厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

同種造血幹細胞移植における移植免疫反応に関与する要因の解析
移植片対白血病効果と組織適合性抗原

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研究要旨：同種造血幹細胞移植は自家移植や同系移植に比べ移植後の白血病の再発が少ないことが臨床的に示され、この白血病抑制効果を移植片対白血病効果（graft-versus-leukemia:GVL）効果と呼んでいる。ドナーと患者の組織適合性抗原の違いが如何に GVL 効果に影響を及ぼしているかを解明することを目的とし、非血縁者間骨髄移植を受けた白血病症例を対象に HLA 抗原の違いと白血病再発との関連を Cox regression model を用いた多変量解析法で解析した。この結果、（１）HLA-C 不適合症例では白血病再発率が低下し、（２）とくに急性リンパ性白血病で低下が著しかった。（３）HLA-C 型から推測できる NK 細胞受容体 KIR2DL ligand 不適合症例では反対に白血病再発が高率であることが明らかになった。さらに、（４）HLA-DPB1 不適合症例で有意に再発率が低く、とくに慢性骨髄性白血病で低下が著しかった。（５）HLA-A, -B, -DRB1, -DQB1 の違いでは白血病再発率に有意の差はなかった。上記知見は、関与する標的細胞上のＨＬＡ抗原の種類と白血病病型が重要であることを示しており、同種移植においてドナー由来のエフェクター細胞が白血病細胞を攻撃するＧＶＬの機序を解明するための基本的な情報を得ることができた。さらに、これら抗原を標的とする特異的細胞免疫療法開発の基礎データとして重要であると考えられた。

A. 研究目的

HLA-A, B, C, DRB1, DQB1, DPB1 遺伝子型適合度と臨床成績、とくに白血病の再発との関連を解析することにより、移植片対白血病効果（ＧＶＬ効果）の機序の解明と特異的細胞免疫療法の開発の基礎データを得ることを目的とする。

B. 研究方法

日本骨髄バンクで実施された白血病症例で HLA-A, B, C, DRB1, DQB1, DPB1 の DNA タイピングと臨床データが得られた 1790 症例を対象とした。T 細胞除去法を用いた症例と海外ドナー症例は除外した。さらに、NK 細胞受容体である KIR（killer cell Ig-like receptor ：KIR）2 DL の ligand 不適合（GVHD 方向）（KIRG）を HLA-C 型から推測した。KIR2DL1 は標的細胞の HLA-C の C1 エピトープ（Cw2,4,5,6 に共通）を認識し、この ligand 結合により NK 細胞の活性が抑制されることが判明している。同じく、KIR2DL2/3 は C2 エピトープ（Cw1, 3, 7, 8 に共通）と結合する。非血縁者間移植では、ドナーと患者の HLA—C 型が異なる場合に、この ligand 結合が外れる症例がある。GVHD 方向（ドナーのエフェクター細胞は活

性化される組み合わせ）のみの不適合は4.6%の症例、拒絶方向のみの不適合は5.8%の症例、両方向の不適合は0.5%の症例に認められた。

統計解析はCox regression modelsによる多変量解析法を使用し、変数として各HLA型の適合度とKIR ligand適合度、さらに患者・ドナーの年齢、性、性適合度、疾患、移植病期、TBIの有無、GVHD予防法などを用いた。

C. 研究結果

（1）HLA-C不適合症例では白血病再発率が低下した（表1）
Hazard ratio (HR) 0.71 p=0.025

（2）急性リンパ性白血病（ALL）においてHLA-C不適合症例では低下が著しかった（表3）。
HR 0.47 p=0.003

（3）HLA-C型から推測できるNK細胞受容体KIR2DL ligand不適合症例では白血病再発が高率であった（表1）。
HR 2.55 p=0.017

（4）HLA-DPB1不適合症例で有意に再発率が低かった（表1）。
HR 0.68 p=0.001

（5）慢性骨髄性白血病（CML）においてHLA-DPB1不適合症例では低下が著しかった（表4）。

（6）HLA-A, -B, -DRB1, -DQB1の違いでは白血病再発率に有意の差はなかった（表1−4）

D.考察

従来からＨＬＡ不適合でＧＶＬ効果が生じることは判明していたが、責任あるＨＬＡ座は明らかでなかった。本研究で、多数例の均一なＧＶＨＤ予防法を用いた症例を多変量解析したことにより、HLA-CとHLA-DPB1がGVL効果に関与する遺伝子座であることが明らかになった。さらに、NK細胞の受容体であるKIR2DLのリガンド適合度も同時に加えた多変量解析を行うことにより、HLAとKIRの影響を同時に解析することができ、HLA-C不適合の一部症例に生じるKIR 2 DL不適合（NKG）は逆に白血病再発を高めることが判明した。T細胞を介した抗腫瘍効果の機序に加えて、NK細胞受容体を介したNK細胞あるいはT細胞の機序が存在し臨床的に不利に働いていることが判明した。

さらに従来推測されていたHLA-A, Bの GVL効果が認められなかったことは、同種移植反応としてのGVL効果を解明する上で重要な知見と考えられた。

GVL効果が白血病の病型により異なることも新たな知見である。各型のHLA抗原の表出の違い、とくにHLA-CとHLA-DPB1の表出の有無の検索が必要であろう。また、急性骨髄性白血病（AML）ではHLAやKIRの影響がほとんど認められなかったことは、AML細胞が移植免疫反応に直接なんらかの影響を与えていることが推測される。

本研究で得られた知見により、ＧＶＬの機序を解明するための基本的な情報を得ることができた。さらに、これら抗原を標的とする特異的細胞免疫療法開発の基礎データとして重要であると考えられた。

E.結論

ドナーと患者のHLA遺伝子型を本研究班においてレトロスペクティブに同定・解析することにより、同種移植免疫、特にGVL効果に関する新しい知見：HLA-CとHLA-DPB1、KIRとGVL効果を集積することができ、これら抗原を標的とする特異的細胞免疫療法開発の基礎データを得ることができた。

F. 健康危険情報

特になし

G.研究発表

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表1. leukemia patient (all case n=1774)

	HR	95%CI	p-value
HLA A	1.19	0.89-1.58	0.251
HLA B	1.01	0.65-1.59	0.953
HLA C	**0.71**	**0.53-0.96**	**0.025**
HLA DR	1.05	0.73-1.53	0.725
HLA DQ	1.10	0.77-1.58	0.579
HLA DP	**0.68**	**0.55-0.85**	**0.001**
NKG	1.55	0.92-2.63	0.163
NKR	0.73	0.40-1.34	0.313

表2. acute myeloblastic leukemia (n=577)

	HR	95%CI	p-value
HLA A	0.92	0.54-1.58	0.961
HLA B	1.36	0.65-2.88	0.416
HLA C	0.8	0.49-1.30	0.366
HLA DR	0.78	0.40-1.52	0.466
HLA DQ	1.55	0.82-2.95	0.178
HLA DP	0.76	0.52-1.09	0.137
NKG	1.05	0.37-3.01	0.926
NKR	0.52	0.15-1.78	0.305

表3. acute lymphoblastic leukamia (n=616)

	HR	95%CI	p-value
HLA A	1.18	0.76-1.86	0.462
HLA B	0.99	0.48-1.98	0.952
HLA C	**0.47**	**0.28-0.78**	**0.003**
HLA DR	0.91	0.51-1.61	0.737
HLA DQ	1.11	0.63-1.95	0.71
HLA DP	0.92	0.65-1.28	0.604
NKG	**2.55**	**1.18-5.52**	**0.017**
NKR	1.30	0.53-3.19	0.569

表 4. chromic myeloid leukemia (n=596)

	HR	95%CI	p-value
HLA A	1.62	0.89-2.97	0.114
HLA B	0.62	0.22-1.76	0.367
HLA C	1.2	0.62-2.29	0.591
HLA DR	1.25	0.55-2.84	0.59
HLA DQ	0.86	0.39-1.93	0.72
HLA DP	**0.35**	**0.21-0.58**	**<0.001**
NKG	1.23	0.38-3.94	0.727
NKR	0.51	0.14-1.80	0.292

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

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IV. 研究成果の刊行物・別刷

European Journal of Immunology Immunity to infection

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

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

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Introduction

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

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Abbreviations: **CAEBV:** chronic active EBV infection · **CD40-B:** CD40-activated B · **EBNA:** EBV nuclear antigen · **EGFP:** enhanced GFP · **ELISPOT:** enzyme-linked immunospot · **HD:** Hodgkin's disease · **ip-LMP:** low-molecular-weight protein of immunoproteasome subunit · **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

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

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

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

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

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

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

Results

Induction of LMP1-specific CTL using mRNA-transduced APC

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

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

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

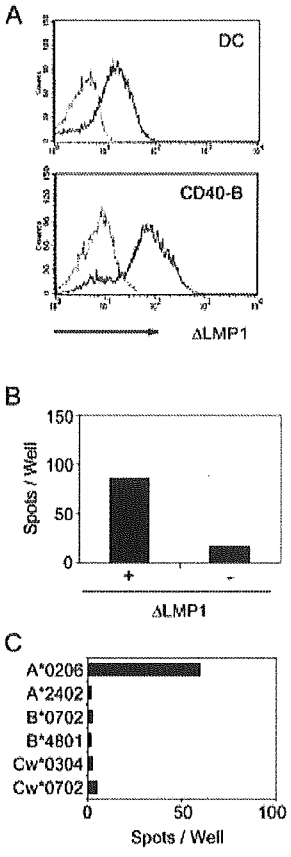

Figure 1. mRNA-transduced DC and CD40-B cells express ΔLMP1 and induce specific T cells. (A) DC and CD40-B cells were transduced with ΔLMP1 mRNA and analyzed for expression of ΔLMP1 by FCM at 36 h post-transduction. The dotted lines show non-transduced, and the solid lines ΔLMP1-transduced cells. (B)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

Identification of the LMP1 epitope

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

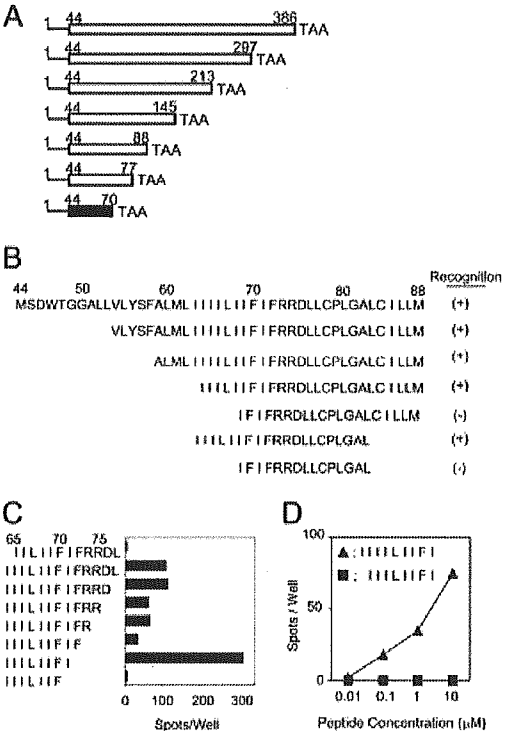

Figure 2. Identification of the LMP1 epitope peptide recognized by H7 CTL clone. (A) A series of C-terminally truncated ΔLMP1 mRNA were generated by *in vitro* transcription. A methionine at amino acid position 44 was used as the initiation codon for all constructs. CD40-B cells transduced with each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

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

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

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

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

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

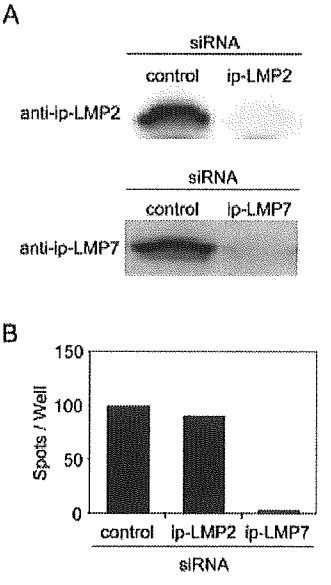

Figure 3. ip-LMP7 is essential for the LMP1 epitope processing. (A) Control siRNA, ip-LMP2 siRNA, or ip-LMP7 siRNA were retrovirally transduced into autologous LCL and the cells were selected for 14 days with puromycin, followed by Western blot analysis of ip-LMP2 (upper panel) and ip-LMP7 (lower panel).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

Cytotoxic activity of the LMP1-specific CTL clone against LCL

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

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

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

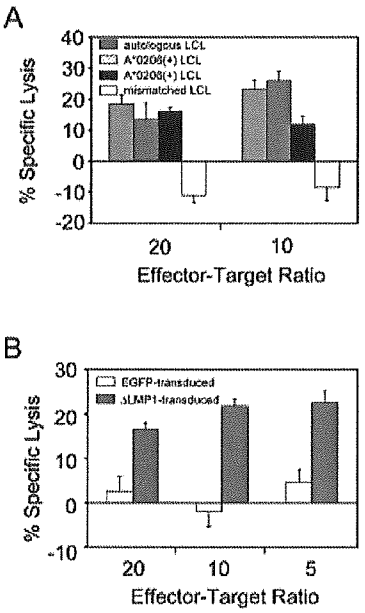

Figure 4. Cytotoxic activity of the LMP1-specific CTL clone H7. (A) Sixteen-hour CTL assays were performed using autologous, HLA-A*0206-shared, and fully HLA-mismatched LCL as target cells. (B) Four-hour CTL assays were performed using ΔLMP1- or EGFP-transfected LCL as target cells. Each bar represents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

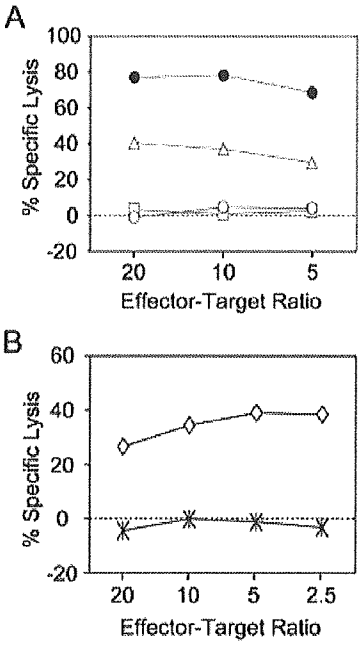

Figure 5. CTL specifically lyse EBV-infected NK cells. (A) Cytolytic activity of the CTL clone H7 was assessed against EBV-carrying NK cells in 16-h CTL assays. Data for two HLA-A*0206-positive NK cell lines (SNK-6 and SNK-10) and one HLA-A*0206-negative NK cell line (HANK-1) are shown with open circles,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

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

Discussion

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

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

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

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

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

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

Materials and methods

Donors and cell lines

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

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-

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

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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

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