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

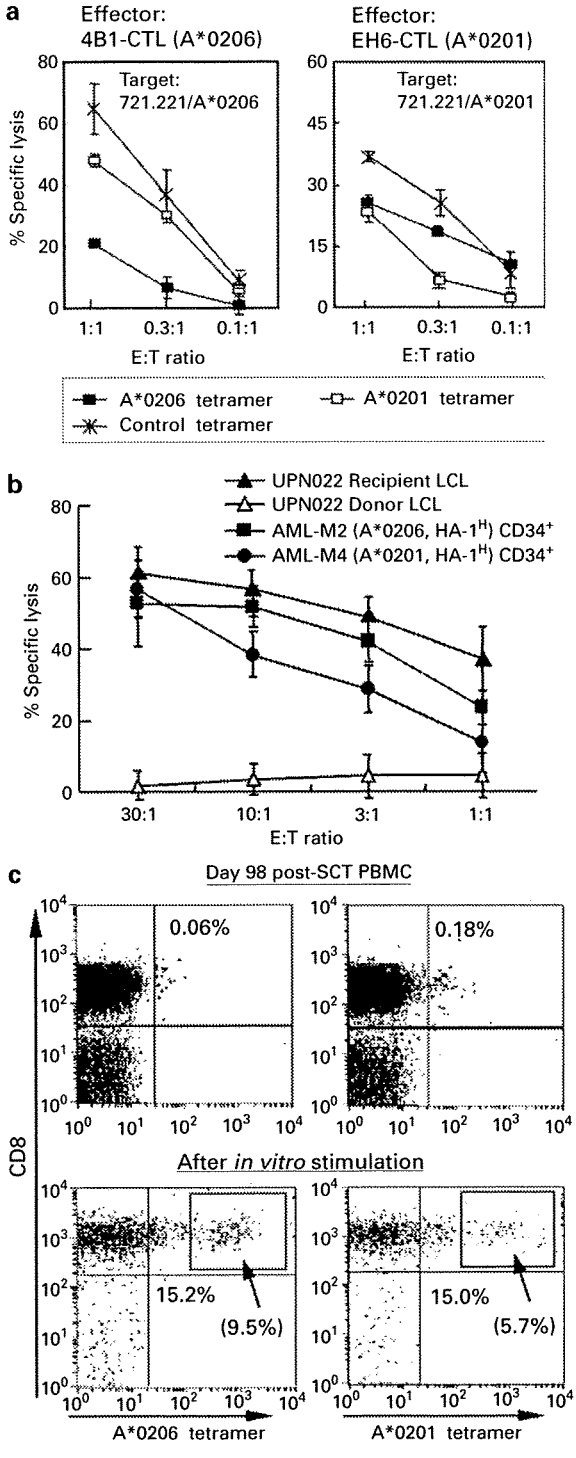

Figure 3 Allele-specific recognition and *in vivo* relevance of HA-1^H-specific T cells. (**a**) Inhibition of cytolytic activity of HA-1^H-specific CTL clones by two different HLA-A2 subtype tetramers. HA-1^H-positive 721.221 LCL transduced with either HLA-A*0206 or HLA-A*0201 cDNA retrovirally were 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[9]) 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

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,

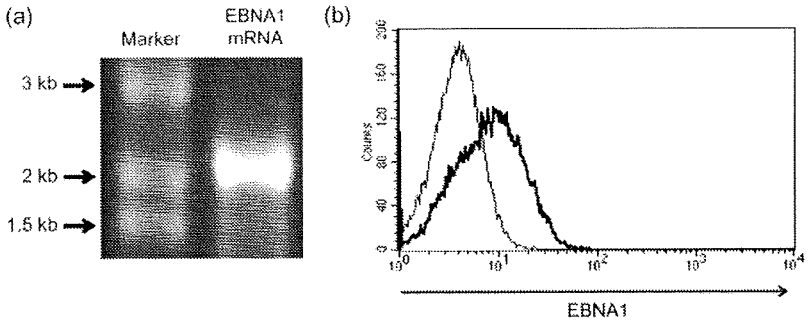

Fig. 1. EBNA1 expression in cells transfected with *in vitro*-transcribed full-length mRNA. (a) *In vitro*-transcribed full-length EBNA1 mRNA produced successfully from an EBNA1-cDNA plasmid. The quality of the EBNA1 mRNA was assessed by gel electrophoresis followed by staining with ethidium bromide. (b) EBNA1 protein expression in EBNA1 mRNA-transfected CD40-B cells. CD40-B cells were transfected with full-length EBNA1 mRNA by electroporation and intracellular staining of EBNA1 protein was performed and analysed by flow cytometry.

the amount of mRNA was sufficient and seen as a single band on the gel (Fig. 1a). Transfection was then performed by electroporation and EBNA1 expression was detected in most CD40-B cells, although the mean fluorescent intensity appeared to be low (Fig. 1b).

Induction of EBNA1-specific CTL lines and clones by using EBNA1 mRNA-transfected APCs

To explore the capacity for T-cell stimulation, autologous $CD8^+$ T lymphocytes were co-cultured with CD40-B cells transfected with full-length EBNA1 mRNA, and IFN-γ-producing cells were enumerated by ELISPOT assay. As shown in Fig. 2(a), $CD8^+$ T lymphocytes of donor Y01 produced IFN-γ spots without *in vitro* stimulation. As $CD8^+$ T lymphocytes of other donors did not produce significant spots on *ex vivo* analysis, the T cells were stimulated weekly with irradiated CD40-B cells that had been transfected with the full-length EBNA1 mRNA. After two rounds of stimulation, $CD8^+$ T lymphocytes of another donor (K04) produced IFN-γ spots upon contact with autologous CD40-B cells transduced with EBNA1 mRNA in the ELISPOT assay (Fig. 2a). These data indicate that the full-length EBNA1 mRNA was translated and that $CD8^+$ T-lymphocyte epitopes are processed and presented on APCs.

Next, EBNA1-specific CTL clones were established by using donor Y01 monocyte-derived DCs transfected with full-length EBNA1 mRNA. The transduced DCs were distributed in 96-well plates and used to stimulate autologous $CD8^+$ T lymphocytes in the presence of IL-7 and IL-12. After three rounds of stimulation, aliquots of each microculture were tested for their ability to secrete IFN-γ specifically upon contact with autologous CD40-B cells transduced with EBNA1 mRNA in the ELISPOT assay. Thirty-two microcultures out of 36 wells were scored as EBNA1-specific (data not shown), and lymphocytes from two well-growing microcultures were cloned by limiting dilution. CTL clones B5 and C6 were thus established, recognizing EBNA1 mRNA-transfected autologous CD40-B cells and autologous LCLs, but not mock-transfected autologous CD40-B cells or HLA-mismatched allogeneic LCLs (Fig. 2).

Identification of the presenting HLA molecules

The donor was typed genetically as HLA-A*2402, -A*3101, -B*1507, -B*3501 and -Cw*0303. To identify the antigen-presenting HLA molecule, a panel of partially HLA-matched LCLs was used to stimulate clones B5 or C6 to produce IFN-γ. In addition to autologous LCLs, allogeneic LCLs expressing HLA-B*3501 were recognized by CTL clone C6 (Fig. 3a), and one LCL with HLA-Cw*0303 and one with -Cw*0304 were recognized by clone B5 (Fig. 3b), demonstrating that HLA-B*3501 is the putative restriction element for clone C6 recognition, whilst both HLA-Cw*0303 and -Cw*0304 act for clone B5.

Identification of EBNA1 antigenic peptides

To identify the epitope region, clones B5 and C6 were stimulated with autologous CD40-B cells incubated with sets of peptides of 20 aa length, overlapping by 13 aa and covering the complete EBNA1 protein sequence without GAr. Because the primary structure of GAr is not likely to be contained in MHC class I epitopes, we did not include this part as an epitope source. Peptide 24 was recognized by clone C6 (Fig. 4). Regarding the HLA-B*3501-restricted epitope, HPVGEADYFEY has been reported previously (Blake *et al.*, 1997). As this epitope sequence is located in the centre of peptide 24 (aa 402–421) (Fig. 5a), we tested whether clone C6 might recognize HPVGEADYFEY-pulsed autologous CD40-B cells and confirmed a response to HPVGEADYFEY-pulsed stimulation (data not shown).

In the case of clone B5, two overlapping peptides, 38 (aa 500–519) and 39 (aa 507–526), were recognized (Fig. 4), sharing the 13 aa sequence VFVYGGSKTSLYN [underlined in Fig. 5(b)]. To predict the optimal epitope binding to HLA-Cw*0303, the program SYFPEITHI was applied. Because the anchor leucine at the C terminus and the auxiliary anchors valine and tyrosine at the third position of epitopes were predicted by the program, we examined the 11mer (VFVYGGSKTSL) and the 10mer (FVYGGSKTSL) (Fig. 5b). Half-maximal recognition of the peptide-pulsed target cells was obtained with 5–10 nM of the 10mer peptide and 1–5 nM of the 11mer (Fig. 5c), suggesting that these two peptides may be the optimal epitopes. The 9mer,

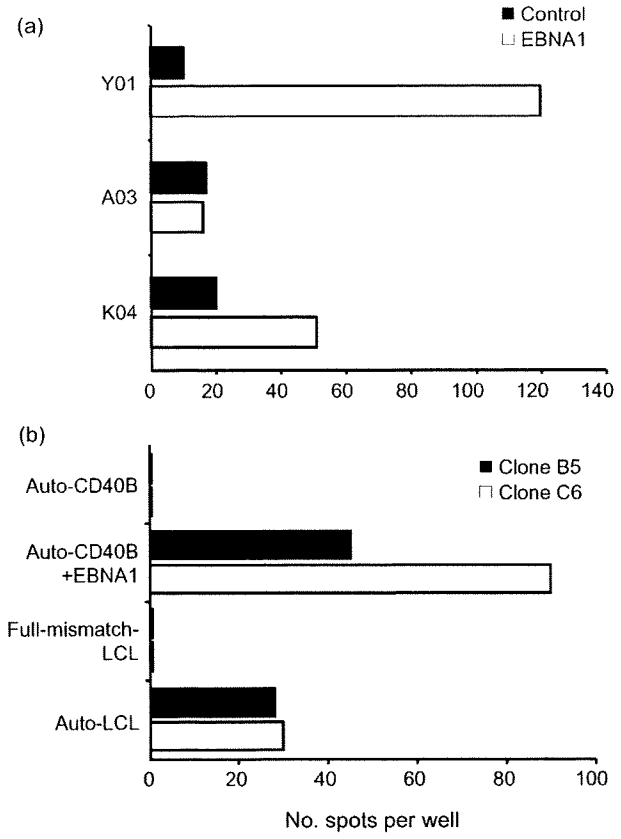

Fig. 2. Presence of anti-EBNA1 T cells in cultures primed with EBNA1 mRNA-transfected APCs. (a) Numbers of IFN-γ-producing cells from $CD8^+$ T lymphocytes without *in vitro* stimulation (Y01) or after two rounds of stimulation (A03 and K04) in ELISPOT assays. Aliquots of 100 000 cells were cultured in single wells with autologous CD40-B cells transduced with full-length EBNA1 mRNA for 20 h. Data from one representative experiment of two are shown. (b) $CD8^+$ T cells from one selected donor were stimulated with autologous DCs transfected with *in vitro*-transcribed EBNA1 mRNA. After three stimulations at weekly intervals, polyclonal $CD8^+$ T cells from two positive cultures were cloned by limiting dilution. Established clones B5 and C6 were then tested for recognition of EBNA1 mRNA-transfected autologous CD40-B cells and autologous LCLs by ELISPOT assay. Five thousand CTLs were seeded in each well. Data from one representative experiment of two are shown.

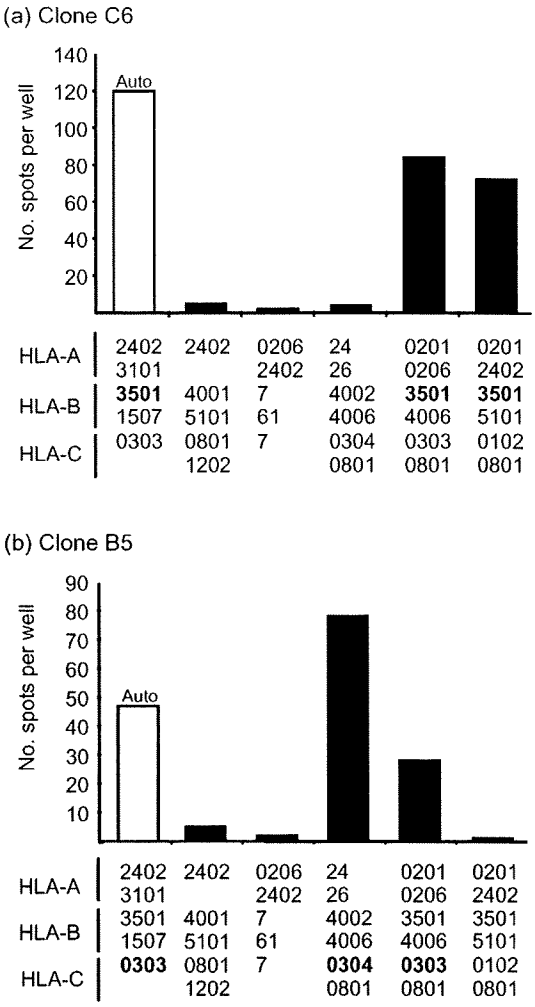

Fig. 3. Identification of presenting HLA molecules for EBNA1-specific CTL clones. (a) HLA-B*3501 molecules function as restriction elements for CTL clone C6. (b) HLA-Cw*0303 and -Cw*0304 molecules function as restriction elements for CTL clone B5. Autologous and allogeneic LCLs were used to stimulate clones B5 or C6 to produce IFN-γ spots. Single allele-matched LCLs were included and cultured with the CTLs (5×10^3) for 20 h. Each bar represents the mean number of spots in duplicate wells.

VYGGSKTSL, was not recognized, even at much higher concentrations.

Tetramers bind to the EBNA1-specific clone B5

As the peptide-dilution assay provided two optimal epitope candidates, we made fluorescently labelled tetramers incorporating the 10mer peptide FVYGGSKTSL or the 11mer VFVYGGSKTSL for further experiments. As shown in Fig. 5(d), these tetramers bound specifically to CTL clone B5. However, the tetramer incorporating the 10mer demonstrated higher avidity for the B5 clone than that incorporating the 11mer, suggesting the 10mer peptide FVYGGSKTSL to be the minimal and optimal epitope for the CTL. Moreover, clone B5 bound strongly to the HLA-Cw*0304 tetramer incorporating the 10mer, showing concordance with the results shown in Fig. 3(b). In addition, we characterized *in vitro*-expanded T cells from two donors by co-staining with MHC–peptide tetramer and CD62L. A proportion of 9.8% of HLA-Cw*0303-FVYGGSKTSL tetramer-positive lymphocytes were CD62-positive in one donor, and 6.5% in the other.

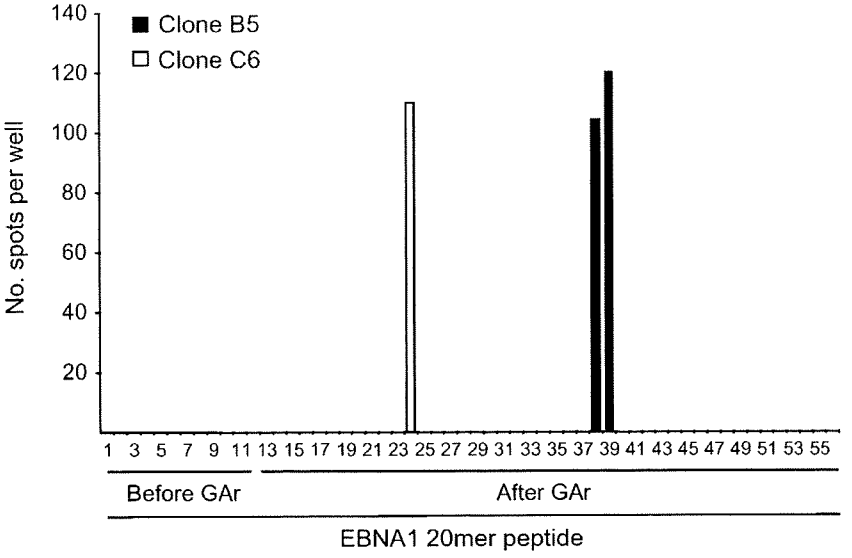

Fig. 4. Identification of overlapping peptides recognized by EBNA1-specific CTL clones. Autologous CD40-B cells (1×10^5 per well) were pulsed with 10 μg ml^{-1} of each of a set of 20mer overlapping peptides encompassing the EBNA1 protein, excluding GAr, and co-cultured with 5×10^2 CTL clone B5 or C6. Production of IFN-γ spots was then measured by ELISPOT assay.

Frequencies of EBNA1 epitope-specific $CD8^+$ T cells in PBMCs of healthy EBV-seropositive donors

We estimated frequencies of EBNA1 epitope-specific $CD8^+$ T cells in healthy EBV-seropositive donors by the mixed lymphocyte–peptide culture method. PBMCs from two donors with HLA-B*3501, one donor with HLA-Cw*0303 and three donors with HLA-Cw*0304 were tested. We could compare the anti-EBNA1 CTLp frequency in two donors with both HLA-B*3501 and HLA-Cw*0303 (HLA-Cw*0304). Representative tetramer staining of negative and positive microcultures from mixed lymphocyte–peptide culture wells is shown in Fig. 6. EBNA1-specific CTLp frequencies of HLA-B*3501-positive donors were 7.2×10^{-6} and 1.8×10^{-4}, and for HLA-Cw*0303 and -Cw*0304 were from 2.5×10^{-5} to $>2.1 \times 10^{-4}$. We did not find any hierarchy between the two EBNA1 epitopes in either of the donors with both HLA-B*3501 and HLA-Cw*0303 (Cw*0304) molecules (Table 1).

Effect of EBNA1-specific CTLs on EBV-infected B-cell growth

Clones B5 and C6 did not lyse autologous LCLs in the chromium-release assay (data not shown). Here, a final set of experiments was performed to ask whether these EBNA1-specific CTLs could affect the long-term growth and survival of EBNA1-expressing LCLs. Autologous and allogeneic LCLs with or without the restricting HLA molecules were seeded in 96-well plates in the presence or absence of responding CTLs. Cultures were then assayed for LCL outgrowth after 4 weeks. At the end, LCL outgrowth was assessed by microscopic inspection and confirmed by CD19 expression by flow cytometry. As shown in Fig. 7(a), both CTL clones clearly inhibited outgrowth of not only autologous LCLs, but also allogeneic LCLs with restricting HLA, suggesting that these CTL clones have the ability to inhibit outgrowth of EBV-positive cells with latency type III.

Recognition by EBNA1-specific CTL clone B5 of HLA-Cw*0303-transduced gastric carcinoma cells expressing EBNA1

Because naturally EBV-positive gastric cell lines are difficult to establish, we generated EBNA1-expressing gastric carcinoma cells designated MKN45-Cw0303-ΔGA-EBNA1 and MKN45-Cw0303-full-EBNA1 to verify that EBV-positive gastric cancer cell lines present the FVYGGSKTSL epitope. To investigate recognition by clone B5, we applied flow cytometry to detect EBNA1-specific CTLs producing IFN-γ. As shown in Fig. 7(b), 2.55% of B5 clone cells produced IFN-γ when co-cultured with MKN45-Cw0303-ΔGA-EBNA1 cells, demonstrating specific recognition of the FVYGGSKTSL epitope on cells transduced with GAr-deleted EBNA1. Otherwise, B5 clone cells did not produce IFN-γ when co-cultured with MKN45-Cw0303-full-EBNA1 cells.

DISCUSSION

Recently, EBNA1-specific CTLs were shown to recognize and lyse HLA-matched LCLs (Lee *et al.*, 2004; Tellam *et al.*, 2004; Voo *et al.*, 2004). In previous studies, such CTLs were generated from PBMCs of healthy donors after *in vitro* stimulation with autologous LCLs or EBNA1 peptide-pulsed PBMCs. As EBNA1 is expressed in all EBV-associated tumours and might be an important target for immunotherapy, we have explored the efficient induction of EBNA1-specific CTLs. Of the different methods used to obtain HLA class I-restricted epitopes on APCs for stimulation, we chose to employ full-length EBNA1 mRNA-transfected DCs. This strategy offers the following advantages: (i) the method is not dependent on knowledge of the HLA haplotype of each donor; (ii) there is complete deletion of antigenicity of the vector-backbone sequence; and (iii) the yield of mRNA *in vitro* transcription is highly reproducible and transduction is very efficient (Van

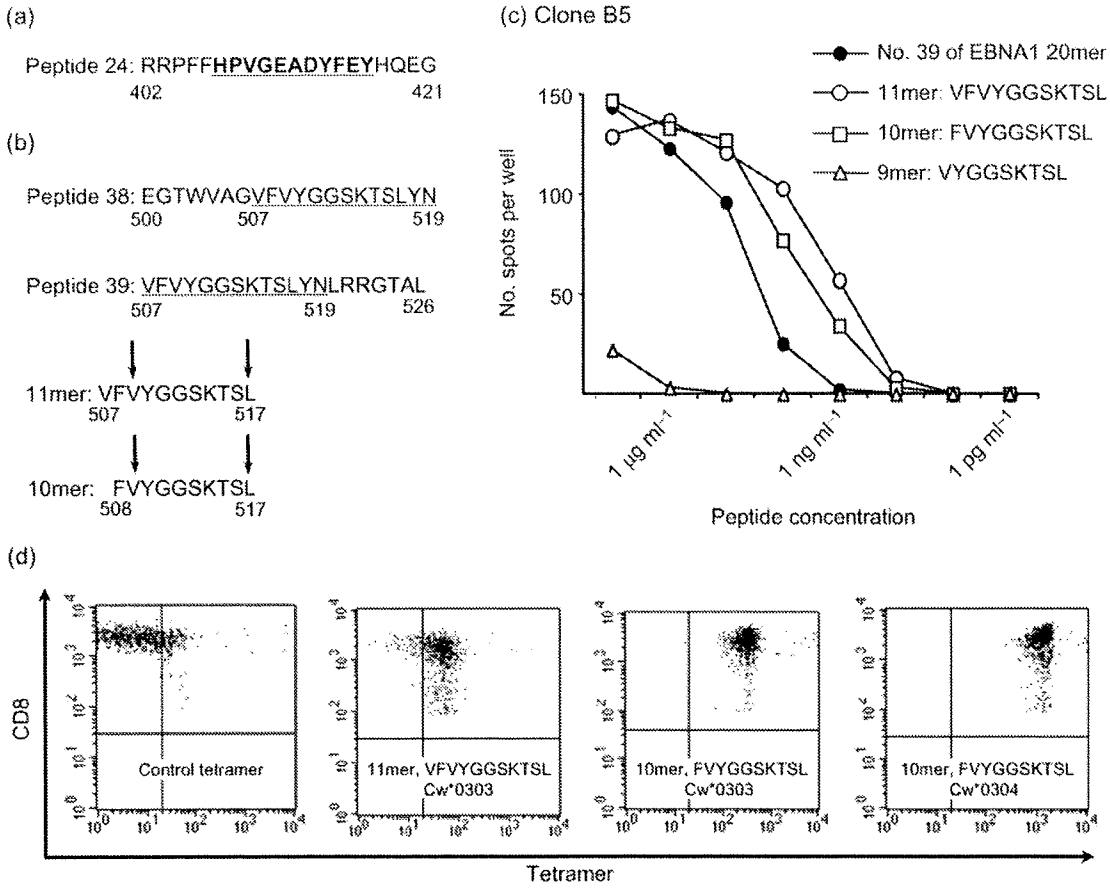

Fig. 5. Identification of optimal EBNA1 antigenic peptides recognized by EBNA1-specific CTL clones. (a) Amino acid sequence of the overlapping peptide recognized by the HLA-B*3501-restricted clone C6. The known epitope HPVGEADYFEY is indicated in bold and underlined. The numbers of the amino acid position in EBNA1 are shown. (b) Amino acid sequences of two consecutive overlapping peptides recognized by clone B5 and the potential optimal epitope sequences. The overlapping sequence between peptides 38 and 39 is underlined. Arrows indicate the primary and auxiliary anchors for HLA-Cw*0303 predicted by the program SYFPEITHI. The numbers of the amino acid positions in the EBNA1 protein are shown. (c) Titration of EBNA1-derived synthetic peptides. Autologous CD40-B cells were incubated for 1 h with 10-fold serial dilutions of synthetic peptides 507–526 (no. 39, 20mer), 507–517 (11mer), 508–17 (10mer) and 509–517 (9mer). CTL clone B5 (200 cells per well) was subsequently added and cultured for 20 h. Each symbol indicates the mean number of spots in duplicate wells. (d) The HLA-Cw*0303-restricted EBNA1-specific CTL clone B5 was stained with PE-conjugated HLA-Cw*0303–FVYGGSKTSL, HLA-Cw*0303–VFVYGGSKTSL or HLA-Cw*0304–FVYGGSKTSL tetrameric complexes and FITC-labelled anti-CD8 antibodies, and analysed by flow cytometry.

Tendeloo *et al.*, 2001). As an antigen, we used GAr-containing full-length EBNA1 instead of a GAr-deleted example to selectively activate CTL populations capable of reacting with epitopes that escape from the inhibitory mechanism governed by EBNA1 encoding GAr. Of note, even a low level of antigen delivery into DCs could induce antigen-specific CTL responses (Grunebach *et al.*, 2003), suggesting that this strategy has the potential to induce a CTL response even when a low density of EBNA1 epitopes is presented on DCs. In this study, we generated HLA-B- and -C-restricted EBNA1-specific CTLs successfully from a single donor, demonstrating that this method is a useful tool for generating EBNA1-specific CTLs, allowing investigation of the contribution of EBNA1 to cell-mediated immune responses in EBV-associated malignancies. Moreover, EBNA1 may have antigenicity when expressed on APCs, even if containing GAr *in vivo*. Finally, this induction method may be applicable for preparing EBNA1-specific CTLs for immunotherapy.

EBNA1 is generally immunologically invisible and only a small number of CTL epitopes have been identified (Blake *et al.*, 1997, 2000; Voo *et al.*, 2004). Of these, five epitopes are HLA-B-restricted and one is presented in the context of HLA-A. We detected two, one from HLA-B and another from HLA-C, from a single donor. To our knowledge, this is the first demonstration of an HLA-C-restricted EBNA1 epitope. To determine the minimal epitope, we compared

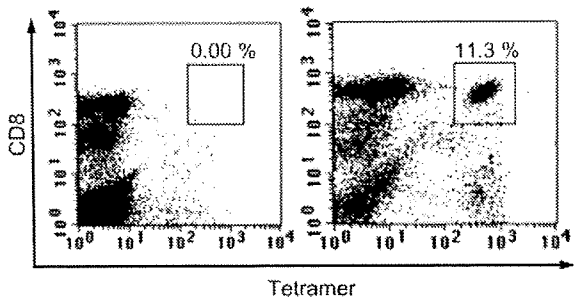

Fig. 6. Mixed lymphocyte–peptide culture analysis to estimate frequencies of C6 CTLp in $CD8^+$ lymphocytes of healthy EBV-seropositive donors. PBMCs from healthy EBV-seropositive donors with HLA-Cw*0303 were distributed at 2×10^5, 1×10^5 or 5×10^4 cells per well in 96-well round-bottomed plates in CTL medium with FVYGGSKTSL peptide (1 μg ml^{-1}) and IL-2 (20 U ml^{-1}). Half of the medium was replaced by fresh medium containing the relevant epitope peptide and IL-2 on day 7 and tetramer staining was performed on day 14. The plots show only data for $CD8^+$ lymphocytes, corresponding to 20–40 % of the cells in representative positive and negative cultures. The proportions of $CD8^+$ lymphocytes labelled specifically with the HLA-Cw*0303–FVYGGSKTSL tetramer are indicated.

the 11mer (VFVYGGSKTSL) and 10mer (FVYGGSKTSL) in a peptide-titration assay and found peptide concentration with half-maximal recognition of the target cells to be almost the same. However, clone C6 bound more strongly to tetramers incorporating the 10mer and we speculate that the N-terminal valine of the 11mer might be trimmed efficiently to yield 10mer in ELISPOT assay medium containing FCS. Moreover, the 10mer FVYGGSKTSL epitope was presented by HLA-Cw*0303 and -Cw*0304 molecules. As these two HLA-C alleles are possessed by > 35 % of Japanese, > 20 % of Northern Han Chinese (Hong *et al.*, 2005) and > 25 % of Caucasians, this new epitope should enable us to analyse cellular immunity to EBNA1 in a broad population. Indeed, we estimated $CD8^+$ T-cell frequencies specific to either FVYGGSKTSL or HPVGEADYFEY in PBMCs of healthy EBV-seropositive donors by the mixed lymphocyte–peptide culture method followed by tetramer staining and found that EBNA1-specific CTLp frequencies of HLA-B*3501- or HLA-Cw*0303 (and -Cw*0304)-positive donors were between 1×10^{-5} and 1×10^{-4}. These data provide useful information for understanding cellular immunity to EBNA1. For determination of frequencies of EBNA1 epitope-specific CTLs, the *ex vivo* ELISPOT assay (Blake *et al.*, 2000) is simple and readily applicable, because frequencies can be predicted at the level of 1×10^{-4} $CD8^+$ lymphocytes.

Adoptive immunotherapy with CTLs has proved feasible for preventing and treating EBV-associated PTLD, HD and NPC (Bollard *et al.*, 2004; Gottschalk *et al.*, 2005; Straathof *et al.*, 2005). With respect to the targets for EBV-specific CTLs, EBNA3s and LMP2 are major EBV latent antigens; EBNA3s are immunodominant and LMP2 is recognized frequently, but is subdominant. In contrast, CTL responses to other antigens (EBNA2, EBNA-LP, LMP1 and EBNA1) seem to be less frequent (Rickinson & Moss, 1997), although EBNA1 can be immunodominant in some EBV-seropositive donors (Blake *et al.*, 2000). Indeed, LCL-activated EBV-specific CTL lines from NPC patients for adoptive immunotherapy demonstrate stronger responses to the immunodominant EBNA3s than against LMP1 and LMP2 (Straathof *et al.*, 2005). In addition, tetramer and functional

Table 1. Frequencies of anti-EBNA1 CTL precursors

Donor	HLA-B*3501				HLA-Cw*0303/0304			
	$CD8^+$ (%)	PBMCs per well	Positive/tested wells	Frequency (among $CD8^+$)	$CD8^+$ (%)	PBMCs per well	Positive/tested wells	Frequency (among $CD8^+$)
Y01*	38	2×10^5	32/32	$> 1.8 \times 10^{-4}$	32	1×10^5	20/32	3.5×10^{-5}
		1×10^5	32/32			5×10^4	15/32	
		5×10^4	32/32			2.5×10^4	13/32	
T02†	32	2×10^5	10/32	7.2×10^{-6}	32	2×10^5	32/32	$> 2.1 \times 10^{-4}$
		1×10^5	7/32			1×10^5	32/32	
		5×10^4	5/32			5×10^4	32/32	
A03†		NA‡			23	2×10^5	20/32	2.5×10^{-5}
						1×10^5	13/32	
						5×10^4	12/32	
K04†		NA			19	2×10^5	18/32	2.7×10^{-5}
						1×10^5	13/32	
						5×10^4	11/32	

*HLA-Cw*0303.
†HLA-Cw*0304.
‡NA, Not available.

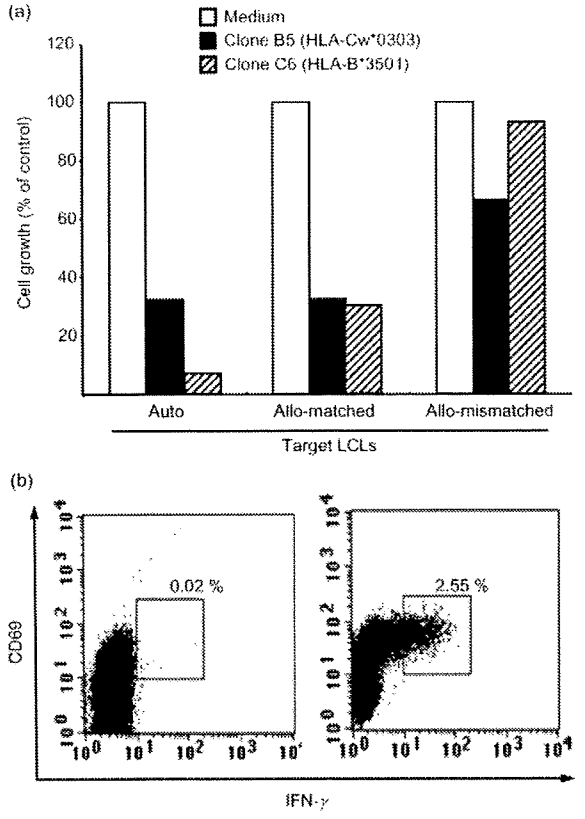

Fig. 7. Effects of EBNA1-specific CTLs on EBNA1-expressing cells. (a) EBNA1-specific CTL clones inhibit *in vitro* outgrowth of HLA-matched LCLs. Target LCLs (2×10^4) were cultured in triplicate wells of round-bottomed 96-well plates with EBNA1-specific CTL clones (1×10^4) or medium alone (control). 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. The B-cell (LCL) identity of the outgrowing cultures was confirmed by analysis of CD19 expression by flow cytometry. Data from one representative experiment of two are shown. (b) Detection of IFN-γ-producing anti-EBNA1 CTLs recognizing HLA-Cw*0303-positive cancer cells expressing EBNA1. B5 clones (5×10^5) were incubated with 2×10^6 MKN45-Cw0303 cells or MKN45-Cw0303-ΔGA-EBNA1 cells for 6 h, in the presence of brefeldin A for the last 5 h. After incubation, the cell suspensions were fixed with 4% paraformaldehyde in PBS and then permeabilized with IC Perm and stained with PE–cyanin-5.1-labelled anti-CD8, PE-labelled anti-CD69 or FITC-labelled anti-human IFN-γ mAbs. Stained cells were analysed by flow cytometry. Fifty thousand events were acquired for each analysis. The proportions of IFN-γ^+ CD69$^+$ CTLs among CD8$^+$ lymphocytes are indicated for one representative experiment of two performed.

analyses have shown that LMP2-specific CTLs are present in the infused CTLs used for adoptive immunotherapy and might have antiviral activity in patients with a good response to immunotherapy for HD (Bollard *et al.*, 2004). Interestingly, the CTL line from one NPC patient who attained a complete response was shown to contain a relatively large T-cell population for an EBNA1-derived CTL epitope (Straathof *et al.*, 2005). This suggests that increased attention should be focused on the contribution of EBNA1-specific CTLs to EBV cellular immunity. In this study, we showed two EBNA1-specific CTL clones to cause strong, specific inhibition of LCL outgrowth *in vitro*, which is consistent with recent observations with HLA-B8- and HLA-B*3501-restricted CTL clones (Tellam *et al.*, 2004; Voo *et al.*, 2004). C6 CTLs failed to respond to an HLA-Cw*0303-expressing gastric cancer cell line transduced with full-length EBNA1, although they produced IFN-γ when GAr-depleted EBNA1 was transduced (Fig. 7b). These data suggest differential antigen-processing machinery and presentation on class I molecules between LCLs and gastric cancer cells.

In conclusion, we have established EBNA1-specific CTL clones from PBMCs of a healthy donor by using EBNA1 mRNA-transfected DCs, and identified a novel CTL epitope of EBNA1 presented by HLA-Cw*0303 and -Cw*0304 molecules. The induction method adapted may be useful for generating EBNA1-specific CTLs and for investigating cellular immunity against EBNA1. Finally, the induced EBNA1-specific CTLs recognized EBNA1-expressing gastric carcinoma cells in the context of HLA-Cw*0303 *in vitro*, suggesting that EBNA1 is an important antigen for the further development of CTL therapy for EBV-associated malignancies.

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High-risk HLA allele mismatch combinations responsible for severe acute graft-versus-host disease and implication for its molecular mechanism

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In allogenic hematopoietic stem-cell transplantation, an effect of HLA locus mismatch in allele level on clinical outcome has been clarified. However, the effect of each HLA allele mismatch combination is little known, and its molecular mechanism to induce acute graft-versus-host disease (aGVHD) remains to be elucidated. A total of 5210 donor-patient pairs who underwent transplantation through Japan Marrow Donor Program were analyzed. All HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 alleles were retrospectively typed in all pairs. The impacts of the HLA allele mismatch combinations and amino acid substitution positions in 6 HLA loci on severe aGVHD were analyzed. A total of 15 significant high-risk HLA allele mismatch combinations and 1 HLA-DRB1-DQB1 linked mismatch combinations (high-risk mismatch) for severe aGVHD were identified, and the number of high-risk mismatches was highly associated with the occurrence of severe aGVHD regardless of the presence of mismatch combinations other than high-risk mismatch. Furthermore, 6 specific amino acid substitution positions in HLA class I were identified as those responsible for severe aGVHD. These findings provide evidence to elucidate the mechanism of aGVHD on the basis of HLA molecule. Furthermore, the identification of high-risk mismatch, that is, nonpermissive mismatch, would be beneficial for the selection of a suitable donor. (Blood. 2007;110:2235-2241)

Introduction

Allogenic hematopoietic stem-cell transplantation (HSCT) from an HLA-matched unrelated (UR) donor has been established as a treatment for hematologic malignancies, when an HLA-identical sibling donor is unavailable.[1,2] When a matched unrelated donor was not found in the donor registry, a partially HLA-matched unrelated donor was one of the candidates for alternative donor. But the higher risk of immunologic events, especially graft-versus-host disease (GVHD), was an important drawback. Extensive recent research has accumulated evidence of the role of each HLA locus mismatch on clinical outcome for UR-HSCT,[3-9] which has made it easy to search and select a partially matched donor. To further expand options for donor selection, our next challenge is to identify permissive and nonpermissive mismatch combinations of each HLA allele. Although there were some divisional trials with small populations,[10,11] a large-scale cohort is essential for comprehensive analysis to identify nonpermissive mismatch combinations that are significant risk factors for severe acute graft-versus-host disease (aGVHD).

In this study, we identified nonpermissive HLA mismatch allele combinations of all major 6 HLA loci, and their responsible positions of amino acid substitution for aGVHD.

Patients, materials, and methods

Patients

A total of 5210 donor-patient pairs who underwent transplantation through the Japan Marrow Donor Program (JMDP) with T-cell–replete marrow from a serologically HLA-A, -B, and -DR antigen-matched donor between January 1993 and January 2006 were analyzed in this cohort study. Patients who received a transplant of harvested marrow outside Japan (n = 51) or were unavailable for blood sample (n = 428) were not eligible for this study of a total of 5689 consecutively registered patients.

Patient characteristics are shown in Table S1, available on the *Blood* website (see the Supplemental Materials link at the top of the online article). The final clinical survey of these patients was completed by June 1, 2006. Informed consent was obtained from patients and donors in accordance with the Declaration of Helsinki, and approval of the study was obtained from the Institutional Review Board of Aichi Cancer Center and JMDP.

HLA typing of patients and donors

Alleles at the HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 loci were identified by the methods described previously.[4,5] Six HLA locus alleles were typed in all 5210 pairs. HLA genotypes of HLA-A, -B, -C, -DQB1, and -DPB1 allele of patient and donor were reconfirmed by the Luminex microbead method (Luminex 100 System; Luminex, Austin, TX). For convenience, we showed the frequency of HLA alleles that existed with

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more than a 5% allele frequency in the current Japanese data set and less than a 1% allele frequency in white populations[12] in Table S2.

Matching of HLA allele between patient and donor

For the analysis of aGVHD, HLA allele mismatch among the donor-recipient pair was scored when the recipient's alleles were not shared by the donor (GVH vector). We also used GVH vectors for the analysis of overall survival (OS) to indicate OS of aGVHD high-risk or low-risk group.

Evaluation of acute GVHD

Occurrences of aGVHD were graded with grade 0, I, II, III, and IV according to established criteria.[13] Grades III and IV were defined as severe aGVHD.

Definitions of amino acid substitution

Amino acid sequences of HLA-A, -B, -C, -DR, -DQ, and -DP molecules were obtained from IMGT/HLA sequence database.[14] For example, Tyr9A-Phe9A indicated amino acid substitutions of position 9 in HLA-A molecule at which the donor had tyrosine and the patient phenylalanine. Substituted amino acids in HLA class I were summarized in Tables S3-S5.

Definition of nonpermissive HLA combinations

We defined the nonpermissive HLA allele combination as a significant risk factor for severe aGVHD, because severe aGVHD was a solid marker for alloreactivity in HSCT and was the main contributor to transplantation-related mortality.[15,16]

Definition of hydropathy scale

The hydropathy scale proposed by Kyte and Doolittle[17] evaluates the hydrophilicity and hydrophobicity of 20 amino acids to estimate the protein structure. Hydrophobic amino acid has a plus value and hydrophilic amino acid a minus value, and their absolute value indicates the grade of each property.

Statistical analysis

Cumulative incidences of aGVHD were assessed by the method described elsewhere to eliminate the effect of competing risk.[18,19] The competing event regarding aGVHD was defined as death without aGVHD. A log-rank test was applied to assess the impact by the factor of interest. Multivariable Cox regression analyses[20] were conducted to evaluate the impact of HLA allele mismatch combination, and the positions and types of amino acid substitution (for example, alanine, arginine, asparagines) of HLA molecules.

The HLA mismatch combination was evaluated for each locus separately, and the HLA match and HLA one-locus mismatch in every locus were analyzed. For example, A0206-A0201 mismatch combination meant that the donor has HLA-A*0206, recipient has HLA-A*0201, and another HLA-A allele of each donor and recipient was identical. This mismatch was compared with the HLA-A allele match. The mismatch combination of which the number of pairs was less than 10 was lumped together as "other mismatch." This is because, according to the computer simulation by Peduzzi et al,[21] it is generally accepted that regression analysis for a variable having fewer than 10 events might give an unreliable estimation. The model was constructed with mismatch combinations, mismatch status in other loci (match, 1 locus mismatch, and 2 locus mismatches as ordinal variable), and potential confounders. Confounders considered were sex (donor-recipient pairs), patient age (linear), donor age (linear), type of disease, risk of leukemia relapse (standard, high, and diseases other than leukemia), GVHD prophylaxis (cyclosporine [CSP] vs FK 506 [FK]), ATG (ATG vs no ATG), and preconditioning (total body irradiation [TBI] vs non-TBI). We used these confounders in all analyses in this paper to keep results comparable.

The impact of positions and types of amino acid substitution in HLA molecules was evaluated in pairs with HLA one-locus mismatch in HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 separately. The amino acid positions we analyzed were all those at which amino acid was substituted in each locus. We analyzed the impact of each amino acid substitution on each position separately. Multivariable Cox models including positions and types of amino acid substitution, mismatch status in other loci (match, 1 locus mismatch, and 2 locus mismatches as ordinal variable), and confounders described in "Statistical analysis" were constructed.

We applied a P value of less than .005 as statistically significant to eliminate false-positive associations. All the analyses were conducted by STATA version 9.2 (Stata, College Station, TX).

Validation of statistical analysis

We validated the statistical analysis using 2 methods, traditional training-and-test method and bootstrap resampling method, in HLA-A analysis to confirm the usability of bootstrap resampling. In the traditional training-and-test method, donor-recipient pairs were divided at random in 2 equally scaled groups, group A and group B. When consistent results were obtained in both analyses, we considered the results as validated. In the bootstrap resampling method,[22] we estimated the measure of association with the resampled data repeatedly drawn from the original data. Although around 100 to 200 bootstrapped samplings are generally sufficient,[23] we explored 500, 1000, 5000, 10 000, and 50 000 bootstrappings in analysis of HLA-A mismatch combinations. We confirmed that an analysis using more than 5000 bootstrappings made the results stable. Because there was high concordance between these 2 methods (Table S6), we adopted bootstrap resampling using 10 000 bootstrap samples for all analyses in this paper as the method for validation. This is because traditional training-and-test methods do not work efficiently when small subgroups are considered as in this paper. Only when the results of base analysis and validating analysis using bootstrap resampling were significant concurrently were the results of the analysis judged to be statistically significant. When the result of base analysis was significant but the result of validating analysis using bootstrap resampling was not, we indicated this by adding an asterisk next to the P value of the base analysis.

Results

Impact of HLA allele mismatch combinations on severe aGVHD

Hazard ratios (HRs) of HLA allele mismatch combinations in HLA-A and -C on severe aGVHD are shown in Table 1 (HLA-B, -DR, -DQ, and -DP are available in Table S7).

In HLA-A locus mismatch combinations, A*0206-A*0201 (HR: 1.78; CI, 1.32-2.41), A*0206-A*0207 (HR: 3.45; CI: 2.09-5.70), A*2602-A*2601 (HR: 3.35; CI: 1.89-5.91), and A*2603-A*2601 (HR: 2.17; CI: 1.29-3.64), were significant risk factors for severe aGVHD.

In HLA-C locus mismatch combinations, 7 combinations were significant risk factors for severe aGVHD; those were as follows: Cw*0401-Cw*0303 (HR: 2.81; CI: 1.72-4.60), Cw*0801-Cw*0303 (HR: 2.32; CI: 1.58-3.40), Cw*0303-Cw*1502 (HR: 3.22; CI: 1.75-5.89), Cw*0304-Cw*0801 (HR: 2.34; CI: 1.55-3.52), Cw*1402-Cw*0304 (HR: 3.66; CI: 2.00-6.68), Cw*1502-Cw*0,304 (HR: 3.77; CI: 2.20-6.47), and Cw*1502-Cw*1402 (HR: 4.97; CI: 3.41-7.25). To summarize, high-risk HLA allele mismatch combinations for severe aGVHD, that is, nonpermissive mismatch combinations, of all major 6 HLA loci were listed in Table 2. A total of 15 nonpermissive HLA allele mismatch combinations (4 in HLA-A, 1 in HLA-B, 7 in HLA-C, 1 in HLA-DRB1, and 2 in HLA-DPB1) and 1 HLA-DRB1-DQB1 linked mismatch combination (Table 2 legend) were identified.

We divided donor-recipient pairs into 4 groups according to the number of nonpermissive mismatches: (1) full match (in HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1) group; (2) zero nonpermissive mismatch (with mismatches other than nonpermissive mismatches)

Table 1. Multivariable analysis of impact of mismatch pairs for sever aGVHD in HLA-A and -C

Mismatch combination, donor-patient	N	HR (95% CI)	P
A locus match	4510	1	NA
A0201-A0206	138	1.23 (0.87-1.73)	.223
A0206-A0201	131	1.78 (1.32-2.41)	< .001
A0201-A0207	28	0.83 (0.34-2.03)	.699
A0207-A0201	20	1.12 (0.42-3.02)	.809
A0201-A0210	11	1.57 (0.58-4.23)	.367
A0206-A0207	27	3.45 (2.09-5.70)	< .001
A0207-A0206	22	0.71 (0.23-2.24)	.571
A2402-A2420	60	0.64 (0.32-1.30)	.225
A2420-A2402	30	1.18 (0.56-2.49)	.66
A2601-A2602	24	0.64 (0.26-1.58)	.34
A2602-A2601	21	3.35 (1.89-5.91)	< .001
A2601-A2603	34	1.37 (0.73-2.57)	.326
A2603-A2601	35	2.17 (1.29-3.64)	.003
A2602-A2603	10	1.23 (0.30-4.98)	.763
A2603-A2602	12	1.50 (0.48-4.68)	.485
A other mismatch	97	1.47 (1.00-2.15)	.047
C locus match	3685	1	NA
C0102-C0303	30	2.83 (1.50-5.32)	.001*
C0303-C0102	38	1.05 (0.47-2.36)	.899
C0102-C0304	12	1.85 (0.59-5.81)	.287
C0304-C0102	19	0.89 (0.28-2.79)	.854
C0102-C0401	14	1.87 (0.77-4.55)	.164
C0102-C0803	24	1.97 (0.87-4.42)	.099
C0803-C0102	10	1.66 (0.53-5.19)	.383
C0102-C1402	16	3.86 (1.98-7.51)	< .001*
C1402-C0102	13	0.46 (0.06-3.33)	.45
C0303-C0304	83	1.08 (0.63-1.85)	.761
C0304-C0303	62	0.83 (0.41-1.68)	.614
C0303-C0401	31	1.73 (0.89-3.36)	.103
C0401-C0303	42	2.81 (1.72-4.60)	< .001
C0303-C0702	25	1.16 (0.52-2.62)	.706
C0702-C0303	18	2.16 (0.96-4.85)	.062
C0303-C0801	76	1.07 (0.63-1.84)	.782
C0801-C0303	80	2.32 (1.58-3.40)	< .001
C0303-C1502	25	3.22 (1.75-5.89)	< .001
C0304-C0401	15	3.02 (1.34-6.79)	.007
C0401-C0304	12	6.22 (3.07-12.5)	< .001*
C0304-C0702	26	2.35 (1.16-4.76)	.017
C0702-C0304	33	1.22 (0.58-2.59)	.59
C0304-C0801	69	2.34 (1.55-3.52)	< .001
C0801-C0304	47	1.64 (0.98-2.76)	.057
C0304-C1402	28	3.06 (1.68-5.60)	< .001*
C1402-C0304	23	3.66 (2.00-6.68)	< .001
C0304-C1502	53	1.82 (1.08-3.05)	.023
C1502-C0304	27	3.77 (2.20-6.47)	< .001
C0801-C0102	10	2.88 (0.92-9.03)	.068
C0801-C0803	27	1.55 (0.69-3.48)	.284
C0803-C0801	26	2.04 (1.04-3.99)	.037
C0801-C1502	36	1.59 (0.79-3.21)	.19
C1502-C0801	23	2.28 (1.07-4.85)	.031
C1402-C1502	55	1.67 (1.01-2.77)	.043
C1502-C1402	50	4.97 (3.41-7.25)	< .001
C other mismatch	347	1.69 (1.34-2.14)	< .001

A0206-A0201 mismatch combination meant that the donor has HLA-A*0206, recipient has HLA-A*0201 and another HLA-A allele of each donor and recipient was identical. Each mismatch pair in HLA-A was compared with the HLA-A allele match, and each mismatch pair in HLA-C was compared with the HLA-C allele match. Confounders considered were sex (donor-recipient pairs), patient age (linear), donor age (linear), type of disease, risk of leukemia relapse (standard, high and diseases other than leukemia), GVHD prophylaxis, (CSP vs. FK), ATG (ATG vs. no ATG) and preconditioning (TBI vs non-TBI).

HR denotes hazard ratio; CI, confidence interval; NA, not applicable.

*The result of base analysis was significant, but the result of validating analysis using bootstrap resampling was not. The results of the analysis were thus judged not to be statistically significant.

Table 2. Nonpermissive allele mismatch combinations for severe aGVHD

Mismatch combination, donor-patient	N	HR (95% CI)	P
A0206-A0201	131	1.78 (1.32-2.41)	< .001
A0206-A0207	27	3.45 (2.09-5.70)	< .001
A2602-A2601	21	3.35 (1.89-5.91)	< .001
A2603-A2601	35	2.17 (1.29-3.64)	.003
B1501-B1507	19	3.34 (1.85-5.99)	< .001
C0303-C1502	25	3.22 (1.75-5.89)	< .001
C0304-C0801	69	2.34 (1.55-3.52)	< .001
C0401-C0303	42	2.81 (1.72-4.60)	< .001
C0801-C0303	80	2.32 (1.58-3.40)	< .001
C1402-C0304	23	3.66 (2.00-6.68)	< .001
C1502-C0304	27	3.77 (2.20-6.47)	< .001
C1502-C1402	50	4.97 (3.41-7.25)	< .001
DR0405-DR0403	53	2.13 (1.28-3.53)	.003
(DR1403-DQ0301)-(DR1401-DQ0502)	19	2.81 (1.44-5.51)	.002
DP0301-DP0501	49	2.41 (1.49-3.89)	< .001
DP0501-DP0901	71	2.03 (1.30-3.16)	.002

Analysis method is the same as in Table 1. We surveyed specific linked mismatches between nonpermissive mismatches elucidated. As a result, obvious specific linked mismatches exist only between DRB1*1403- DRB1*1401 and DQB1*0301- DQB1*0502. Therefore, we could not evaluate which mismatch combination impacted aGVHD, and we considered this linked mismatch did so. On the other hand, because other nonpermissive mismatch combinations had no specific link with the others, we judged other than DRB1*1403- DRB1*1401 and DQB1*0301- DQB1*0502 nonpermissive mismatches solely impacted aGVHD. (DR1403-DQ0301)-(DR1401-DQ0502) linked mismatch meant that the donor has HLA-DRB1*1403-HLADQB1*0301 and the recipient has HLA-DRB1*1401-HLADQB1*0502.

HR indicates hazard ratio; CI, confidence interval.

group; (3) 1 nonpermissive mismatch (with or without mismatches other than nonpermissive mismatches) group; and (4) 2 or more nonpermissive mismatches (with or without mismatches other than nonpermissive mismatches) group, and analyzed for association with severe aGVHD. This analysis excluded pairs with 2 locus mismatches in the same locus. Patient characteristics according to the number of nonpermissive mismatches are shown in Table 3. The curve of cumulative incidence of severe aGVHD is shown in Figure 1A. Multivariable analysis revealed that severe aGVHD occurred with almost equal frequency between the full match group and zero nonpermissive mismatch group, and was significantly associated with the number of nonpermissive mismatches (Table 4). Relative risk of significant factor for aGVHD and OS is shown in Table S8. In terms of the mortality due to aGVHD according to the number of nonpermissive mismatches, one nonpermissive mismatch group and 2 or more nonpermissive mismatch groups showed higher mortality (19.7% and 15.8%, respectively) than full match group and zero nonpermissive mismatch group (8.5% and 11.4%, respectively).

Impact of positions and types of amino acid substitutions of HLA molecules for severe aGVHD

One specific amino acid substitution at position 9 in HLA-A molecule and 6 specific amino acid substitutions at positions 9, 77, 80, 99, 116, and 156 in HLA-C molecule were significant risk factors for severe aGVHD: Tyr9A-Phe9A (HR: 1.66; CI: 1.19-3.32), Tyr9C-Ser9C (HR: 1.66; CI: 1.23-2.25), Asn77C-Ser77C (HR: 1.87; CI: 1.46-2.39), Lys80C-Asn80C (HR: 1.87; CI: 1.46-2.39), Tyr99C-Phe99C (HR: 1.64; CI: 1.21-2.22), Leu116C-Ser116C (HR: 3.40; CI: 2.20-5.25), and Arg156C-Leu156C (HR: 1.48; CI: 1.15-1.90) (Table 5). The amplitude of hydropathy scales were 4.1, 0.5, 2.7, 0.4, 4.1, 4.6, and 8.3, respectively. Although all

Table 3. Patient characteristics according to number of nonpermissive mismatches

Group	Total	Full match	Zero nonpermissive mismatch	One nonpermissive mismatch	Two or more nonpermissive mismatches
Total	4050	712	2670	602	66
Patient age, median y	30	32	30	29	29
Sex, donor/patient, no. patients					
Male/male	1673	312	1096	237	28
Male/female	785	134	518	119	14
Female/male	769	115	524	117	13
Female/female	823	151	532	129	11
Disease, no. patients					
ALL	981	162	668	139	12
ANLL	1075	196	698	158	23
CML	703	119	453	115	16
Hereditary disease	85	14	56	15	0
MDS	476	91	304	72	9
Malignant lymnphoma	349	69	229	48	3
Multiple myeloma	42	8	29	4	1
Severe aplastic anemia	247	33	175	37	2
Other disease	92	20	58	14	0
Risk of leukemia relapse,* no. patients					
Standard risk	1308	249	857	181	21
High risk	1451	228	962	231	30
Diseases other than leukemia	1291	235	851	190	15
GVHD prophylaxis, no. patients					
Cyclosporin-based	2198	402	1444	319	33
Tacrolimus-based	1852	310	1226	283	33
ATG, no. patients					
ATG	323	48	215	53	7
Non-ATG	3727	664	2455	549	59
Preconditioning, no. patients					
TBI regimen	3117	539	2071	449	58
Non-TBI regimen	933	173	599	153	8

ALL indicates acute lymphoblastic leukemia; ANLL, acute non-lymphoblastic leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; and TBI, total body irradiation.

*Standard risk for leukemia relapse was defined as the status of the 1st CR of AML and ALL and the 1st CP of CML at transplant, while high risk was defined as a more advanced status than standard risk in AML, ALL, and CML, and diseases other than leukemia was defined as other than ALL, ANLL, and CML.

amino acid positions substituted in each HLA locus were analyzed, amino acid substitutions of any other HLA-A and -C positions were not significant risk factors. As for HLA-B, DRB1, DQB1, and DPB1, there was no significant association between the positions of amino acid substitution and severe aGVHD. Impact for OS about positions and types of amino acid substitutions that were significant risk factors for aGVHD was shown in Table S9.

Discussion

Extensive recent research has accumulated evidence of the role of each HLA locus mismatch on clinical outcome for UR-HSCT.[3-9] Our next concern is identifying the combinations of HLA allele mismatch and the positions of amino acid substitution of the HLA molecules responsible for aGVHD. In the present study, multivariable analysis revealed that 15 combinations of HLA allele mismatch and 1 HLA-DRB1-DQB1 haplotype mismatch significantly increase the occurrence of severe aGVHD (Table 2), and most of them increased the mortality rate after transplantation (data not shown). Thus, these mismatch combinations of HLA allele might be called nonpermissive clinically. We speculated that the effect of HLA locus mismatch was a reflection and summation of these HLA allele mismatch combinations. Discrepancies of responsible HLA locus for aGVHD between ethnically diverse transplantations might be explained by the proportions of nonpermissive mismatch combinations in each HLA locus. The same study in other populations would be needed to clarify this question as well as the severity of aGVHD. Interestingly, the full match group and zero nonpermissive mismatch group showed an almost equal occurrence of severe aGVHD, though pairs in zero nonpermissive mismatch group had one or more mismatches other than nonpermissive mismatches. And HR was elevated with the increase in the number of nonpermissive mismatches (Figure 1A; Table 4), while the number of nonpermissive mismatches also had a significant effect on OS after transplantation (Figure 1B; Table 4). These findings indicated at least that nonpermissive mismatches should be avoided in donor selection for UR-HSCT, and that the order of donor selection based on this nonpermissive mismatch would be useful, instead of that based on HLA locus mismatch. We also speculated that there are permissive mismatches in mismatches other than nonpermissive mismatches. It is therefore an important task in the future to identify permissive mismatches for partially HLA-matched donor selection. On the other hand, we do not deny the possibility that some mismatch combinations not classified as nonpermissive may actually be potential nonpermissive ones. Misclassification might happen because of insufficient statistical power due to the relatively small number of subjects in subcategories.

At present, there have been only a few reports indicating that the transplant-related immunologic reactions and clinical outcomes were caused by the HLA allele mismatch combinations. Macdonald

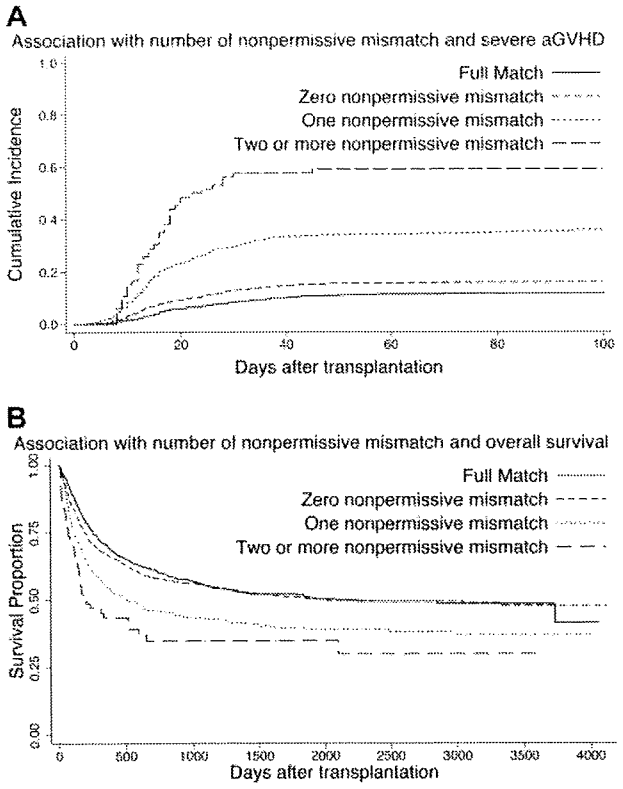

Figure 1. Impact of number of nonpermissive mismatches on severe aGVHD and overall survival. (A) Cumulative incidence of severe aGVHD according to number of nonpermissive mismatches. — indicates full match (in HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1) group; ----, zero nonpermissive mismatch (with mismatches other than nonpermissive mismatches) group; ····, one nonpermissive mismatch (with or without mismatches other than nonpermissive mismatches) group; and – – –, 2 or more nonpermissive mismatches (with or without mismatches other than nonpermissive mismatches) group. (B) Kaplan-Meier estimates of survival according to number of nonpermissive mismatches. Each group was divided as described for panel A.

et al[24] reported that cytotoxic T lymphocytes (CTLs) discriminate between HLA-B*4402 and HLA-B*4403, and induce strong alloresponses, but the stronger T-cell alloreactivity is observed toward HLA-B*4403 compared with HLA-B*4402 in vitro. Zino et al[10] and Fleischhauer et al[11] attempted to develop an algorithm for prediction of nonpermissive HLA-DPB1 mismatches. The present report is the first to provide far more precise and detailed evidence for numerous HLA allele mismatch combinations for severe aGVHD.

Table 5. Multivariable analysis of impact of amino acid substitution on HLA class I molecules for severe aGVHD

Posisiton and kind of amino acid substitution, donor-recipient	HS	N	Event†	HR (95% CI)	P
HLA-A locus					
Tyr9A-Phe9A	4.1	163	64	1.66 (1.19-2.32)	.003
Asn116A-Asp116A	0	32	15	2.25 (1.26-4.01)	.005*
HLA-C locus					
Tyr9C-Ser9C	0.5	146	59	1.66 (1.23-2.25)	.001
Asn77C-Ser77C	2.7	205	90	1.87 (1.46-2.39)	< .001
Lys80C-Asn80C	0.4	205	90	1.87 (1.46-2.39)	< .001
Tyr99C-Phe99C	4.1	146	59	1.64 (1.21-2.22)	.001
Leu116C-Ser116C	4.6	53	30	3.40 (2.20-5.25)	< .001
Arg156C-Leu156C	8.3	251	88	1.48 (1.15-1.90)	.002

HLA-B. -DRB1, -DQB1 -DPB1 locus had no significant substitutions. The impact of positions and types of amino acid substitution in HLA molecules was evaluated in pairs with HLA one-locus mismatch in HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 separately. For example, Tyr9A-Phe9A indicated amino acid substitutions of position 9 in HLA-A molecule at which donor had tyrosine and patient phenylalanine. The impacts of positions and kinds of amino acid substitutions in each HLA molecule were evaluated in pairs with HLA one locus mismatch in each HLA locus separately. Pairs which substituted specific amino acid at each position were compared with amino acid matched pairs at that position.

HS indicates hydropathy scale; HR, hazard ratio; CI, confidence interval; Tyr, tyrosine; Phe, phenylalanine; Asn, asparagine; Asp, asparatic acid; Ser, serine; Lys, lysine; Leu, leucine; and Arg, arginine.

*Result of base analysis was significant but result of validating analysis using bootstrap resampling was not. Results of analysis were thus judged not to be statistically significant.

†Measured in number of occurrences of severe acute GVHD.

In this study, substitutions of specific amino acids at positions 9, 77, 80, 99, 116, and 156 were elucidated as a significant risk factor for severe aGVHD. We speculated that the responsibility of positions 77 and 80 in HLA-C for severe aGVHD was associated with ligand matching of NK-cell receptor (KIR2DL). Although the role of KIR2DL in acute GVHD has been controversial,[25] a recent JMDP analysis demonstrated that KIR2DL ligand mismatched pairs in GVH vector induced severe aGVHD in UR-HSCT with T-cell–replete marrow.[9] The ligand of KIR2DL is located at positions 77 and 80, which are completely linked in HLA-C molecule. And almost all pairs in this study with Asn77C-Ser77C and Lys80C-Asn80C substitutions have a KIR2DL mismatch in GVH vector.

Except for positions 77 and 80, which are associated with KIR2DL ligand in HLA-C, positions 9, 99, 116, and 156 were elucidated. Positions 9, 99, and 116 are located in the beta-plated

Table 4. Multivariable analysis of impact of number of nonpermissive mismatches on severe aGVHD and overall survival

			Univariate analysis		Multivariate analysis		Bootstrap (10000)	
	N	Event*	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
For severe aGVHD								
Full match group	972	129	1.00	NA	1.00	NA	1.00	NA
Zero nonpermissive mismatch group	2446	411	1.21 (0.95-1.54)	.111	1.00 (0.75-1.32)	.996	1.00 (0.74-1.33)	.996
One nonpermissive mismatch group	571	211	2.88 (2.20-3.78)	< .001	2.22 (1.62-3.04)	< .001	2.22 (1.63-3.02)	< .001
Two or more nonpermissive mismatch group	61	36	5.62 (3.77-8.39)	< .001	3.68 (2.33-5.80)	< .001	3.68 (2.33-5.80)	< .001
For overall survival								
Full match group	972	400	1.00	NA	1.00	NA	1.00	NA
Zero nonpermissive mismatch group	2446	1021	1.10 (0.98-1.23)	.091	1.06 (0.94-1.20)	.315	1.06 (0.94-1.20)	.299
One nonpermissive mismatch group	571	309	1.55 (1.34-1.78)	< .001	1.51 (1.30-1.76)	< .001	1.51 (1.29-1.77)	< .001
Two or more nonpermissive mismatch group	61	39	2.12 (1.54-2.90)	< .001	2.25 (1.65-3.08)	< .001	2.25 (1.65-3.08)	< .001

Each group was compared with Full match group. Confounders considered were sex (donor-recipient pairs), patient age (linear), donor age (linear), type of disease, risk of leukemia relapse (standard, highand diseases other than leukemia), GVHD prophylaxis, (CSP vs. FK), ATG (ATG vs. no ATG) and preconditioning (TBI vs. non-TBI).

HR indicates hazard ratio; CI, confidence interval; Boot strap (10000), bootstrap resampling using 10000 bootstrapping.

*For severe aGVHD, "Event" refers to number of occurrences; for overall survival, number of deaths.