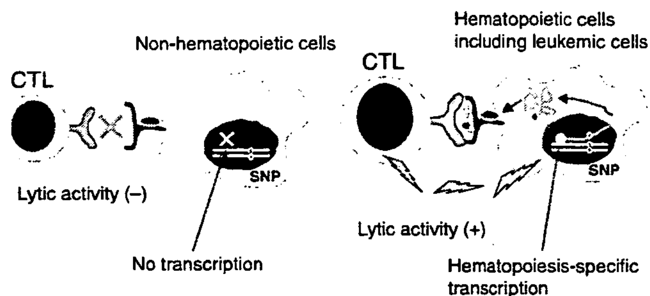

Fig. 1. Selective induction of graft-versus-leukemia/lymphoma (GVL) effects. Donor T cells sensitized to minor histocompatibility antigen complex (mHag) epitopes encoded by polymorphic genes, the expression of which is limited to hematopoietic cells, including hematological malignant cells, are responsible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

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

Mechanisms of mHag epitope generation

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

Currently known mHag

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

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

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

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

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

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

Hematopoietic cell-restricted mHag epitopes

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

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

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

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

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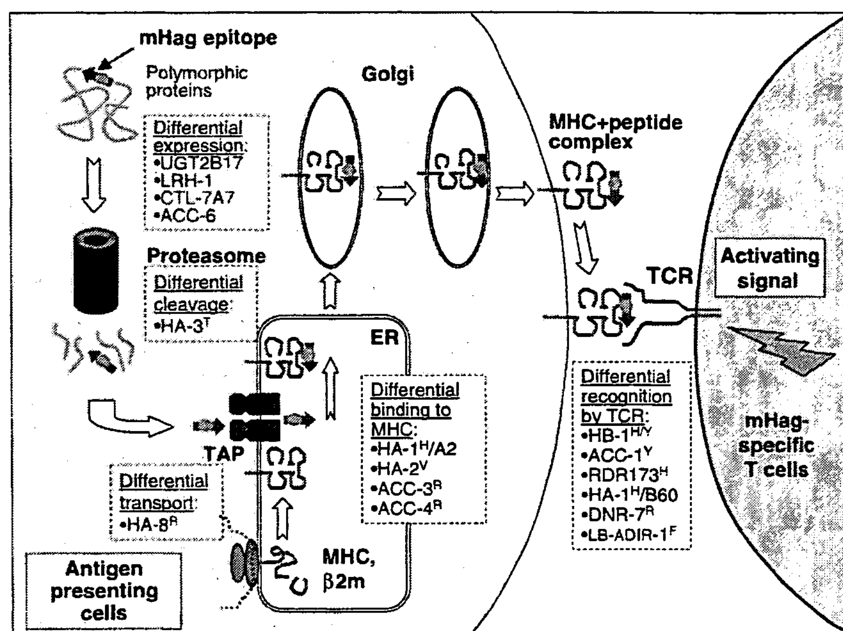

Fig. 2. Mechanisms involved in the generation of antigenicity of minor histocompatibility antigen complex (mHag) epitopes. In antigen-presenting cells, there is different machinery involved in the presentation of cellular proteins onto major histocompatibility complex (MHC) molecules on cell surfaces.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

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

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

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

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

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

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

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

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

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

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

The HLA-B7-restricted RDR17$3^H$ mHag was identified using expression cloning,[31] like HB-1^H,[29] ACC-4^R and ACC-5^R.[27] The *ECGF1* gene encoding this mHag is expressed in hematopoietic cells and some solid tumors, as expected from its gene name, angiogenic endothelial-cell growth factor-1.[31] Clinical applicability of this mHag in Asians is again not high because of its HLA-B7 restriction.[37]

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

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

ACC-6 (HMSD^A/B44). ACC-6 is a hematopoietic cell-specific mHag the authors have found just recently.[19] Encoded by a splice variant of a pseudogene termed *HMSD* (*H*istocompatibility (*M*inor) *S*erpin *D*omain containing), it does not include polymorphic amino acids, like LRH-1 and RDR17$3^H$ mHag,[17,31] as detailed above. This was the first novel mHag demonstrated to be formed by alternative splicing due to SNP in the consensus sequence critical for spliceome binding. The expression of *HMSD* is limited to leukemic cells, particularly of myeloid origin, myeloma cells, and some activated normal hematopoietic cells, including mature dendritic cells. *In vivo* immunogenicity was also proved by the presence of T cells specific for ACC-6 (0.8% of all $CD3^+$ cells at the peak time) using a quantitative polymerase chain reaction (PCR) method with clone-specific primers and a probe,[19] because HLA-B44 is notorious as a molecule unsuitable for tetramer construction. The observed magnitude of T-cell response appears comparable with those observed for HA-1^H-specific T-cell responses following DLI against recurrent leukemia.[17,20,31] In addition, as with LRH-1 mHag,[17] both alleles of *HMSD* can encode substantial polypeptides whose amino acid sequences are distinct due to a frameshift, so that it is possible that different HLA alleles may present other epitopes encoded by not only *HMSD-v* but also the original *HMSD* (encoding a 193-mer polypeptide). Furthermore, eradication of leukemic stem cells by the CTL was confirmed using severe immunodeficient common γ-chain knockout NOD/SCID (NOG) mice,[47] transplanted with CD34-selected acute myeloid leukemic cells with or without preincubation with either ACC-6-specific or irrelevant CTL clones.[19] These findings collectively suggest that ACC-6 is a promising target for immunotherapy at least against recurring myeloid leukemia post-HCT.

Clinical application of mHag

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

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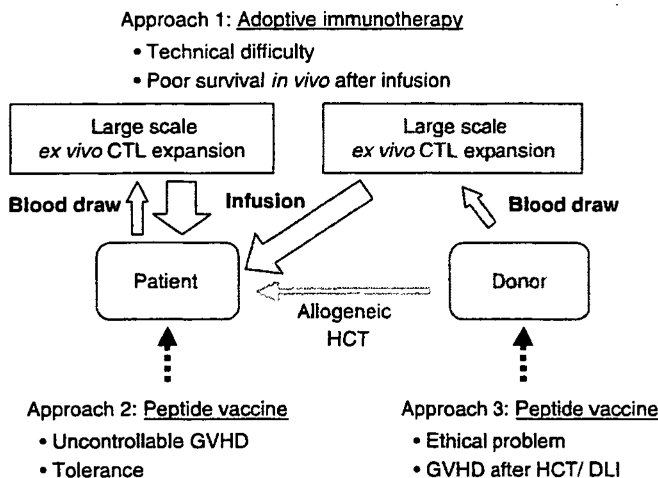

Fig. 3. Modalities of minor histocompatibility antigen complex (mHag)-based immunotherapy. Initially, adoptive immunotherapy with cytotoxic T cells (CTL) specific for mHag was developed largely due to the cell therapeutic nature of allogeneic cell transplantation (Approach 1). CTL are induced from either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

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

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

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

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

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

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

Conclusions

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

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ORIGINAL ARTICLE

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

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

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

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

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

Materials and methods

Patients, cell culture and clones

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

Synthetic peptides and in vitro *CTL induction*

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

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

Genotyping of HA-1 polymorphisms

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

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

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

Epitope reconstitution assay

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

HLA peptide binding assay

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

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

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

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

Results

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

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

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

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

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

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

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

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

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

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

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

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

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

Phenotypic and functional analyses using tetramers

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

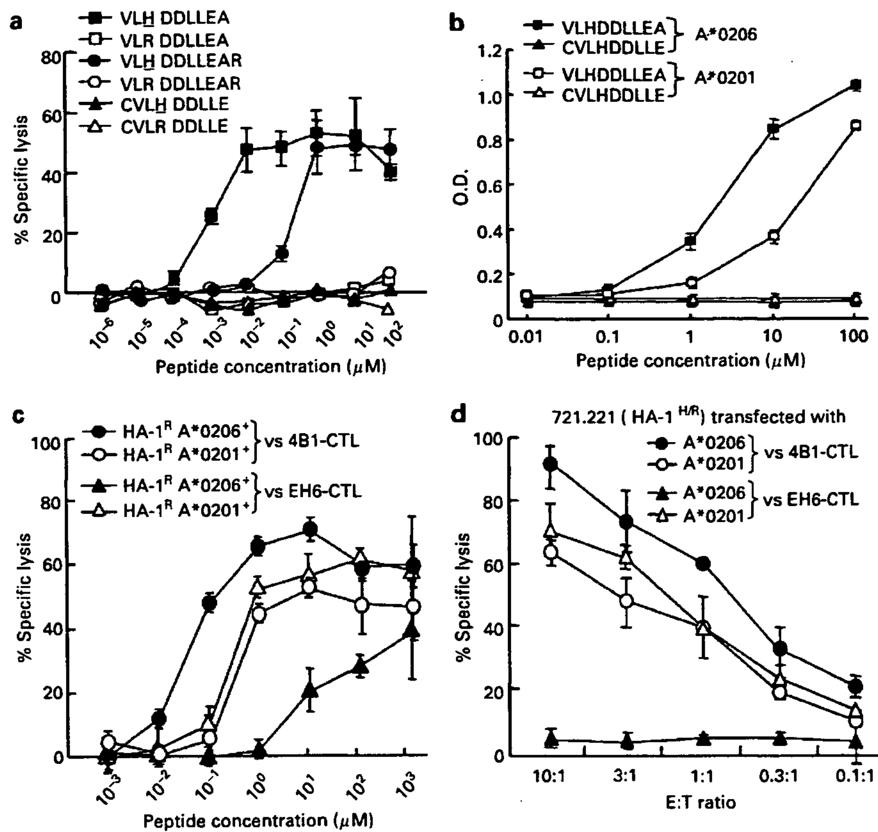

Figure 1 Identification of minimal epitope by epitope reconstitution and peptide-HLA binding assays. (**a**) UPN022 donor LCL were labeled with ^{51}Cr and distributed to wells of 96-well round-bottomed plates, pulsed with serial dilutions of the indicated peptides for 30 min at room temperature, and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

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

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

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

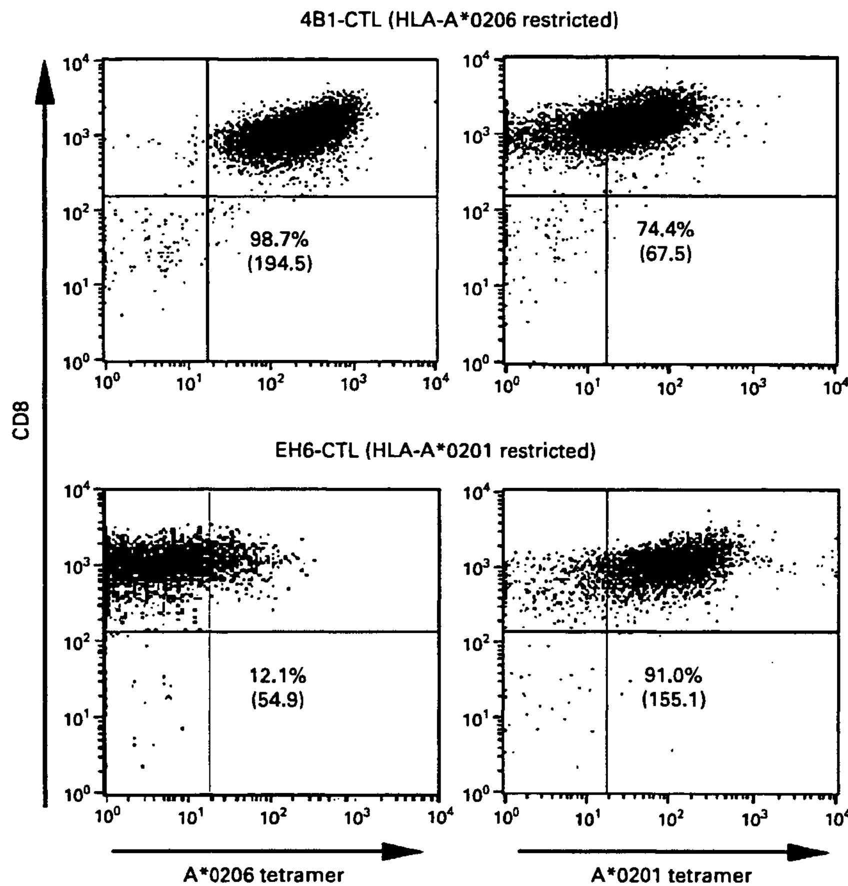

Figure 2 Representative staining profiles of CTL clones by tetramers. 4B1-CTL (upper panels) and EH6-CTL (lower panels) were stained with VLHDDLLEA peptide-incorporated HLA-A*0206 (left column) and HLA-A*0201 (right column) tetramers for 15 min at room temperature. Cells were washed and counterstained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

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

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

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

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

Discussion

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

Clinical association of the emergence of CTLs specific for HA-1^H/A*0201 and the induction of remission following donor lymphocyte infusion in relapsed leukemia patients has been elegantly shown using tetramer analysis.[31] After successfully using a reverse immunological approach to identify HLA-B60 as the allele that presents the HMHA1-derived peptide KECVLHDDLL, we sought to identify other HLA alleles common in Asian populations associated with this mHA to expand the definition of the SCT recipient population that may benefit from immunotherapy based on HA-1 disparate transplantation.

In this study, we adopted two straightforward strategies. First, we used as responder cells post-SCT CD3$^+$ cells from patients receiving HA-1^R to HA-1^H disparate SCT. Because these cells have potentially been primed *in vivo* by certain HLA molecules that are able to present endogenously processed mHA peptides, such memory T cells should be more easily expanded than naïve T cells. Second, we used a long (29-mer) peptide with the polymorphic His at the middle to cover the possibility that putative epitope(s) are presented not only by HLA class I but also by class II. This strategy is based on an epitope mapping approach using overlapping peptides, which has been applied to identifying epitopes from an entire protein sequence of interest regardless of HLA type,[32,33] although up to 20-mer peptides are recommended for efficient stimulation for an intracellular cytokine staining approach.[33] As a result of induction and cloning attempts from four post-SCT blood samples, we obtained CTL clones that could recognize the HA-1^H/A*0201 peptide, VLHDDLLEA, which was endogenously processed and presented on HLA-A*0206 molecules. In addition, the *in vivo* presence of a bona fide HA-1^H-specific T cell response was indeed confirmed by tetramer analysis. These results support the recent report demonstrating that not only professional APCs but also non-professional APCs such as B cells or even peripheral blood lymphocytes can process and present properly trimmed peptides in the context of HLA class I, although the mechanism involved has not been fully elucidated.[34] Based on our observations, it is likely that PHA-activated CD4$^+$ blasts can handle long peptides up to 29 amino acids in length. However, further studies into whether CD4$^+$ blasts may also present processed antigens on class II molecules and stimulate cognate responder CD4$^+$ T cells are necessary. Finally, whether HLA-A*0206 is the sole allele that can present

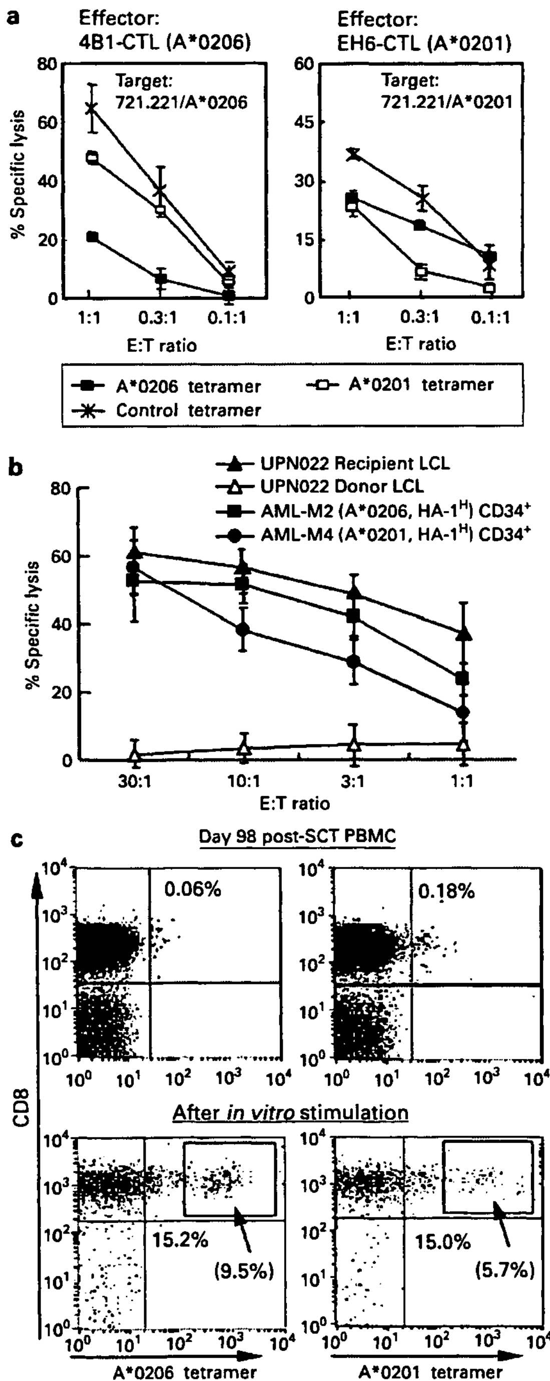

Figure 3 Allele-specific recognition and *in vivo* relevance of HA-1^H-specific T cells. (a) Inhibition of cytolytic activity of HA-1^H-specific CTL clones by two different HLA-A2 subtype tetramers. HA-1^H-positive 721.221 LCL transduced with either HLA-A*0206 or HLA-A*0201 cDNA retrovirally were labeled with ^{51}Cr and distributed to wells of 96-well round-bottomed plates as targets. Indicated CTL clones were incubated with 40 μg/ml of HLA-A*0206/HA-1^H, HLA-A*0201/HA-1^H or control (HLA-A*2402/ACC-1^Y) tetramers at room temperature for 15 min, then washed and added to the above target cells at indicated E:T ratios. After 4 h of coculture, the supernatants were harvested and counted. Data shown are representative of four independent experiments and the experiments were performed in duplicate. (b) Cytolytic activity of HLA-A*0206-restricted HA-1^H-specific 4B1-CTL against primary leukemic blasts. HA-1^H-positive CD34-selected leukemic blasts from one HLA-A*0206-positive patient and one HLA-A*0201-positive patient with acute myeloid leukemia were assayed in a standard 4 h ^{51}Cr release assay. Data shown are representative of two independent experiments and the experiments were performed in duplicate. The HLA-A*0201-restricted EH6-CTL recognized HLA-A*0201$^+$ but not HLA-A*0206$^+$ blasts (data not shown). (c) *In vivo* presence of HA-1^H-specific, HLA-A*0206-restricted T cells. PBMCs obtained at day 98 after SCT from an HLA-A*0201$^+$ and HLA-A*0206$^+$ patient, UPN011, who received HA-1 disparate marrow transplant from an unrelated, HLA-A, -B, -DR-matched donor were analyzed by HLA-A*0206 and HLA-A*0201 tetramers, respectively (upper panels). The rest of PBMCs were stimulated with HA-1^H-pulsed APCs and assayed for tetramer positivity (lower panels). The percentage in the parentheses indicate CD8$^+$ cells that were stained as bright as 4B1-CTL and EH6-CTL, respectively (shown in boxes).

Table 3 Amino-acid sequence of TCRV- to J-regions of HA-1^H-specific CTL clones restricted by different HLA-A2 subtypes

Clone	V-region		N-region	J-region	
TCRA chain					
4B1-CTL[a]	V3	ALYFCAVRD	IG	SGAGSYQLTFGKGTKLSVIP	J28
EH6-CTL	V13-2	PGDSAVYFC	CAE	GSSGGGADGLTFGKGTHLIIQP	J45
TCRB chain					
4B1-CTL[a]	V7-9	AMYLCASS	LVGG	EKLFFGSGTQLSVL	J1-4
EH6-CTL	V7-9	AMYLCAS	TGGTV	YNEQFFGPGTRLTVL	J2-1

Abbreviations: CTL = cytotoxic T lymphocyte; HLA = human leukocyte antigen; TCR = T-cell receptor for antigen; TCRA = TCR alpha; TCRB = TCR beta.
[a]4B1-CTL and 3B11-CTL were considered to be derived from the same origin based on their complementarity-determining region 3 (CDR3) sequence. Nomenclature is based on the international ImMunoGeneTics (IMGT) information system. The LV amino-acid pair in the TCRB N-region, which was also shown to be interindividually shared by HLA-A*0201-restricted HA-1^H-specific CTL clones,[27] is underlined.

His-containing peptide derived from the *HMHA1* gene in the two patients studied requires further investigation.

Because our approach involved CTL cloning, HA-1^H-specific T cell clones restricted by other than HLA-A*0206 might be missed if they comprise only minor populations in the polyclonal T cell lines. Nevertheless, to our knowledge, this is the first demonstration that the HLA-A*0206 molecule is able to bind and efficiently present HA-1^H/A*0201 peptide, VLHDDLLEA, to cognate T cells. It was unexpected because the peptide sequence possessed none of the preferred anchor motifs for HLA-A*0206.[25,35] This information and the absence of HLA-A*0206 in general Caucasian populations might be the reasons why this allele has not been focused on until now. Because HLA-A*0206 is the second or third most common HLA-A2 subtype worldwide (unpublished data from the 12th IHW[13]), the opportunity to utilize HA-1^H mHA for immunotherapy has been nearly doubled in eastern Asian populations. We found that 4B1-CTL failed to recognize LCL from an HA-1^H-positive individual expressing HLA-A*0207 (another allele frequently expressed in the Asian population). However, this finding does not necessarily rule out the possibility that HLA-A*0207 may also present HA-1^H. Direct induction of HA-1^H-specific CTL from an HLA-A*0207$^+$ patient receiving an HA-1^H disparate transplant could be attempted in future studies. Our findings imply that a reverse immunological approach relying on computer algorithm-based prediction may overlook potential candidate peptides. It is still necessary to optimize culture conditions such as varying peptide length, adjusting the stimulation period for minimizing *in vitro* clonal contraction and incorporating positive selection before cloning to maximize the chance to identify multiple clones.[36]

It would be interesting to know whether HLA-A*0201-restricted HA-1^H-specific CTLs raised by other researchers also fail to recognize the HA-1^H/A*0201 peptide, VLHDDLLEA, presented on HLA-A*0206 and whether our HLA-A*0206-restricted CTL clones that lysed HLA-A*0201$^+$ and HA-1^H LCL are relatively uncommon. To this end, it would be necessary to randomly raise CTL clones by single cell-sorting T cells strongly stained with either HLA-A*0206/HA-1^H or HLA-A*0201/HA-1^H tetramer. Alternatively, use of CD8-binding site-mutated tetramer[37] may provide more stringent staining, especially for HLA-A*0201/HA-1^H tetramer. However, the observation that HLA-A*0201/HA-1^H tetramer crossreacted with A*0206-restricted 4B1-CTL, although mean fluorescence intensity was three-fold lower, is concordant with the lytic activity of the 4B1-CTL clone against 721.221/A*0201 carrying the HA-1^H allele. The explanation for the less stringent recognition of HLA-A*0206-restricted 4B1-CTL and stringent recognition of HLA-A*0201-restricted EH6-CTL requires crystallographic analysis. The shared TCRBV7-9 usage between the two CTL clones and the shared Leu–Val amino-acid pair in the *N*-region between 4B1-CTL and the CTL clones specific for HLA-A*0201-restricted HA-1^H mHA[27,28] are probably more than a mere coincidence, suggesting a strong selection pressure of TCRBV and *N*-region as potential direct interaction sites with the HA-1^H/A*0201 peptide, VLHDDLLEA.

In summary, our data demonstrate that the HA-1^H/A*0201 peptide, VLHDDLLEA, is also presented by HLA-A*0206 and is fully immunogenic *in vivo*. This finding will expand our understanding of the patient population expressing HLA-A*0206 who may benefit from HA-1^H disparate SCT.

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Full-length EBNA1 mRNA-transduced dendritic cells stimulate cytotoxic T lymphocytes recognizing a novel HLA-Cw*0303- and -Cw*0304-restricted epitope on EBNA1-expressing cells

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Epstein–Barr virus (EBV)-encoded nuclear antigen 1 (EBNA1) is an attractive target for immunotherapy against EBV-associated malignancies because it is expressed in all EBV-positive cells. Although CD8$^+$ cytotoxic T-lymphocyte (CTL) epitope presentation is largely prevented by its glycine–alanine-repeat domain (GAr), the use of mRNA-transduced dendritic cells (DCs) would offer the advantage of priming EBNA1-specific CTLs. After stimulation with GAr-containing EBNA1-transduced monocyte-derived DCs, two EBNA1-specific CTL clones, B5 and C6, were isolated successfully from a healthy donor. These CTLs recognize peptides in the context of HLA-B*3501 and HLA-Cw*0303, respectively. A novel epitope, FVYGGSKTSL, was then identified, presented by both HLA-Cw*0303 and -Cw*0304, which are expressed by >35 % of Japanese, >20 % of Northern Han Chinese and >25 % of Caucasians. The mixed lymphocyte–peptide culture method revealed that FVYGGSKTSL-specific CTL-precursor frequencies in HLA-Cw*0303- or -Cw*0304-positive donors were between 1×10^{-5} and 1×10^{-4} CD8$^+$ T cells. Moreover, both CTL clones inhibited growth of HLA-matched EBV-transformed B lymphocytes *in vitro*, and B5 CTLs produced a gamma interferon response to EBNA1-expressing gastric carcinoma cells in the context of HLA-Cw*0303. These data demonstrate that EBNA1 mRNA-transduced DCs may be useful tools for inducing EBNA1-specific CTLs that might be of clinical interest for CTL therapy of EBV-associated malignancies.

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INTRODUCTION

Epstein–Barr virus (EBV), a human gammaherpesvirus that establishes lifelong latency in memory B cells (Babcock *et al.*, 2000), is associated with several different lymphoid and epithelial malignancies, including Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin's disease (HD) and post-transplant lymphoproliferative disorder (PTLD). All EBV-positive malignant cells exhibit one of three latency types, distinguished from each other by the pattern of expressed EBV antigens. In latency type I, only EBV-encoded nuclear antigen 1 (EBNA1) is expressed, as in BL; latent membrane protein 1 (LMP1) and LMP2, as well as EBNA1, are expressed in latency type II, as in HD and NPC. In latency type III, highly immunogenic EBNA3 genes, EBNA3A, EBNA3B and EBNA3C, are expressed together with other EBV latent antigens, as in PTLD (Rickinson & Kieff, 2001).

EBNA1 is required for the maintenance and replication of the viral episome in EBV-transformed cells (Kieff & Rickinson, 2001). Because it is expressed in all EBV-associated tumours, EBNA1 is an attractive target for immunotherapy. However, CD8$^+$ cytotoxic T-lymphocyte (CTL) responses are directed preferentially toward EBNA3s among latent-cycle proteins, and EBNA1 has been believed to be immunologically invisible because of studies indicating that there has been escape from recognition by CTLs (Callan *et al.*, 1998; Khanna *et al.*, 1992; Murray *et al.*, 1992; Steven *et al.*, 1996). A glycine–alanine-repeat domain (GAr) within EBNA1 was found to prevent antigen processing for CTL recognition (Levitskaya *et al.*, 1995). Presence of this GAr was shown to prevent processing by the proteasome, the main catalytic machinery for generation of major histocompatibility complex (MHC) class I epitopes (Blake *et al.*, 1997; Levitskaya *et al.*, 1997). Moreover, the same domain was established to prevent EBNA1 mRNA translation (Yin *et al.*, 2003).

To explore the possibility of targeting EBNA1, EBV-specific CD4$^+$ T-cell responses have been examined and

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EBNA1-specific CD4$^+$ T-cell responses have been shown to be mainly T helper type 1 in nature (Bickham *et al.*, 2001), with direct recognition of EBV-transformed cells (Khanna *et al.*, 1997; Munz *et al.*, 2000). Several MHC class II-restricted EBNA1 epitopes have been identified (Khanna *et al.*, 1995; Kruger *et al.*, 2003; Leen *et al.*, 2001; Paludan *et al.*, 2002; Voo *et al.*, 2002), implying that EBNA1-specific CD4$^+$ T cells may play a role in controlling tumour growth *in vivo*. Surprisingly, recent studies demonstrated that EBNA1-specific CD8$^+$ CTLs moderately lyse EBV-transformed lymphoblastoid cell lines (LCLs) and suppress LCL outgrowth *in vitro* (Lee *et al.*, 2004; Tellam *et al.*, 2004; Voo *et al.*, 2004). Defective ribosomal products of EBNA1 were shown to be the sources of EBNA1 CTL epitopes presented on the cell surfaces.

In this study, we induced CD8$^+$ EBNA1-specific CTL clones from peripheral blood mononuclear cells (PBMCs) by using GAr-containing EBNA1 mRNA-transfected dendritic cells (DCs) as antigen-presenting cells (APCs). Antigen-transduced DCs have been demonstrated to prime antigen-specific CTLs efficiently *in vitro* (Grunebach *et al.*, 2003; Heiser *et al.*, 2000; Muller *et al.*, 2004; Nair *et al.*, 1998) and *in vivo* (Heiser *et al.*, 2002; Nair *et al.*, 2000; Su *et al.*, 2003; Zeis *et al.*, 2003). Furthermore, we identified a novel human leukocyte antigen (HLA)-C-restricted CTL epitope and confirmed EBNA1 recognition by CTL clones using tetrameric MHC–peptide complexes (tetramer). Finally, we estimated frequencies of EBNA1-specific CTL precursors (CTLp) in PBMCs of healthy EBV-seropositive donors and assessed effects of the CTL clones on EBNA1-expressing cells *in vitro*.

METHODS

Donors and cell lines. The study design and purpose, approved by the institutional review board of Aichi Cancer Center, Nagoya, Japan, were explained fully to all blood donors and informed consent was obtained. CD40-activated B (CD40-B) cells were generated from PBMCs of blood donors as described previously (Kondo *et al.*, 2002; Schultze *et al.*, 1997). Briefly, PBMCs were cultured with irradiated human CD40L-transfected NIH3T3 cells (referred as to t-CD40L; kindly provided by Dr Gordon Freeman, Dana-Farber Cancer Institute, Boston, MA, USA), recombinant interleukin-4 (IL-4) (Genzyme) and cyclosporine A (Sandoz) in the culture medium. Expanding CD40-B cells were stimulated twice a week. LCLs were prepared by transforming PBMCs with B95-8 cell-culture supernatant as described previously (Kuzushima *et al.*, 1999) and cultured in RPMI 1640 medium (Sigma) supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine, 50 U penicillin ml^{-1}, 50 μg streptomycin ml^{-1} and 50 μg kanamycin ml^{-1} (referred to as complete culture medium). HLA-Cw*0303-expressing gastric carcinoma cells [MKN45 (referred to as MKN45-Cw0303) cells] were generated from MKN45 cells by retroviral transduction using Phoenix GALV cells (kind gifts from Dr Kiem, Fred Hutchinson Cancer Research Center, Seattle, WA, USA, and Dr Nolan, Stanford University, Stanford, CA, USA). Retroviral transduction of HLA genes was performed as described previously (Akatsuka *et al.*, 2002; Kondo *et al.*, 2002). EBNA1 (without GAr)-expressing MKN45-Cw0303 cells (referred to as MKN45-Cw0303-ΔGA-EBNA1) were then generated from MKN45-Cw0303 cells by lentivirus transduction (Bai *et al.*, 2003). For this, an EBNA1-coding sequence without GAr (EBNA1 codons 92–323) was inserted into the lentivirus self-inactivating vector (CSIICMV-MCS; kindly provided by Dr Hiroyuki Miyoshi, RIKEN BioResource Center, Tsukuba, Japan) (Bai *et al.*, 2003). MKN45-Cw0303 cells expressing full-length EBNA1 (referred to as MKN45-Cw0303-full-EBNA1) were also generated from MKN45-Cw0303 cells by mRNA transduction. Transduction of *in vitro*-transcribed full-length EBNA1 mRNA was performed as described above. MKN45-Cw0303 and MKN45-Cw0303-ΔGA-EBNA1 cells were cultured in complete culture medium with 1.0 μg puromycin ml^{-1}.

Preparation of DCs. DCs were prepared as described previously (Dauer *et al.*, 2003; Romani *et al.*, 1994; Sallusto & Lanzavecchia, 1994). Briefly, CD8$^+$ T cells were isolated from PBMCs by using CD8 MicroBeads (Miltenyi Biotec) and stored at −135 °C. The CD8-depleted PBMCs were suspended in 4 ml RPMI 1640 medium supplemented with 5 % human serum (MP Biomedicals), 2 mM L-glutamine, 50 U penicillin ml^{-1}, 50 μg streptomycin ml^{-1} and 50 μg kanamycin ml^{-1} (referred to as DC medium) and incubated for 2 h in six-well plates at 37 °C. Non-adherent cells were removed by gentle pipetting and adherent cells were cultured in DC medium in the presence of 50 ng granulocyte–macrophage colony-stimulating factor ml^{-1} (GM-CSF; Osteogenetics) and 10 ng IL-4 ml^{-1} (Osteogenetics). On days 2 and 4, half of the medium was replaced with fresh DC medium containing GM-CSF and IL-4. On day 6, DCs were collected and electroporated for mRNA transduction.

Production and transduction of *in vitro*-transcribed mRNA. To generate *in vitro*-transcribed EBNA1 mRNA, a pcDNA/EBNA1 vector was constructed. The coding sequence for EBNA1 was obtained by extraction of total RNA from B95-8-transformed LCLs using an RNeasy kit (Qiagen) and, after reverse transcription, EBNA1 cDNA was amplified by PCR with specific primers as follows: EBNA1 forward primer, 5'-AAGCTTGCCACCATGTCTGACGAGGGGCCAGGTACAG; reverse primer, 5'-GAATTCTCACTCCTGCCCTTCCTCACCCTC. The full-length EBNA1 fragment was then ligated into pcDNA3.1(+) (Invitrogen) using its *Hin*dIII and *Eco*RI sites (pcDNA/EBNA1). Clones were sequenced to verify their identity. Resulting plasmid DNA was linearized and transcribed *in vitro* by using an mMESSAGE and mMACHINE kit (Ambion) according to the manufacturer's instructions. A 3'-poly(A) tail was added by using poly(A) polymerase (Ambion) followed by purification with an RNeasy kit. The resulting mRNA was visualized by using the Reliant RNA gel system (Cambrex). DCs and CD40-B cells were transfected with mRNA by electroporation. First, they were washed twice with serum-free RPMI 1640 medium and suspended at a final concentration of 2.5×10^7 cells ml^{-1}. After mixing with 20 μg mRNA in 40 μl RPMI 1640 medium, they were then electroporated in a 2 mm cuvette by using an Electro Square Porator ECM 830 (Harvard Apparatus), under conditions of 450 V and 500 μS for DCs and 350 V and 350 μS for CD40-B cells. DCs were subsequently cultured in DC medium supplemented with GM-CSF and IL-4 for 3 h, followed with tumour necrosis factor alpha (PeproTech), IL-1β (PeproTech) and prostaglandin E_2 (Cayman Chemical) for maturation. CD40-B cells were seeded immediately onto irradiated t-CD40L cells and, after 36–48 h, these cells were used as APCs.

EBNA1 staining. EBNA1 mRNA-transfected CD40-B cells were collected and fixed with 4 % paraformaldehyde in PBS for 10 min at room temperature. After washing with PBS, cells were permeabilized with PBS containing 0.5 % Tween 20 and reacted with anti-EBNA1 rabbit polyclonal antibodies (kindly provided by Dr Tatsuya Tsurumi, Aichi Cancer Center Research Institute, Nagoya, Japan) for 30 min at 4 °C. After washing with PBS, cells were stained with fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG (Beckman Coulter) for 30 min at 4 °C. The stained cells were analysed by FACSCalibur (BD Biosciences) using CELLQUEST software (BD Biosciences).

CTL induction. The stored CD8$^+$ T cells were thawed, washed and co-cultured with irradiated (33 Gy) autologous EBNA1 mRNA-transfected DCs in 200 µl RPMI 1640 medium supplemented with 10% human serum, 2 mM L-glutamine, 50 U penicillin ml^{-1}, 50 µg streptomycin ml^{-1} and 50 µg kanamycin ml^{-1} (referred to as CTL medium) in the presence of 5 ng IL-7 ml^{-1} (R&D Systems) and 5 ng IL-12 ml^{-1} (R&D systems) at 5% CO_2 in a humidified incubator. On days 8, 16 and 23, T cells were restimulated with EBNA1 mRNA-transfected and irradiated DCs. One day after each restimulation, IL-2 (Shionogi) was added to a final concentration of 20 U ml^{-1}. To establish T-cell clones, limiting dilution of polyclonal CTLs was performed (Kuzushima *et al.*, 2001). In brief, polyclonal CD8$^+$ T cells were seeded at 1 cell per well in round-bottomed 96-well plates containing CTL medium with a monoclonal antibody (mAb) specific to CD3 (30 ng ml^{-1}; Ortho Biotech), 1×10^5 irradiated (33 Gy) PBMCs and 2×10^4 irradiated (55 Gy) LCLs. The next day, IL-2 was added to each well (50 U ml^{-1}). After 2 weeks culture, growing wells were split into two replicates and used as effectors in ELISPOT assays against either autologous EBNA1 mRNA-transfected CD40-B cells or autologous LCLs. Positive wells were transferred into flasks and expanded with anti-CD3 mAb, irradiated feeder cells and IL-2.

ELISPOT assays. ELISPOT assays were performed as described previously (Kuzushima *et al.*, 2003). Briefly, CD8$^+$ T cells were co-cultured with various stimulators in wells of Multiscreen-HA plates (Millipore) coated with anti-human gamma interferon (IFN-γ) mAb (Pierce Biotechnology). As stimulators, (i) autologous EBNA1 mRNA-transfected CD40-B cells or non-transfected CD40-B cells and (ii) autologous or allogeneic LCLs (1×10^5 cells per well) were seeded into each well. For peptide-titration assays and overlapping-peptide assay, serial concentrations of synthetic peptides were pulsed to autologous CD40-B cells for 1 h at room temperature. After probing with anti-human IFN-γ rabbit polyclonal antibodies (Pierce Biotechnology) followed by exposure to horseradish peroxidase-labelled anti-rabbit IgG antibody (Genzyme) and substrate, the plates were washed and dried. IFN-γ spots were enumerated under a dissecting microscope. The numbers of spots were confirmed by three investigators.

Peptides. Peptides were purchased from Bio-Synthesis Inc. The whole EBNA1 protein, excluding the GAr, deduced from the prototype B95-8 DNA sequence (GenBank accession no. V01555) was covered by 20 aa long synthetic peptides overlapping by 13 aa (total of 56 peptides). In addition, potential epitope peptides VYGGSKTSL (509–517), FVYGGSKTSL (508–517) and VFVYGGSKTSL (507–517) were synthesized, as predicted by the program SYFPEITHI (http://www.syfpeithi.de/) (Rammensee *et al.*, 1999). The known EBNA1 epitope peptide HPVGEADYFEY (Blake *et al.*, 1997) was also synthesized.

Tetramer production and staining. HLA-Cw*0303 and -Cw*0304 cDNA clones were used as templates to amplify sequences encoding the extracellular domains of HLA-Cw*0303 and -Cw*0304 heavy chains with primers C03F (5′-AACCATGGGCAGCCATTCTATGCGCTATTTTTACACCGCTGTGTCCCGGCC-3′) and C03R (5′-AAGGATCCTGGCTCCCATCTCAGGGTGAGG-3′). C03F contains several base changes designed to optimize protein expression in *Escherichia coli* BL21 (DE3) pLysS. The PCR product was digested with *Nco*I and *Bam*HI and cloned into a vector containing a BirA biotinylation site in frame with the 3′ end of the HLA sequence. Recombinant HLA-B*3501 protein was produced by using pGMT7-B35 (a kind gift of Dr McMichael, Weatherall Institute of Molecular Medicine, Oxford, UK). Recombinant MHC molecules were folded *in vitro* with β2-microglobulin and epitope peptides FVYGGSKTSL or VFVYGGSKTSL. Soluble complexes, purified by gel filtration, were biotinylated by using the BirA enzyme (Avidity LCC). Phycoerythrin (PE)-labelled tetramers were produced by mixing these biotinylated complexes with PE-labelled streptavidin (Molecular Probes). Tetramer staining was performed as follows. CTL clones (2×10^5) were incubated with tetramers at a concentration of 0.1 mg ml^{-1} and FITC–anti-CD8 mAb (Caltag) at 4 °C for 15 min. After washing twice, stained cells were fixed in 0.5% paraformaldehyde and analysed by flow cytometry.

Mixed lymphocyte–peptide culture. Mixed lymphocyte–peptide culture was performed as described by Coulie *et al.* (2001) with modifications. PBMCs were isolated by density-gradient centrifugation and suspended in CTL medium. An aliquot was used for enumeration of CD8$^+$ cells. The cells were then distributed at 2×10^5, 1×10^5 or 5×10^4 cells per well in 96-well round-bottomed plates in 200 µl medium in the presence of epitope peptide (1 µg ml^{-1}) and IL-2 (20 U ml^{-1}). Thirty-two cultures of each PBMC density were set up. On day 7, half of the medium was replaced by fresh medium containing the peptide and IL-2. Tetramer staining was performed on day 14. We counted the tetramer-positive microcultures and estimated the anti-EBNA1 CTLp frequency by limiting-dilution evaluation using the L-Calc program (Stem Cell Technologies).

Outgrowth-inhibition assay. Outgrowth-inhibition assays were performed as described by Lee *et al.* (2004) with modifications. Briefly, target LCLs were seeded into round-bottomed 96-well plates at 2×10^4 cells per well in triplicate. EBNA1-specific CTL clones (1×10^4 cells per well) or CTL medium alone (as a control) were added to target-cell cultures. All cultures were maintained weekly by changing half of the medium. After 4 weeks culture, the number of LCLs in the culture at each setting was counted. Cell growth (percentage of control) was calculated as [no. LCLs from the culture with CTLs (clone B5 or clone C6)]/[no. LCLs from the culture without CTLs (medium)] × 100. B-cell identity of grown cells was confirmed by staining with PE–cyanin 5-labelled anti-CD19 and PE-labelled anti-CD8 mAbs (Beckman Coulter) and analysis by flow cytometry.

Detection of IFN-γ-producing CD8$^+$ T cells by flow cytometry. EBNA1-specific CTL recognition was measured as described previously (Kuzushima *et al.*, 2001) with slight modifications. Briefly, 5×10^5 B5 CTLs were incubated with 2×10^6 MKN45-Cw0303, MKN45-Cw0303-ΔGA-EBNA1 or MKN45-Cw0303-full-EBNA1 cells in 1 ml complete culture medium in a culture tube at 37 °C for 6 h, in the presence of brefeldin A (Sigma) during the last 5 h. After the incubation, the cell suspensions were fixed with 4% paraformaldehyde in PBS. After washing with PBS, cells were permeabilized with IC Perm (BioSource International) and stained with PE–cyanin-5.1-labelled anti-CD8 (Beckman Coulter), PE-labelled anti-CD69 (Immunotech Coulter) and FITC-labelled anti-human IFN-γ (BD Biosciences) mAbs. Stained cells were analysed by flow cytometry. Live gating of the CD8$^+$ subset was performed and 50 000 events were acquired for each analysis.

RESULTS

EBNA1 expression in full-length EBNA1 mRNA-transfected CD40-B cells

To generate EBNA1-expressing APCs, we first produced full-length EBNA1 mRNA with a poly(A) tail from the pcDNA/EBNA1 plasmid by using an *in vitro* transcription system. The yield of capped mRNA was low, probably due to the presence of GAr (comprising GC-rich sequences), and this was not overcome fully by the change of reaction temperature or by adding single-stranded DNA-binding protein to the reaction mixture (data not shown). However,