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European Journal of Immunology Immunity to infection

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

Ayako Demachi-Okamura[1], Yoshinori Ito[1], Yoshiki Akatsuka[1], Kunio Tsujimura[1], Yasuo Morishima[2], Toshitada Takahashi[1] and Kiyotaka Kuzushima[1]

[1] Division of Immunology, Aichi Cancer Center Research Institute, Nagoya, Japan
[2] Department of Cell Therapy, Aichi Cancer Center Hospital, Nagoya, Japan

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

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Introduction

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

Correspondence: Dr. Kiyotaka Kuzushima, Division of Immunology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan
Fax: +81-52-764-2990
e-mail: kkuzushi@aichi-cc.jp

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

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

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

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

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

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

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

Results

Induction of LMP1-specific CTL using mRNA-transduced APC

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

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

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

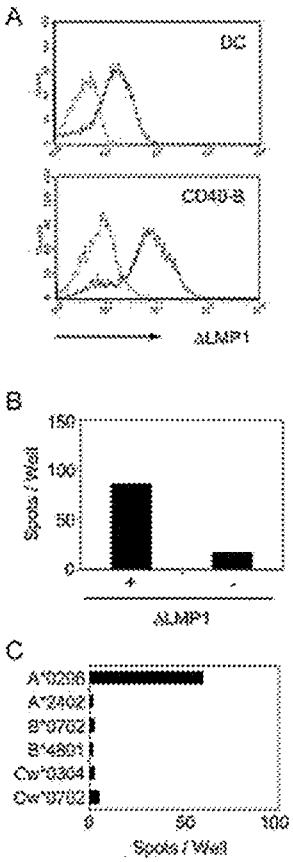

Figure 1. mRNA-transduced DC and CD40-B cells express ΔLMP1 and induce specific T cells. (A) DC and CD40-B cells were transduced with ΔLMP1 mRNA and analyzed for expression of ΔLMP1 by FCM at 36 h post-transduction. The dotted lines show non-transduced, and the solid lines ΔLMP1-transduced cells. (B)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

Identification of the LMP1 epitope

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

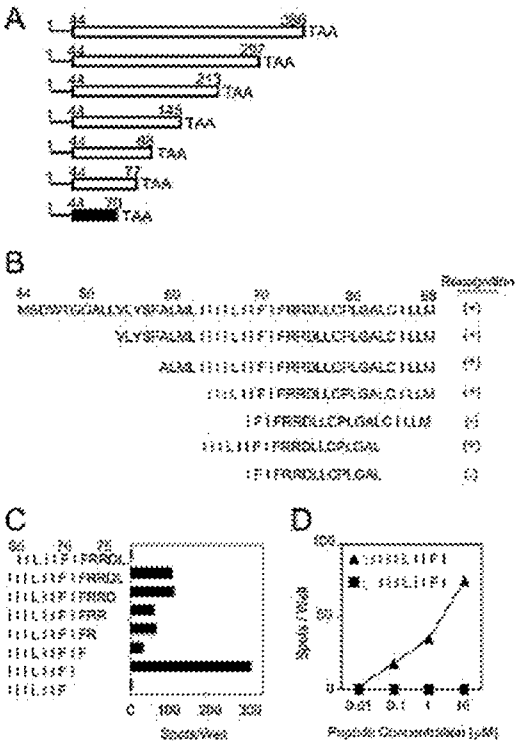

Figure 2. Identification of the LMP1 epitope peptide recognized by H7 CTL clone. (A) A series of C-terminally truncated ΔLMP1 mRNA were generated by *in vitro* transcription. A methionine at amino acid position 44 was used as the initiation codon for all constructs. CD40-B cells transduced with each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

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

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

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

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

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

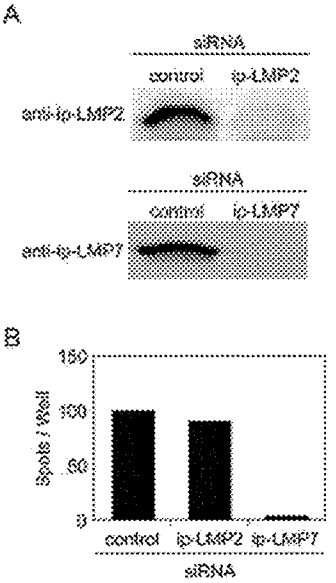

Figure 3. ip-LMP7 is essential for the LMP1 epitope processing. (A) Control siRNA, ip-LMP2 siRNA, or ip-LMP7 siRNA were retrovirally transduced into autologous LCL and the cells were selected for 14 days with puromycin, followed by Western blot analysis of ip-LMP2 (upper panel) and ip-LMP7 (lower panel).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

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Cytotoxic activity of the LMP1-specific CTL clone against LCL

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

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

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

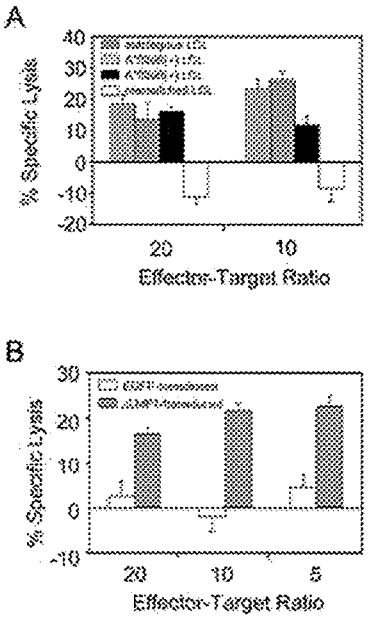

Figure 4. Cytotoxic activity of the LMP1-specific CTL clone H7. (A) Sixteen-hour CTL assays were performed using autologous, HLA-A*0206-shared, and fully HLA-mismatched LCL as target cells. (B) Four-hour CTL assays were performed using ΔLMP1- or EGFP-transfected LCL as target cells. Each bar represents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

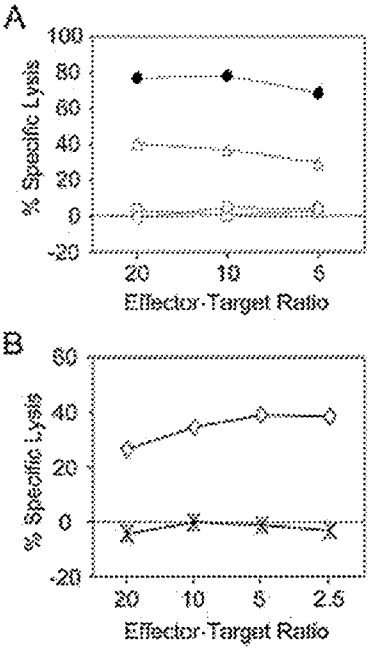

Figure 5. CTL specifically lyse EBV-infected NK cells. (A) Cytolytic activity of the CTL clone H7 was assessed against EBV-carrying NK cells in 16-h CTL assays. Data for two HLA-A*0206-positive NK cell lines (SNK-6 and SNK-10) and one HLA-A*0206-negative NK cell line (HANK-1) are shown with open circles,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

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

Discussion

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

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

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

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

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

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

Materials and methods

Donors and cell lines

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

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Preparation of APC

DC were prepared as previously described with slight modification [51]. Briefly, $CD8^+$ T cells were isolated from PBMC using CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and stored at –135°C until use. The CD8-depleted PBMC were suspended in 4 mL RPMI 1640 medium supplemented with 5% human serum (MP Biomedicals, Aurora, OH), 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and 50 μg/mL kanamycin (referred to as DC medium), and incubated for 2 h in wells of 6-well plates at 37°C. Nonadherent cells were removed by gentle pipetting, and the adherent cells were cultured in DC medium in the presence of 50 ng/mL GM-CSF (Osteogenetics, Wuerzburg, Germany) and 10 ng/mL IL-4 (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, DC were collected and electroporated for mRNA transduction. CD40-B cells were generated as described previously [50, 52] using NIH/3T3 human CD40 ligand cells (kindly provided by Dr. Freeman, Dana-Farber Cancer Institute, Boston, MA).

Plasmid construction

To construct ΔLMP1 [20], PCR was performed using a sense primer 5′-aagcttgccaccATGAGTGACTGGACTGGA-3′, an antisense primer 5′-ttgaattcttagtcatagtagcttagctga-3′, and EBV strain B95-8 (NCBI accession No. V01555) cDNA as a template. The resultant DNA fragment was cloned into pcDNA 3.1(+) (Invitrogen, Carlsbad, CA) using its *Hind*III and *Eco*RI sites (pcDNA/ΔLMP1). For constructing further C-terminal and N-terminal deletion mutants of the ΔLMP1 gene, truncated fragments were prepared by PCR using pcDNA/ΔLMP1 as a template, and cloned into the pcDNA3.1(+). To construct some plasmids encoding short LMP1 peptide fragments, each pair of complementary oligonucleotides were annealed and cloned into restriction enzyme-cut pcDNA3.1(+). ΔLMP1 and EGFP [50] cDNA were cloned into the pMSCVpuro retroviral vector (BD Biosciences Clontech, Palo Alto, CA) to generate pMSCVpuro/ΔLMP1 and /EGFP, respectively.

shRNA interference retrovirus vectors were constructed using the RNAi-Ready pSIREN-RetroQ vector (BD Biosciences Clontech). The following siRNA targets were selected in this study: ip-LMP2, AAGUGAAGGAGGUCAGGUAUA, and ip-LMP7, AGAUUAACCCUUACCUGCUTT. The shRNA constructs included a TTCAAGAGA loop separating the sense and antisense sequences followed by a 5T termination signal. These constructs were synthesized as two complementary DNA oligonucleotides, annealed, and ligated between the *Bam*HI and *Eco*RI sites of the vector. In addition, a negative control siRNA annealed oligonucleotide (BD Biosciences Clontech) was inserted into the same vector and used as a control. The cloned genes were sequenced to verify their identity.

Retrovirus production and infection

To establish retrovirus-producing cells, pMSCVpuro/ΔLMP1, pMSCVpuro/EGFP and RNAi-Ready pSIREN-RetroQ-based vectors were packaged in PT67 cells (BD Biosciences Clontech) using Lipofectamine 2000 (Invitrogen). LCL were infected with the retroviral supernatant in the presence of 8 μg/mL polybrene (Sigma Chemical Co.), spun at 1000 × g at 32°C for 1 h, and incubated at 37°C for 2 days. Thereafter, these LCL were cultured in the presence of 0.8 μg/mL puromycin for 14 days. Expression of EGFP and ΔLMP1 was analyzed by FCM. Expression of ip-LMP2 and ip-LMP7 was assessed by Western blotting as described previously [53, 54].

Production and transduction of *in vitro* transcribed mRNA

Fragments containing the T7 promoter region and the ΔLMP1 coding region were prepared by PCR using pcDNA/ΔLMP1 as a template. The amplified DNA was used as a template for *in vitro* transcription of 5′-capped mRNA using a mMESSAGE mMACHINE kit (Ambion, Austin, TX) according to the manufacturer's instructions. The 3′ polyA tail was added using polyA polymerase (Ambion) followed by purification with an RNeasy kit (QIAGEN, Tokyo, Japan).

Prior to electroporation, DC and CD40-B cells were washed twice with serum-free RPMI 1640 medium and suspended to a final concentration of 2.5×10^7 cells/mL. Cells in 40 μL were mixed with 20 μg of mRNA, and electroporated in a 0.2-cm cuvette using an Electro Square Porator ECM 830 (Harvard Apparatus, Holliston, MA). The conditions were 450 V and 500 μS for DC and 350 V and 350 μS for CD40-B cells. After electroporation, DC were cultured in DC medium supplemented with GM-CSF and IL-4 for 3-h, then exposed to TNF-α (PeproTech, Rocky Hill, NJ), IL-1β (PeproTech) and prostaglandin E2 (Cayman Chemical Company, Ann Arbor, MI) for maturation. CD40-B cells were immediately seeded onto irradiated NIH/3T3 human CD40 ligand cells, and after 36–48 h used as APC.

Cell staining and FCM

Intracellular staining of LMP1 Ag was performed as previously described with slight modification [20]. Briefly, electroporated cells were collected and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After washing with PBS, cells were permeabilized with IC Perm (BioSource International, Camarillo, CA) and reacted with an mAb recognizing the C terminus of LMP1 (CS1-4; DAKO Cytomation, Glostrup, Denmark) for 30 min at 4°C. After washing with PBS, cells were stained with fluorescein isothiocyanate-labeled anti-mouse IgG (H+L) (Immunotech, Marseille, France) for 30 min at 4°C. The stained cells were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA) and CellQUEST software (BD Biosciences).

CTL induction

Stored $CD8^+$ T cells were thawed, washed, and co-cultured with irradiated (33 Gy) autologous ΔLMP1 mRNA-transduced DC in 2 mL RPMI 1640 medium supplemented with 10% human serum, 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and 50 μg/mL kanamycin, in the presence of 25 ng/mL IL-7 (R&D Systems, Minneapolis, MN) and 5 ng/mL IL-12 (R&D Systems) at 5% CO_2 in a humidified incubator. On days 8 and 15, T cells were restimulated with ΔLMP1 mRNA-

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transduced and γ-irradiated DC and CD40-B cells, respectively. One day after each restimulation, IL-2 (Shionogi, Osaka, Japan) was added to a final concentration of 20 U/mL. To establish T cell clones, limiting dilution of polyclonal CTL was performed using round-bottomed 96-well plates as previously described [55]. After 2 wk of culture, growing wells were split into three replicates and used as effectors in the CTL assay against either ΔLMP1 mRNA- or EGFP mRNA-transduced autologous CD40-B cells. Wells were scored as positive when the counts per minute from ΔLMP1 mRNA-transduced CD40-B cells exceeded the mean counts per minute from EGFP mRNA-transduced CD40-B cells by three standard deviations. Positive wells were transferred into flasks and expanded as previously described [55].

ELISPOT assay

ELISPOT assays were performed as described earlier [48, 50, 55]. Briefly, $CD8^+$ T cells were co-cultured with various stimulators in wells of Multiscreen-HA plates (MAHA S4510; Millipore, Billerica, MA) coated with anti human IFN-γ mAb (M700A; Pierce Biotechnology, Philadelphia, PA). As stimulators, HLA-A*0206-positive or -negative LCL (1×10^5 cells/well) or HLA-A*0206-expressing HEK-293T (referred to as A0206-293T) cells (5×10^4 cells/well) transfected with plasmids using Lipofectamin 2000 (Invitrogen) 48 h earlier were seeded into each well. For peptide titration assays, serial concentrations of synthetic peptides (Greiner, Frickenhausen, Germany) were pulsed to A0206–293T cells for 1 h at room temperature. After probing with anti-rabbit polyclonal IFN-γ antibody (P700; Pierce Biotechnology), followed by exposure to horseradish peroxidase-labeled anti-rabbit IgG antibody (Genzyme, Cambridge, MA) and spots visualization, the plates were washed and dried. IFN-γ spots were enumerated using a dissecting microscope.

CTL assay

Target cells were labeled with 50 μCi ^{51}Cr for 1.5 h at 37°C, washed, and mixed with CTL at the indicated effector-to-target ratios in 96-well plates. After incubation for 4 or 16 h at 37°C, the radioactivity in the supernatants was counted in a γ-counter. The percentage specific ^{51}Cr release was calculated as 100 × (experimental release – spontaneous release) / (maximum release – minimum release).

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Three Immunoproteasome-Associated Subunits Cooperatively Generate a Cytotoxic T-Lymphocyte Epitope of Epstein-Barr Virus LMP2A by Overcoming Specific Structures Resistant to Epitope Liberation

Yoshinori Ito,[1] Eisei Kondo,[2] Ayako Demachi-Okamura,[1] Yoshiki Akatsuka,[1] Kunio Tsujimura,[1] Mitsune Tanimoto,[2] Yasuo Morishima,[3] Toshitada Takahashi,[1] and Kiyotaka Kuzushima[1]*

Division of Immunology, Aichi Cancer Center Research Institute, Nagoya, Japan[1]; Department of Internal Medicine II, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan[2]; and Department of Cell Therapy, Aichi Cancer Center Hospital, Nagoya, Japan[3]

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The precise roles of gamma interferon-inducible immunoproteasome-associated molecules in generation of cytotoxic T-lymphocyte (CTL) epitopes have yet to be fully elucidated. We describe here a unique epitope derived from the Epstein-Barr virus (EBV) latent membrane protein 2A (LMP2A) presented by HLA-A*2402 molecules. Generation of the epitope, designated $LMP2A_{222–230}$, from the full-length protein requires the immunoproteasome subunit low-molecular-weight protein 7 (ip-LMP7) and the proteasome activator 28-α subunit and is accelerated by ip-LMP2, as revealed by gene expression experiments using an $LMP2A_{222–230}$-specific CTL clone as a responder in enzyme-linked immunospot assays. The unequivocal involvement of all three components was confirmed by RNA interference gene silencing. Interestingly, the $LMP2A_{222–230}$ epitope could be efficiently generated from incomplete EBV-LMP2A fragments that were produced by puromycin treatment or gene-engineered shortened EBV-LMP2A lacking some of its hydrophobic domains. In addition, epitope generation was increased by a single amino acid substitution from leucine to alanine immediately flanking the C terminus, this being predicted by a web-accessible program to increase the cleavage strength. Taken together, the data indicate that the generation of $LMP2A_{222–230}$ is influenced not only by extrinsic factors such as immunoproteasomes but also by intrinsic factors such as the length of the EBV-LMP2A protein and proteasomal cleavage strength at specific positions in the source antigen.

Cytotoxic T lymphocytes (CTLs) recognize short peptide products processed from target proteins and presented by major histocompatibility complex (MHC) class I molecules. The first step in protein processing in the cytosol is cleavage by proteasomes, proteolytic complexes playing a critical role in the antigen processing pathway. Resultant peptides are translocated by the transporters associated with antigen processing into the endoplasmic reticulum, where they assemble with newly synthesized MHC class I molecules for transportation to the cell surface (12, 28, 33). Proteasome catalytic activity is exerted by the 20S core proteasome, a cylindrical structure composed of four stacked rings. The outer two rings consist of seven different α subunits, and the inner rings consist of seven different β-type subunits. Enzymatic activity is mediated by three of the β subunits, designated β1 (Y/δ), β2 (Z/MC14), and β5 (X/MB1) (33). Exposure of cells to gamma interferon (IFN-γ) during immune responses alters the proteasome activity qualitatively and quantitatively with the induction of three newly synthesized immunoproteasome β subunits, low-molecular-weight protein 2 (ip-LMP2) or β1i, multicatalytic endopeptidase complex-like 1 (MECL-1) or β2i, and ip-LMP7 or β5i. These become incorporated interdependently and replace the three constitutive β subunits in newly assembled immunoproteasomes (14, 22, 24). The expression of ip-LMP7 and/or ip-LMP2 is known to alter the proteasomal cleavage specificity for virus- and tumor-associated antigens (15, 39). Furthermore, the incorporation of ip-LMP7 is sufficient to alter cleavage properties of proteasomes although the role of its catalytic site remains unclear (8, 36, 38, 40). The expression of ip-LMP2 alone or with ip-LMP7 is also reported to change cleavage specificity (1, 19, 23), and effects of the two subunits have been observed in each subunit's knockout mice (4, 7, 45).

Besides its effects on immunoproteasomes, IFN-γ up-regulates expression of the proteasome activator 28 (PA28), which consists of two different subunits, α and β, that form a heptameric ring that binds to proteasomes and is thought to increase their rate of cleavage (39). Regarding contributions to epitope liberation, effects of the α subunit have been observed (10, 41) but findings are limited regarding the β subunit (41). Elucidating differential effects of the three immunoproteasome subunits and two PA28 subunits is clearly important for a better understanding of generation of CTL epitopes.

Recently, defective ribosomal products (DRiPs) from newly synthesized proteins, which are rapidly ubiquitylated and degraded by proteasomes, were shown to be the main sources of antigenic peptides (29, 35, 48, 49); this suggests that antigen structures are critical for efficient processing by proteasomes. While almost every amino acid residue can serve as a cleavage site, there are certain preferences (25, 27, 44). Because the C terminus of the CTL epitope is precisely determined by the proteasome, cleavage strength at specific positions, such as that immediately flanking the C terminus of the epitope, really affects epitope generation (14, 34). In fact, mutation in the

* Corresponding author. Mailing address: Division of Immunology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan. Phone: 81-52-764-2990. Fax: 81-52-764-2990. E-mail: kkuzushi@aichi-cc.jp.

flanking region of epitopes has been shown to impair the processing by proteasomes (2, 37). Thus, structural features play an important role in epitope liberation and could influence the working of the five immunoproteasome-associated subunits.

We have previously shown the generation of an HLA-A*2402-restriced CTL epitope in the Epstein-Barr virus (EBV) latent membrane protein 2A (EBV-LMP2A), amino acids 222 to 230 (referred to as $LMP2A_{222-230}$), to be dependent on IFN-γ exposure (18). Differential expression of ip-LMP2, MECL-1, ip-LMP7, PA28α, and PA28β in various combinations has allowed us to selectively address the role of each subunit in the processing of the epitope independently of other IFN-γ-inducible proteins, and we have established that the generation of $LMP2A_{222-230}$ is cooperatively controlled by interplay among ip-LMP2, ip-LMP7, and PA28α. Moreover, these observations were supported by the results of RNA interference experiments. We have now extended our studies to demonstrate that LMP2A structural factors influence epitope liberation in various target cells.

MATERIALS AND METHODS

CTL clones and epitopes. EBV-specific CTL lines and clones were established as described earlier (18). Briefly, EBV-specific T-cell lines were generated from peripheral blood mononuclear cells after stimulation with HLA-A*2402-transfected, TAP-negative T2-A24 cells (18) pulsed with each epitope peptide or autologous EBV-carrying lymphoblastoid cell lines (LCLs). After several rounds of stimulation, CTL clones were established by a limiting dilution method. A polyclonal CTL line that was specific to the epitope $LMP2A_{419-427}$ (TYGPVFMCL) (21) was designated $LMP2A_{419-427}$-CTL, and CTL clones that were specific to the epitope $LMP2A_{222-230}$ (IYVLVMLVL) (18) were designated $LMP2A_{222-230}$-CTL.

Cell lines. T2-A24 cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 800 μg/ml of G418 (Invitrogen Corp., Carlsbad, CA). HLA-A*2402-positive LCLs and PT67 cells (BD Bioscience Clontech, Palo Alto, CA), retroviral packaging cell lines, were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml of kanamycin (referred to as LCL medium). HLA-A*2402-positive LCLs expressing short hairpin RNA (shRNA) were maintained in LCL medium in the presence of 0.8 μg/ml of puromycin. HEK293 T cells (referred to as 293T; American Type Culture Collection, Manassas, VA) and HLA-A*2402-positive dermal fibroblast cell lines were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Expression vectors. Plasmids expressing various lengths of EBV-LMP2A and EBNA3A, from full-length proteins to minimal epitopes, were constructed as described previously (16, 18). Full-length HLA-A*2402, ip-LMP2, ip-LMP7, MECL-1, PA28α, and PA28β were amplified by reverse transcriptase (RT)-PCR from HLA-A*2402-positive LCLs, cloned into the pcDNA3.1(+) vector (Invitrogen Corp.), and sequenced. A plasmid containing a mutant EBV-LMP2A gene with alanine substituted for leucine at position 231 was constructed by PCR-based mutagenesis as described previously (16). This single amino acid substitution was intended to increase the proteasome cleavage strength, as predicted with the Prediction Algorithm for Proteasomal Cleavages I program (PAProC version 1.0; http://www.paproc.de/) (17, 26).

Transduction of 293T cells. The plasmids encoding HLA-A*2402 and EBV-LMP2A and at least one of those expressing ip-LMP2, ip-LMP7, MECL-1, PA28α, or PA28β were transfected into 293T cells using TransIT-293 transfection reagents (Mirus, Madison, WI). Briefly, 3×10^4 cells were transfected with 100 ng of each plasmid and 0.2 μl TransIT reagent per 100 ng DNA in various combinations in 96-well plates. After 24 h, these cells were used as stimulators in the enzyme-linked immunospot (ELISPOT) assay.

shRNA interference retrovirus vectors. The following small interfering RNA targets were selected in this study: ip-LMP2, AAGUGAAGGAGGUCAGGU AUA; ip-LMP7, AGAUUAACCCUUACCUGCUTT; and PA28α, AAGCCA ACUUGAGCAAUCUGA. shRNA constructs included a TTCAAGAGA-loop separating the sense and antisense sequences followed by a 5T termination signal. These constructs were synthesized as two cDNA oligonucleotides, annealed, and ligated between the BamHI and EcoRI sites of the RNAi-Ready pSIREN-RetroQ vector (BD Biosciences Clontech). In addition, oligonucleotides with sequences selected by the company (BD Biosciences Clontech) as a negative control for gene silencing were annealed and inserted into the same vector.

Retrovirus production and infection. PT67 cells were plated on six-well culture plates, and a 4-μg aliquot of each retrovirus vector plasmid was transfected with Lipofectamine 2000 (Invitrogen Corp), according to the manufacturer's instructions. After culture in the presence of 2.5 μg/ml of puromycin for 14 days, the cells were incubated in medium without puromycin for another 48 h. The culture supernatant was collected, and debris was removed by centrifugation at $1,000 \times g$ for 10 min. A total of 1×10^6 LCLs were suspended in 1 ml of the virus-containing culture supernatant in each well of a 12-well plate, and polybrene was added to a final concentration of 10 μg/ml. Plates were centrifuged at $1,000 \times g$ at 32°C for 1 h and incubated at 37°C in a humidified incubator. The LCLs were then cultured in medium containing puromycin for 14 days. Expression of ip-LMP2, ip-LMP7, or PA28α in these LCLs was analyzed by Western blotting and RT-PCR for gene silencing.

Western blotting. Western blotting was performed as described previously with slight modifications (42). Briefly, aliquots of 130 μg protein were applied to sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis, blotted onto Immobilon-P membranes (Millipore Corporation, Bedford, MA), blocked with phosphate-buffered saline containing 10% low-fat dry milk and 0.1% Tween 20 overnight at 4°C, and probed with rabbit polyclonal antibodies specific to ip-LMP2, ip-LMP7, and PA28α (Affinity, Mamhead, United Kingdom), followed by peroxidase-conjugated goat anti-rabbit immunoglobulin G (Zymed, San Francisco, CA). Proteins were visualized using an ECL Western blot detection system (Amersham Biosciences, Buckinghamshire, United Kingdom).

RT-PCR. Total RNA was extracted from LCLs and reverse transcription was performed in 20-μl reactions containing random hexamers and 1-μg aliquots. The specific primer sets used to detect ip-LMP2, ip-LMP7, and PA28α were as follows: ip-LMP2 forward, 5′-GGTGGTGAACCGAGTGTTTGA-3′; ip-LMP2 reverse, 5′-GCCAAAACAAGTGGAGGTTCC-3′; ip-LMP7 forward, 5′-GAT TGCAGCAGTGGATTCTCG-3′; ip-LMP7 reverse, 5′-GACATGGTGCCA AGCAGGTAA-3′; PA28α forward, 5′-ACCAAGACAGAGAACCTGCTCG-3′; and PA28α reverse, 5′-GGCCTTCAGATTGCTCAAGTTG-3′.

ELISPOT assays. ELISPOT assays were performed as previously described (18). In brief, a MultiScreen-HA plate (Millipore) was coated with anti-human IFN-γ monoclonal antibody (Endogen, Rockford, IL) and used as the assay plate. The following stimulator cells in 100 μl of LCL medium were seeded into the wells: (i) 293T cells cotransfected with plasmids expressing HLA-A*2402 and those expressing various lengths of EBV-LMP2A (in some experiments, cells were treated with puromycin at 1 μg/ml for 30 min); (ii) 293T cells cotransfected with plasmids encoding HLA-A*2402 and EBV-LMP2A and at least one of those two expressing ip-LMP2, ip-LMP7, MECL-1, PA28α, or PA28b; and (iii) LCLs transduced by retrovirus vectors expressing shRNA for either ip-LMP2, ip-LMP7, or PA28a.

$LMP2A_{222-230}$-CTLs or $LMP2A_{419-427}$-CTLs in 100 μl medium were introduced into each well and incubated for 20 h. To visualize spots, anti-human IFN-γ polyclonal antibody (Endogen), horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Zymed) and substrate were used. All assays were performed in duplicate.

RESULTS

$LMP2A_{222-230}$ is not presented on target cells expressing full-length EBV-LMP2A. The $LMP2A_{419-427}$-CTL responded to 293T cells pulsed with the epitope peptide and to those expressing full-length EBV-LMP2A cotransfected with HLA-A*2402. However, the $LMP2A_{222-230}$-CTL responded to 293T cells expressing the minimal epitope, but not full-length EBV-LMP2A, cotransfected with HLA-A*2402 (Fig. 1).

As we reported previously, IFN-γ-treated fibroblasts transduced with full-length EBV-LMP2A were recognized by $LMP2A_{222-230}$-CTL, showing $LMP2A_{222-230}$ to be an IFN-γ-dependent epitope. This suggested that IFN-γ-induced immunoproteasome and PA28 subunits generate $LMP2A_{222-230}$ in the target cells (18).

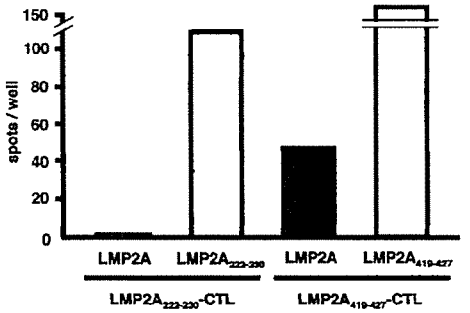

FIG. 1. EBV-specific CTL recognition of target cells measured by the ELISPOT assay. The epitope $LMP2A_{222-230}$ is not presented on 293T cells expressing full-length EBV-LMP2A while $LMP2A_{419-427}$ is presented on these cells. $LMP2A_{222-230}$-CTL is the clone specific for $LMP2A_{222-230}$, and $LMP2A_{419-427}$-CTL is a polyclonal $CD8^+$ T-cell line specific to $LMP2A_{419-427}$. 293T cells were cotransfected with plasmids expressing HLA-A*2402 and full-length EBV-LMP2A or pulsed with the epitope peptide. $CD8^+$ T cells (200/well) were cultured with the indicated stimulators for 20 h. Data from one representative experiment out of three are shown. Each bar demonstrates the average number of spots in duplicate wells.

Generation of $LMP2A_{222-230}$ requires the immunoproteasome subunit ip-LMP7 and PA28α and is enhanced by ip-LMP2. To investigate whether immunoproteasome-associated molecules are involved in generating the $LMP2A_{222-230}$ epitope, we examined the effect of each proteasome immunosubunit (ip-LMP2, ip-LMP7, and MECL-1) and PA28 subunit (PA28α and PA28β) in 293T cells that dominantly have a standard proteasome. First, 293T cells were cotransfected with plasmids encoding HLA-A*2402, the full-length EBV-LMP2A, and immunoproteasome-associated molecules in various combinations, as shown in Fig. 2A. We then evaluated epitope liberation using the ELISPOT assay. Surprisingly, three molecules were found to be involved in the generation of $LMP2A_{222-230}$: ip-LMP7 and PA28α subunits were required, and the ip-LMP2 subunit enhanced its recognition (Fig. 2A). We confirmed the expression of ip-LMP2, ip-LMP7, and PA28α by Western blotting (Fig. 2B).

Inhibition of ip-LMP2, ip-LMP7, and PA28α expression in LCLs by RNA interference decreases the generation of $LMP2A_{222-230}$ in target cells. LCLs predominantly have immunoproteasomes (24), and the $LMP2A_{222-230}$-CTL have recognized HLA-A*2402-positive LCLs, as we reported previously (18). To examine whether ip-LMP2, ip-LMP7, or PA28α is most directly involved in the generation of $LMP2A_{222-230}$, we evaluated the epitope liberation in LCLs in which the expression of each subunit was separately inhibited using a gene-silencing technique. HLA-A*2402-positive LCLs were infected with retrovirus vectors expressing shRNAs for ip-LMP2, ip-LMP7, or PA28α and assessed for the expression of each subunit by Western blotting (Fig. 3A, B, and C) and RT-PCR (data not shown). Then, generation of the $LMP2A_{222-230}$ epitope was probed with epitope-specific CTL using the ELISPOT assay. As expected, epitope liberation was clearly decreased with the inhibition of ip-LMP2, ip-LMP7, or PA28α expression (Fig. 3A, B, and C), demonstrating definitive involvement of all three molecules in the generation of $LMP2A_{222-230}$. To test whether the generation of IFN-γ-independent EBV-LMP2 epitope was influenced in these

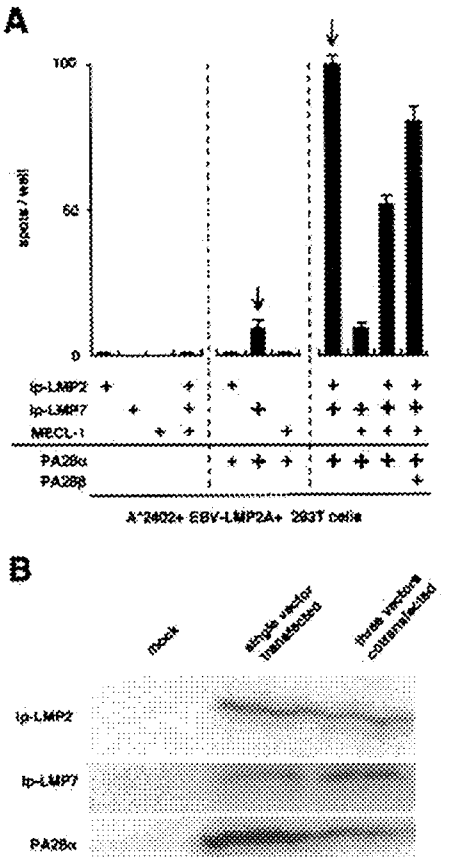

FIG. 2. Involvement of immunoproteasome and PA28 subunits in the generation of $LMP2A_{222-230}$ as analyzed by the ELISPOT assay. (A) Generation of $LMP2A_{222-230}$ requires three immunoproteasome-associated molecules. 293T cells were cotransfected with plasmids encoding HLA-A*2402, full-length EBV-LMP2A, and at least one immunoproteasome-associated molecule (ip-LMP2, ip-LMP7, MECL-1, PA28α, and PA28β). $LMP2A_{222-230}$-CTLs were cultured with stimulators for 20 h as described in Materials and Methods. Data from one representative experiment out of three are shown. Data are means plus or minus standard deviation (SD) of spots in duplicate wells. The arrows indicate noteworthy results. +, presence of immunoproteasome or subunit. (B) Expression of ip-LMP2, ip-LMP7, and PA28α in 293T cells transfected with corrresponding expression vectors. 293T cells were transfected with each of three plasmids or with all these vectors. Expression of each subunit was analyzed by Western blotting. "Single vector-transfected" represents the 293T cells transfected with each plasmid encoding ip-LMP2, ip-LMP7, or PA28α and "three vectors cotransfected" represents the 293T cells cotransfected with the three plasmids. Results of one representative experiment out of two are shown.

LCLs transfected with shRNA expression vectors for ip-LMP2, ip-LMP7, or PA28α, we investigated the generation of $LMP2A_{419-427}$ using the ELISPOT assay. We found that there were no significant differences in the processing of this epitope. (data not shown).

Incomplete or shortened EBV-LMP2A results in efficient generation of $LMP2A_{222-230}$ in target cells. Recently, DRiPs have been reported to be major sources of CTL epitopes (43, 46), suggesting that incomplete antigen proteins allow efficient processing. To test this possibility with regard to $LMP2A_{222-230}$, 293T

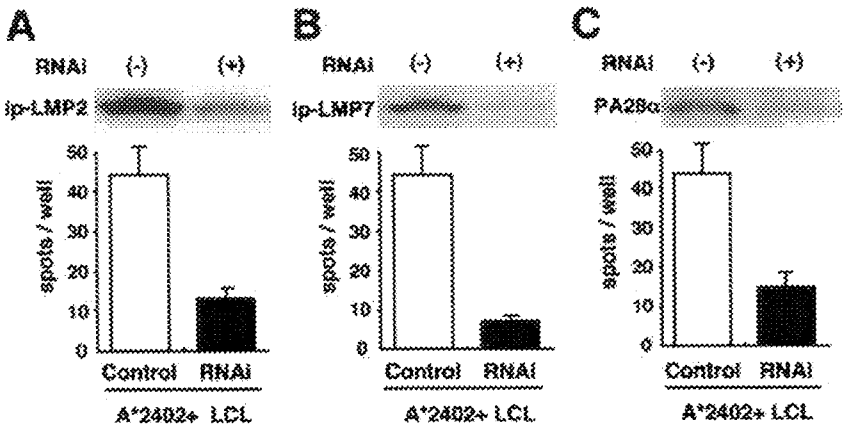

FIG. 3. Generation of $LMP2A_{222-230}$ is inhibited by RNA interference (RNAi) products targeting ip-LMP2 (A), ip-LMP7 (B), or PA28α (C) expression. Immunoproteasome-expressing HLA-A*2402-positive LCLs were infected with retrovirus vectors expressing shRNA for ip-LMP2, ip-LMP7, or PA28α. As a control stimulator, an LCL infected with a retrovirus vector expressing a nonsilencing shRNA was used. Inhibition of each subunit expression in shRNA-expressing LCLs was analyzed by Western blotting. $LMP2A_{222-230}$-CTL (5×10^3 cells/well) was cultured with each shRNA-transduced LCL for 20 h. Results of one representative experiment out of two are shown. Data are means plus or minus SD of spots in duplicate wells. −, absence of immunoproteasome or subunit; +, presence of immunoproteasome or subunit.

cells transduced with HLA-A*2402 and full-length EBV-LMP2A together with ip-LMP7 and/or PA28α were treated with puromycin for 30 min to generate short-lived premature proteins (6, 13, 47). We then analyzed the generation of $LMP2A_{222-230}$ by ELISPOT assay. As shown in Fig. 4A, puromycin treatment remarkably augmented $LMP2A_{222-230}$-CTL recognition on the cells expressing ip-LMP7 and PA28α. Interestingly, puromycin was capable of substituting either effect of ip-LMP7 and PA28α.

Next, we introduced truncated EBV-LMP2A of different lengths starting from isoleucine at position 222, the first amino acid of $LMP2A_{222-230}$, into expression vectors (Fig. 4B). The generation of $LMP2A_{222-230}$ was studied in 293T cells cotransfected with vectors encoding HLA-A*2402 and each truncated EBV-LMP2A without immunoproteasomes and PA28 subunits. Interestingly, the shortest EBV-LMP2A antigen was processed most efficiently and all truncated EBV-LMP2A antigens could be processed to generate $LMP2A_{222-230}$ without the aid of immunoproteasomes and PA28 (Fig. 4C). These data clearly demonstrated that the efficiency of $LMP2A_{222-230}$ generation is, at least in part, dependent on the length of the source protein.

Substitution of amino acid immediately flanking the C terminus of $LMP2A_{222-230}$, increasing the proteasome cleavage strength, results in efficient generation of $LMP2A_{222-230}$ in target cells. Finally, we investigated whether the amino acid cleavage strength at a specific position affects the processing of the $LMP2A_{222-230}$ epitope. To determine the cleavage strength of each amino acid in EBV-LMP2A, the program PAProC was used (http://www.paproc.de/). We focused on the cleavage strength, which is critical for epitope generation, of amino acids in the position immediately flanking the C termini of CTL epitopes (14, 34). First, we constructed a plasmid containing a mutant full-length LMP2A gene in which alanine replaced leucine at position 231; this was predicted to increase the cleavage strength after leucine at position 230, i.e., the C terminus of $LMP2A_{222-230}$ (Fig. 5A). It was thought that this change would facilitate $LMP2A_{222-230}$ generation by proteasomes. Target 293T cells were cotransfected with vectors encoding HLA-A*2402, ip-LMP7, PA28α, and the mutant EBV-LMP2A, and $LMP2A_{222-230}$-CTL recognition was evaluated using the ELISPOT assay. A remarkable increase was evident for cells expressing ip-LMP7 and PA28α (Fig. 5B), suggesting the processing of $LMP2A_{222-230}$ to be accelerated by the amino acid substitution at the specific position in the EBV-LMP2A antigen.

DISCUSSION

IFN-γ induces cells to express the proteasome subunits ip-LMP2, MECL-1, and ip-LMP7, leading to the formation of immunoproteasomes and the proteasome activator subunits PA28α and PA28β, comprising the activator complex. Early experiments with IFN-γ-treated cells demonstrated the generation of a number of epitopes to be affected by immunoproteasomes and PA28 (15, 32, 44). Immunoproteasomes have various cleavage site preferences as well as cleavage rates for the generation of some epitopes, while PA28 up-regulates epitope liberation via conformational changes within the proteasome 20S complex. Following the discovery that the influenza virus matrix-derived epitope required ip-LMP7 expression for its generation (3), the involvement of immunoproteasomes and PA28 subunits with different CTL epitopes received much attention. The results of the studies that investigated the effect of at least two immunoproteasome-associated molecules in the generation of CTL epitopes are summarized in Table 1 (1, 8, 10, 19, 23, 36, 38, 40, 41). The combination patterns of the five immunoproteasome-associated subunits fall into three categories. (i) PA28 alone, (ii) ip-LMP7 alone, and (iii) both ip-LMP2 and ip-LMP7 exerted the epitope generation. It has been hypothesized that immunoproteasomes and PA28 cooperate in antigen processing, but direct experimental evidence has hitherto been lacking. In this study, we found that the $LMP2A_{222-230}$ epitope has two unique features. First, coexpression of ip-LMP7 and one PA28 subunit is necessary for its

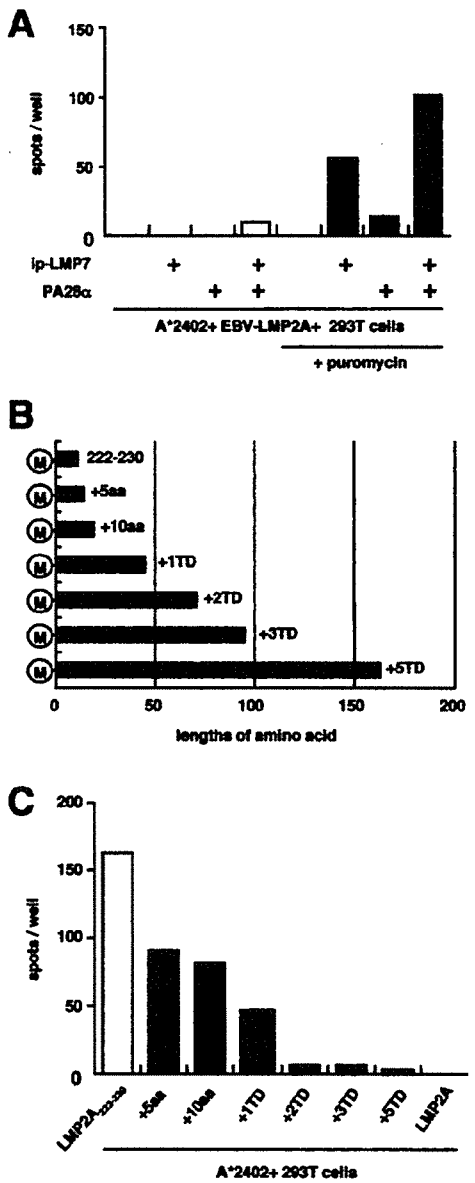

FIG. 4. Generation of $LMP2A_{222-230}$ from incomplete and shortened EBV-LMP2A as analyzed by ELISPOT assay. (A) $LMP2A_{222-230}$-CTL recognition of puromycin-treated (1 μg/ml for 30 min) or untreated target cells expressing EBV-LMP2A. 293T cells, cotransfected with plasmids encoding HLA-A*2402, full-length EBV-LMP2A, ip-LMP7, and/or PA28, were cultured with $LMP2A_{222-230}$-CTL (1×10^4 cells/well) for 20 h. For Ip-LMP7 and PA28 lanes, "+" indicates the presence of the immunoproteasome or subunit. (B) The length and structure of the truncated EBV-LMP2A fragments. Numbers of transmembrane domains (TDs) following $LMP2A_{222-230}$ in the fragments are shown; e.g., +3TD indicates fragment $LMP2A_{222-230}$ plus three transmembrane domains. Each TD located from the C terminus of $LMP2A_{222-230}$ is serially numbered. M, methionine. (C) $LMP2A_{222-230}$-CTL recognition of target cells expressing truncated EBV-LMP2A fragments in the absence of immunoproteasomes. The 293T cells were cotransfected with expression vectors encoding HLA-A*2402 and one truncated EBV-LMP2A and cultured with $LMP2A_{222-230}$-CTL (1×10^3 cells/well) for 20 h. Numbers of amino acids and transmembrane domains (TDs) following $LMP2A_{222-230}$ in the fragments are shown; e.g., +5aa indicates fragment $LMP2A_{222-230}$ plus five C-terminal amino acids. Results of one representative experiment out of two are shown. Each bar represents the average number of spots in duplicate.

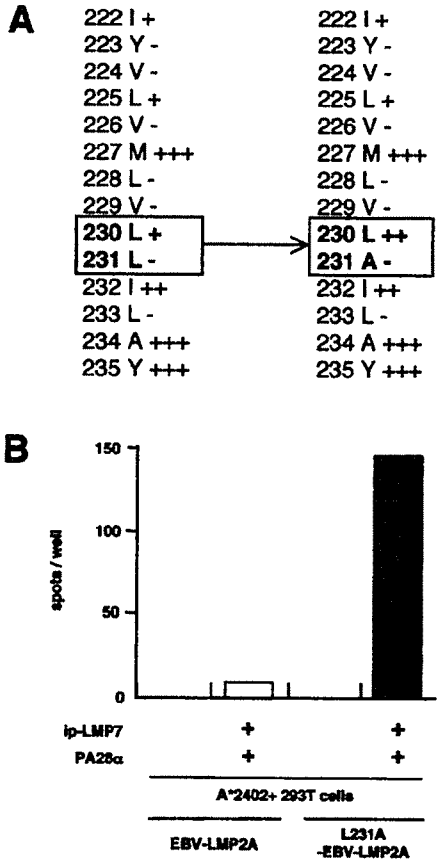

FIG. 5. Comparison of generation of $LMP2A_{222-230}$ from native and mutant EBV-LMP2A with alanine substituted for leucine at position 231 (referred as to L231A-EBV-LMP2A). (A) Partial amino acid sequences of EBV-LMP2A and L231A-EBV-LMP2A. The position numbers, single code letters for amino acids and predicted cleavage strengths are shown. Predictions by the program PAProC are scored as follows: −, no cleavage behind this position; +, ++, +++, cleavage behind this position, with a hint of the strength indicated by the number of +'s. (B) Generation of $LMP2A_{222-230}$ from EBV-LMP2A and L231A-EBV-LMP2A, analyzed by ELISPOT assay. 293T cells were cotransfected with plasmids encoding HLA A*2402, ip-LMP7, PA28α, and full-length EBV-LMP2A or L231A-EBV-LMP2A and cultured with $LMP2A_{222-230}$-CTL for 20 h. Results of one representative experiment out of two are shown. Each bar represents the average number of spots in duplicate. For Ip-LMP7 and PA28 lanes, "+" indicates the presence of the immunoproteasome or subunit.

generation. Second, ip-LMP2 has additional effects on epitope liberation. These data suggest that the processing of an IFN-γ-inducible epitope is controlled differentially by multiple immunoproteasome-associated subunits. To our best knowledge, this is the first documentation of molecular evidence of such cooperation.

Incorporation of the immunoproteasome is reported to be cooperative. The ip-LMP7 is required for immunoproteasome formation and maturation (9, 14). MECL-1 is incorporated if ip-LMP2 is present, while MECL-1 dependency for the incorporation of ip-LMP2 is under dispute (5, 11). Moreover, this cooperativity in forming proteasome complexes results in altered cleavage properties. In the present study, the generation

TABLE 1. Effects of immunoproteasomes and PA28 subunits by species on epitope generation[a]

Species	Source	Antigen	Epitope location	Epitope sequence	Immunoproteasome or subunit					Reference
					ip-LMP2	MECL1	ip-LMP7	PA28α	PA28β	
Mouse	Murine CMV	pp89	168–176	YPHFMPTNL	−	ND[b]	−	+	ND	11
Mouse	Influenza virus	NP	146–154	TYGRTRALV	−	ND	−	+	ND	11
Human	Influenza virus	Matrix	58–66	GILGFVFTL	−	ND	+	ND	ND	9
Human	HIV	RT	346–354	VIYQYMDDL	−	ND	+	ND	ND	38
Human	HBV	HBcAg	141–151	STLPETTVVRR	−	−	+	ND	ND	39
Human	Melanoma	MAGE-3	114–122	AELVHFLLL	−	−	+	ND	ND	36
Human	Melanoma	TRP2	360–368	TLDSQVMSL	−	−	−	+	+	41
Human	EBV	LMP2A	356–364	FLYALALLL	+	−	+	−	−	20
Human	Melanoma	Melan-A	26–35	ELAGIGILTV	+	−	+	ND	ND	23
Human	LCMV	gp	33–41	KAVYNFATC	+	ND	+	−	−	1
Human	LCMV	gp	276–286	SGVENPGGYCL[c]	+	ND	+	−	−	1
Human	EBV	LMP2A	222–230	IYVLVMLVL	+	−	+	+	−	Present study

[a] CMV, cytomegalovirus; HIV, human immunodeficiency virus; HBV, hepatitis B virus; LCMV, lymphocytic choriomeningitis virus; −, absence; +, presence.
[b] ND, not done.
[c] Generation of this epitope was inhibited by the expression of either ip-LMP7 or ip-LMP2.

of $LMP2A_{222-230}$ is enhanced by ip-LMP2 expression. This effect may be exerted through the functions of ip-LMP7 and PA28α, which induce the cleavages properties on the epitope generation.

In this study, we developed a retrovirus vector producing shRNA to confirm the effects of ip-LMP2, ip-LMP7, and PA28α in the generation of $LMP2A_{222-230}$. Generally, the use of chemically synthesized small interfering RNA or expression plasmids for shRNA is a more feasible way to test the involvement of target molecules but we believe that the retrovirus system has advantages in our case, because the effects of RNA interference in the target cells proved stable. After an epitope binds to MHC molecules and is presented on the cell surface, the complex exists for some time. Since there is a wide range in the life spans of the MHC-epitope complex, it is difficult to infer the sufficient duration to maintain inhibition of immunoproteasome-associated subunits to examine their effects in peptide liberation. In our retrovirus system, LCLs were cultured in medium containing puromycin for 14 days after retrovirus vector infection, and we assessed $LMP2A_{222-230}$ presentation on the surface. This procedure should exclude false-positive results that are observed with the ELISPOT assay.

DRiPs are thought to be important sources of CTL epitopes (29, 35, 49), as in the case of EBNA1, for example, for which epitopes are not readily generated from stable mature EBNA1 because of the glycine-alanine repeat domain within the protein (43, 46). EBV-LMP2A has 12 hydrophobic integral membrane sequences, and this hydrophobic-rich structure may inhibit epitope liberation (20). To address the question of whether the incomplete EBV-LMP2A might be superior to the mature complete EBV-LMP2A for epitope generation, we treated target cells with puromycin, which generates short-lived premature termination products from newly synthesized proteins (6, 13, 47). Interestingly, $LMP2A_{222-230}$ production was accelerated in puromycin-treated 293T cells expressing ip-LMP7 or PA28α, in contrast to the limited yield without puromycin treatment, even when the subunits were coexpressed. The data suggest that puromycin treatment is not sufficient to generate $LMP2A_{222-230}$ epitopes via constitutive proteasomes, but rather affects epitope generation by enhancing the effect of ip-LMP7 and PA28α. Next, we expressed a panel of shorter EBV-LMP2A fragments encompassing $LMP2A_{222-230}$ in target cells and compared their recognition to that of $LMP2A_{222-230}$-CTL. Each fragment started from the N terminus of $LMP2A_{222-230}$, as shown in Fig. 4B. This strategy should focus on the cleavage efficiency of the C-terminal side, which is performed exclusively by proteasomes (14, 34). We found that shorter EBV-LMP2A fragments were processed more efficiently. Therefore, the length of the source antigen may be a critical factor. Addition of two consecutive hydrophobic transmembrane domains substantially abrogated the epitope presentation. The obstacles presented by the intrinsic structure of EBV-LMP2A may be overcome by the effects of ip-LMP7 and PA28α in the generation of the $LMP2A_{222-230}$ epitope.

The cleavage efficiency at each amino acid varies widely in antigen proteins (25, 27, 44), and this may explain why one epitope is generated efficiently by proteasomes while another is not, even when processed from the same protein. Previous work showed that even a single amino acid substitution of asparagine for the aspartic acid immediately flanking the C terminus of the Moloney murine leukemia virus epitope SSWDFITV resulted in its abrogation (2). The program PAProC predicts that the cleavage strength of the C-terminal leucine in EBV-LMP2A is weak (17, 26), and substitution of an amino acid to increase the cleavage strength (from "+" to "++", as shown in Fig. 5A) resulted in remarkable up-regulation of $LMP2A_{222-230}$ liberation in cells expressing ip-LMP7 and PA28α.

EBV-LMP2A is thought to be an important antigen in EBV-related malignancies and is targeted by CTLs that recognize multiple epitopes located throughout the membrane-spanning molecules (20, 30, 31). Interestingly, EBV-LMP2A epitopes can be divided into two groups: (i) hydrophobic examples located in the transmembrane domain and processed in a TAP-independent manner and (ii) intertransmembrane hydrophilic epitopes, which are TAP-dependent (20). In addition, the generation of one hydrophobic epitope, $LMP2A_{356-364}$, requires ip-LMP7 and ip-LMP2 (19). Moreover, we here demonstrated that the processing of $LMP2A_{222-230}$ requires immunoproteasome subunits ip-LMP7 and PA28α and is enhanced by immunoproteasome subunit ip-LMP2. These two epitopes belong to

the former group, although the effects of immunoproteasome and PA28 subunits on other epitopes remain to be investigated. Potentially, EBV-LMP2A is a good model for determining the mechanisms by which immunoproteasomes and PA28 affect CTL epitope generation.

In conclusion, the present investigation provided evidence for differential roles of ip-LMP2, ip-LMP7, and PA28α in the generation of the $LMP2A_{222-230}$ epitope, which was most efficiently generated from incomplete EBV-LMP2A fragments and a mutated LMP2A gene with improved cleavage characteristics in cells expressing ip-LMP7 and PA28α. Although the precise function of each of the three subunits could not be clarified, we showed the generation of $LMP2A_{222-230}$ to be controlled by multiple factors. Further investigations on the differential effects of immunoproteasome-associated subunits could provide important information for understanding the presentation of viral and tumor antigens for CTL recognition.

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Bone marrow may be a reservoir of long-lived memory T cells specific for minor histocompatibility antigen

It has been shown that minor histocompatibility antigens (mHAgs) can function as targets for the graft-*versus*-leukaemia effect (Goulmy, 1997) following human leucocyte antigen (HLA)-identical allogeneic haematopoietic cell transplantation (HCT) and donor lymphocyte infusion (Marijt *et al*, 2003). We previously identified two haematopoietic-specific mHAgs, ACC-1 and ACC-2 (Akatsuka *et al*, 2003), and demonstrated that T cells specific for ACC-1 were detected in the peripheral blood (PB) up to 7 month postHCT in a patient from whom the original ACC-1-specific cytotoxic T cell (CTL) clone had been generated (Nishida *et al*, 2004). As it has recently been proposed that bone marrow (BM) can function as a secondary lymphoid organ and contribute to long-term T cell memory for pathogens and malignant disease (reviewed in Di Rosa & Pabst, 2005), this study was conducted to investigate whether T cells specific for mHAgs could feasibly be generated from BM, rather than PB, long after HCT.

Another patient who received an HLA-identical, ACC-1-disparate HCT for chronic myelomonocytic leukaemia was identified. At 14 months postHCT, she had complete donor chimerism in PB and remained disease free. After informed consent, we examined the phenotype and proliferative capacity of ACC-1-specific T cells in mononuclear cells (MCs) obtained from the patients' PB and BM. Three-colour flow cytometry detected a 3·5-fold higher percentage of ACC-1-specific T cells among the $CD8^+$ population in BM than PB (0·72% vs. 0·21%), with a trend of more $CD62L^+$ cells in the BM (Fig 1A, upper panels). After $CD8^+$ cell selection by immunomagnetic beads, $tetramer^+$ cells were further stained with antibodies

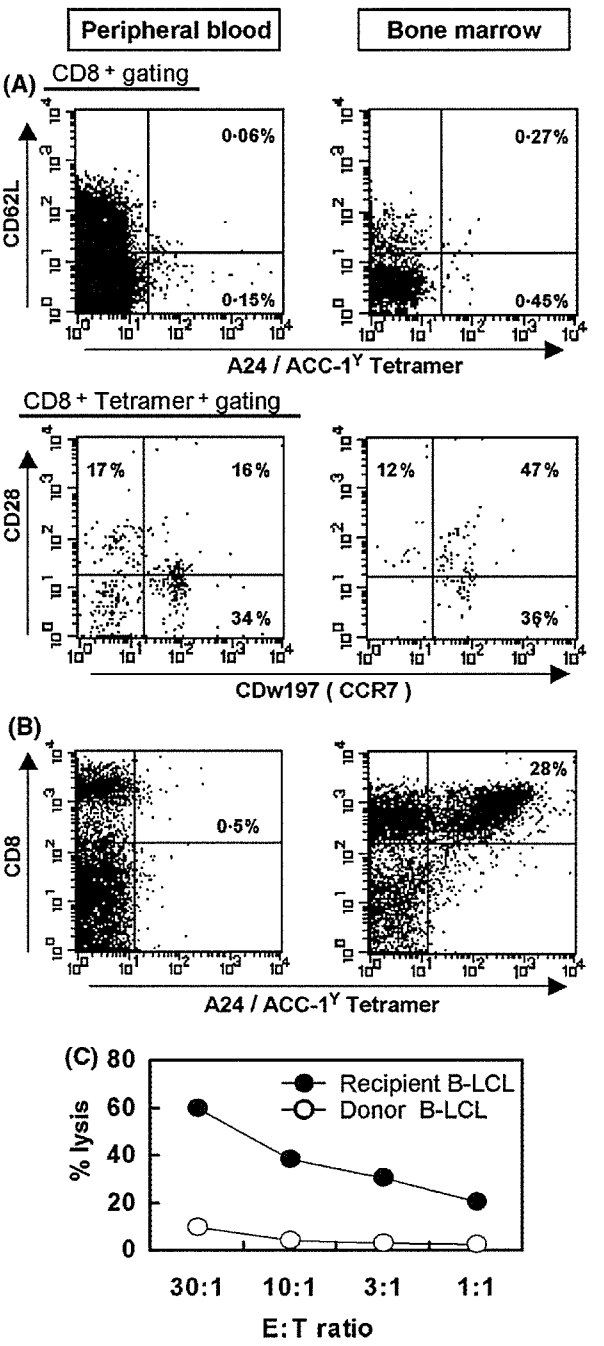

Fig 1. Characterisation of $CD8^+$ T cells specific for the ACC-1 minor histocompatibility antigen in peripheral blood (PB) and bone marrow (BM) at 14 months following human leucocyte antigen (HLA)-identical allogeneic haematopoietic cell transplantation. (A) Mononuclear cells (MC) were isolated from PB and BM and stained with fluorescence-conjugated monoclonal antibodies and HLA-A24/ACC-1 tetramer (Nishida *et al*, 2004). In lower panels, $CD8^+$ MC were first sorted and then stained as above. Percentages shown are of the gated T cells as indicated. (B) Remaining MC were stimulated with 0·1 μmol/l ACC-1 peptide (DYLQYVLQI) directly added to cell suspension on day 0 and 7 in RPMI 1640 medium supplemented with 6% pooled human serum. On day 14, donor-derived OKT3-activated $CD4^+$ blasts, pulsed with the same concentration of peptide, were added as antigen-presenting cells. Interleukin-2 (10 U/ml) was added on days 1 and 4 after the second and third stimulations. Growing T cells were stained as above. (C) Cytolytic activity of the T cell line generated from BM in the right panel of Fig 1B is shown.

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