *in vitro* and *in vivo* [21, 22, 38–42]. In addition, this provides a very safe tool for human clinical studies, as mRNA is not immunogenic, has a relatively short half-life, and lacks the potential for integration into the host genome.

We here demonstrated that DC and CD40-B cells expressing ΔLMP1 mRNA induce LMP1-specific CTL from PBMC of one healthy donor among five tested. We have successfully induced EBNA1-specific $CD8^+$ T cells stimulated with APC transduced with full-length EBNA1 mRNA, including a structure encoding the glycine-alanine repeat domain which prevents Ag processing [34], from four out of four EBV-seropositive donors tested (manuscript in preparation). Thus we speculate that the low success rate of LMP1-specific CTL induction in our hands is not due to inefficiency of our method but rather to inherent low CTL precursor frequencies with the Ag, underscoring previous observations [43, 44].

Low CTL precursor frequencies might be related to LMP1-specific $CD4^+$ T cells in PBMC from EBV-seropositive donors producing high levels of IL-10 [45]. To overcome this potentially significant hurdle, especially in active immunization using LMP1 as a target Ag, it would be necessary to inhibit such $CD4^+$ regulatory T cell function and induce protective Th1 and cytotoxic response with the aid of polyguanosine nucleotides [46] or OK432 [47]. Besides, the most important rationale for immunotherapy targeting with LMP1 is, we believe, the evidence that EBV-infected malignant cells do process and present LMP1-derived peptides and are sensitive to cognate CTL. We have presented support for the conclusion that the isolated CTL clone H7 recognizes a very hydrophobic peptide (IIIILIIFI) in the context of HLA-A*0206. So far there has been little information regarding the ability of EBV-infected NK/T cells to function as targets for CTL specific for viral Ag. For the first time, to our knowledge, this study demonstrated that EBV-infected NK cells derived from patients with CAEBV and EBV-associated NK/T lymphoma with enforced expression of restricting HLA molecules can be lysed by LMP1-specific CTL.

During a series of experiments to identify the minimal LMP1 epitope, we have found that the H7 clone did not recognize A0206–293T cells transduced with the pcDNA/ΔLMP1 despite recognizing CD40-B cells transduced with the ΔLMP1 mRNA. We investigated this discrepancy and found that ip-LMP7, an immunoproteasome subunit, but not ip-LMP2, is required for processing and presentation of the LMP1 epitope. For effective CTL-based immunotherapy using the LMP1 epitope, EBV-positive malignant cells may be required to express the ip-LMP7 molecule. As far as we have tested using RT-PCR, expression of ip-LMP7 is positive in all EBV-infected NK cell lines used in the present study (data not shown). Examination of immunoproteasome subunit expression in the malignant cells in HD and NPC could provide important information for prediction of effects of LMP1-specifc CTL-based immunotherapy, although further studies are clearly needed.

EBV-infected NK cell lines SNK-10 and HANK-1 transduced with HLA-A*0206 gene were here found to be recognized by H7, and displayed the identical epitope sequence (IIIILIIFI) with prototype B95-8 (data not shown). No amino acid variation around the epitope was seen in the SNK-6 cell line, which was not lysed by H7, and the reason for its resistance to lysis is unknown. One possibility is insufficient processing and/or presentation of the epitope. In conclusion, the present study demonstrated some lines of EBV-infected NK cells, derived either from lymphomas or CAEBV, to be susceptible to LMP1-specific CTL-mediated lysis, raising hopes for LMP1-based immunotherapeutic approaches.

Materials and methods

Donors and cell lines

The study design and purpose, which had been approved by the institutional review board of Aichi Cancer Center, were fully explained and informed consent was obtained from all blood donors according to the Declaration of Helsinki. EBV-transformed B-LCL were established as described previously [48] and cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum (PAA Laboratories, Pasching, Austria), 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and 50 μg/mL kanamycin. EBV-carrying NK cell lines SNK-6 [6] and SNK-10 [7] were kindly provided by Dr. Shimizu (Tokyo Medical and Dental University, Tokyo, Japan). Another EBV-carrying NK cell line, HANK-1 [33], was generously donated by Dr. Kagami (Aichi Cancer Center Hospital). All three were cultured as previously described [6]. HEK-293T cells (American Type Culture Collection, Manassas, VA) and Phoenix-GALV cells [49] (kind gifts from Dr. Kiem, Fred Hutchinson Cancer Research Center; and Dr. Nolan, Stanford University, Stanford, CA) were cultured as previously described. Retroviral transduction of HLA genes was performed as detailed earlier [50].

