sets were used, to determine promoter usage. Approximately 50 ng of total RNA (converted to cDNA) was used as template. The amplified products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized by UV light. To detect *BZLF1*, which encodes a transactivator protein and is expressed in lytic cycle infection, nested PCR was performed with the primers described by Prang et al. [24]. To detect the gene for gp350/220, which is also expressed in lytic cycle infection, nested PCR was performed with the outer primers described by Kelleher et al. [25] and newly selected inner primers (5′-CATCACCGGTGACACCAAGT-3′ and 5′-TGCTGGCGAACTGGTGGACA-3′). To detect a housekeeping gene, the human glyceraldehyde-3-phosphate dehydrogenase gene was amplified by single PCR; the sequences of the primer pair were 5′-GAAGGTGAAGGTCGGAGT-3′ and 5′-GAAGATGGTGATGGGATTTC-3′. All of the primer pairs used in the present study were designed to span introns, to avoid amplification of genomic DNA.

Quantification of cytokine gene transcription by use of a real-time PCR assay. Cytokine gene transcription was quantified by use of a real-time PCR assay, as described elsewhere [18]. Transcription of the genes for IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p35, IL15, IFN-γ, and TNF-α was assessed by use of a TaqMan Cytokine Gene Expression Plate 1 (Applied Biosystems). A predeveloped primer/probe (Assays-On-Demand Gene Expression Products; Applied Biosystems) was used to measure IL-13 separately. Each well contained TaqMan primers and probes for assaying human cytokine mRNA and rRNA as an endogenous control. Approximately 25 ng of total RNA (converted to cDNA) was used to quantify the expression of each cytokine gene. All of the assays were conducted in duplicate. To calculate the relative expression of a cytokine gene in cells, the value for the expression of a cytokine gene was divided by that for the internal control rRNA, by use of the comparative threshold cycle method described by the manufacturer (P/N 4306744; Applied Biosystems) [18, 26].

Determination of plasma cytokine concentration by use of ELISA. Plasma cytokine concentrations were determined in all of the patients. Plasma concentrations of IL-1β, IL-4, IL-10, IL-12, IL-13, and IFN-γ were determined by use of sandwich-type ELISA kits (R&D Systems); these assays were conducted in accordance with the manufacturer's instructions [18, 27]. Sample values were determined from a standard curve. The minimum detectable concentrations of IL-1β, IL-4, IL-10, IL-12, IL-13, and IFN-γ were 1, 10, 3.9, 5, 32, and 8 pg/mL, respectively.

Statistical analyses. Statistical analyses were conducted by use of StatView software (version 5.0; SAS Institute). Fisher's exact test or the χ^2 test was used to compare differences in clinical measurements. The Mann-Whitney *U* test was used for statistical comparisons of laboratory data, viral load, cytokine gene expression, and plasma cytokine concentrations. $P < .05$ was considered to be statistically significant.

RESULTS

Characteristics of T cell–type and NK cell–type CAEBV infection. Of the 39 patients with CAEBV infection, 20 had infections that were defined as T cell type, and 19 had infections that were defined as NK cell type. The differences in clinical and laboratory measurements between the 2 types of CAEBV infections are shown in table 1. The patients with T cell–type infection had a poorer prognosis (death rate, 60%); 12 of them died, with the causes of death including hepatic failure ($n = 4$), malignant lymphoma ($n = 2$), and cardiac complications ($n = 2$) (for death by other causes, $n = 4$). Three patients received hematopoietic stem-cell transplantation; 2 are still alive, and 1 relapsed and died shortly afterward. T cell–type infection was strongly characterized by high fever and anemia; other characteristics of T cell–type infection were hepatomegaly and lymphadenopathy, although the statistical significance was marginal. In contrast, the patients with NK cell–type infection had a better prognosis (death rate, 26%); 5 of them died, with the causes of death including complications related to hematopoietic stem-cell transplantation ($n = 3$), sepsis ($n = 1$), and interstitial pneumonia ($n = 1$). Seven patients received hematopoietic stem-cell transplantation; 4 are still alive, and 3 died of transplantation-related complications. NK cell–type infection was characterized by large granular lymphocytosis, hypersensitivity to mosquito bites, and a high IgE concentration. These observed differences between the 2 types of CAEBV infection are in agreement with the results of our previous study [5].

Determination of EBV-infected cells. For 28 patients, fractionation of PBMCs followed by quantitative PCR was used to determine the cell types that were infected (table 2). Using this method, we determined that 15 patients had T cell–type infection, because $CD3^+$ cells contained more EBV DNA than did the other cell populations. In 3 of these patients, mainly $CD4^+$ T cells were infected; in 2 of these patients, mainly $CD8^+$ T cells were infected. In contrast, mainly $CD16^+$ cells (not $CD3^+$ cells) were infected in 13 patients, who were therefore determined to have NK cell–type infection (table 2). In some patients, both $CD16^+$ and $CD19^+$ cells contained more EBV DNA than did unfractionated cells, suggesting that both NK and B cells were infected with EBV.

For the remaining 11 patients, in situ hybridization was used to determine which cell types were infected. For 3 patients with NK cell–type infection, infected cells were identified by fractionating PBMCs and then performing EBER-1 in situ hybridization. EBER-1 was detected in 15%, 25%, and 60% of the $CD56^+$ cells in these 3 patients, indicating that most of the infected cells were NK cells (as described elsewhere [28]). For the other 8 patients, tissue samples were used to identify in-

Table 1. Differences in clinical and laboratory measurements between T cell–type and NK cell–type chronic active Epstein-Barr virus (EBV) infection.

	T cell–type infection (n = 20)	NK cell–type infection (n = 19)	P
Sex, M:F, no.	10:10	11:8	.43
Age at onset, mean ± SD, years	12.6 ± 9.5	8.0 ± 4.9	.19
Death rate, %	**60**	26	**.03**
Time to death from onset, mean ± SD, years	2.9 ± 1.3	5.4 ± 3.8	.27
Symptoms			
Fever >1 day/week, %	**81**	38	**.01**
Hepatomegaly, %	79	53	.09
Lymphadenopathy, %	58	32	.096
Large granular lymphocytosis, %	11	**58**	**.003**
Hypersensitivity to mosquito bites, %	11	**68**	**.0002**
Laboratory data			
WBC count, mean ± SD, cells/μL	5200 ± 5500	6100 ± 3200	.11
Hemoglobin concentration, mean ± SD, g/dL	**10.7 ± 1.2**	12.1 ± 1.9	**.03**
Platelet count, mean ± SD, 10^4 cells/μL	21.4 ± 7.6	19.2 ± 8.5	.52
IgG level, mean ± SD, mg/dL	1990 ± 1440	1590 ± 450	.36
IgE level, mean ± SD, mg/dL	190 ± 220	**5650 ± 6470**	**.002**
EBV-related antibody			
Anti-VCA IgG level, GMT	**2010**	310	**.001**
Anti–EA-DR IgG level, GMT	**610**	70	**.007**
Anti-EBNA level, GMT	27	45	.12
Viral load			
PBMCs, mean ± SD, copies/μg	$10^{3.9 \pm 0.7}$	$\mathbf{10^{4.4 \pm 0.6}}$	**.03**
Plasma, mean ± SD, copies/mL	$10^{2.8 \pm 1.1}$	$10^{2.5 \pm 2.0}$	.87

NOTE. Values in boldface indicate statistically significant results. Either Fisher's exact test or the χ^2 test was used to compare symptoms; the Mann-Whitney U test was used to compare laboratory data and viral load. EA-DR, early antigen–diffuse restricted; EBNA, EBV nuclear antigens; F, female; GMT, geometric mean titer; M, male; PBMCs, peripheral-blood mononuclear cells; VCA, viral capsid antigen; WBC, white blood cell.

fected cells. Double labeling with EBER-1 and surface markers showed that, in 5 patients, most EBER-1–positive cells were $CD3^+$, indicating that they had T cell–type infection. In 3 patients, most EBER-1–positive cells were $CD16^+$ (not $CD3^+$), indicating that they had NK cell–type infection. Some of these results have been described elsewhere [29].

Virologic analyses. EBV-related antibody titers were compared between the 2 types of CAEBV infection. The patients with T cell–type infection had significantly higher titers of anti-VCA and anti–EA-DR IgG (table 1). The anti-EBNA antibody titers were comparable between the 2 types of CAEBV infection. The higher titers of antibody against early and late EBV antigens (i.e., anti–EA-DR IgG and anti-VCA IgG) but not against latent antigen (i.e., anti-EBNA antibody) suggested the possibility of lytic cycle infection in T cells.

To examine whether lytic cycle infection existed in EBV-infected T or NK cells, for 19 patients from whom fresh samples were available (for T cell–type infection, n = 11; for NK cell–type infection, n = 8), reverse-transcription PCR was used to examine EBV gene expression in PBMCs. *BZLF1*, which encodes a transactivator protein and is a hallmark of lytic cycle infection [1], was not detected in any of the 19 patients (table 3); another lytic gene, for gp350/220, was also not detected. *EBNA1*, *LMP1*, and *LMP2A* were detected in PBMCs from nearly one-half of the 19 patients, indicating that they had the latency type II pattern. The Qp promoter, but not the Cp/Wp promoter, was used for *EBNA1* transcription. Representative results are shown in figure 1. Because it was possible that lytic cycle infection was present at sites other than PBMCs, EBV gene expression was examined in autopsy or biopsy samples from 2 patients with T cell–type infection. The *BZLF1* and gp350/220 genes were not detected in the livers, lymph nodes, or spleens of these patients (data not shown). The pattern of EBV gene expression in these tissue samples was also latency type II.

Next, the viral load in peripheral blood was investigated by use of real-time PCR. The viral load in PBMCs was higher in patients with NK cell–type infection (table 1). Interestingly, the viral load in plasma was similar between the patients with each type. To examine the viral load in plasma, plasma samples from selected patients were digested with deoxyribonuclease before extraction of DNA. As a preliminary experiment, pGEM-BALF5, a control plasmid DNA containing an EBV target gene, and the supernatant of an LCL culture containing enveloped virions were tested. The control plasmid DNA was sensitive to deoxy-

Table 2. Determination of Epstein-Barr virus (EBV)–infected cells in peripheral-blood mononuclear cells.

Type of chronic active EBV infection, patient	EBV DNA, copies/μg						Mainly infected cells	Clonality of cells
	Fractionated cells					Unfractionated cells		
	$CD3^+$	$CD4^+$	$CD8^+$	$CD16^+$	$CD19^+$			
T cell								
1	**220,000**	760	**230,000**	100,000	760	210,000	T ($CD8^+$)	Monoclonal
2	**90,000**	ND	ND	17,000	15,000	24,000	T	ND
3	**43,000**	**21,000**	1500	**12,500**	2400	8600	T ($CD4^+$)	Monoclonal
4	**23,000**	ND	ND	1100	1600	14,000	T	Monoclonal
5	**18,000**	ND	ND	6200	0	7300	T	ND
6	**17,000**	ND	ND	4300	5200	16,000	T	ND
7	**12,000**	ND	ND	5700	2700	11,000	T	ND
8[a]	12,000	4500	2500	6500	5000	51,000	T	Monoclonal
9	**11,600**	ND	ND	2700	2000	6100	T	Monoclonal
10	10,000	27,000	4900	1900	1200	42,000	T ($CD4^+$)	Oligoclonal
11	**7600**	ND	ND	80	260	6600	T	ND
12	**6600**	ND	ND	0	900	3900	T	Polyclonal
13	**3600**	ND	ND	840	950	3200	T	ND
14	**400**	**720**	90	250	20	340	T ($CD4^+$)	Monoclonal
15	160	10	**220**	0	20	200	T ($CD8^+$)	Polyclonal
NK cell								
1	120,000	ND	ND	**400,000**	**470,000**	210,000	NK, B	Monoclonal
2	21,000	ND	ND	**170,000**	3200	110,000	NK	Monoclonal
3	7400	ND	ND	**89,000**	17,000	78,000	NK	ND
4	11,000	ND	ND	**86,000**	18,000	75,000	NK	Monoclonal
5	10,000	ND	ND	**54,000**	23,000	36,000	NK	Monoclonal
6	3300	ND	ND	**35,000**	1900	20,000	NK	Monoclonal
7	7600	ND	ND	25,000	1700	28,000	NK	ND
8[a]	1800	ND	ND	**16,000**	**9200**	7000	NK, B	Oligoclonal
9	300	ND	ND	**15,000**	0	2000	NK	ND
10	200	ND	ND	15,000	1800	31,000	NK	ND
11	1600	ND	ND	4500	570	8100	NK	Monoclonal
12	50	ND	ND	**4300**	110	1600	NK	Oligoclonal
13	0	ND	ND	**2700**	**1300**	820	NK, B	ND

NOTE. Values in boldface indicate that EBV DNA was concentrated after fractionation. ND, not done.

[a] Infected cells were confirmed by double labeling of tissue samples.

ribonuclease (percentage of reduction of EBV DNA after digestion, 99.9%), but the LCL supernatant was resistant to the enzyme (percentage of reduction of EBV DNA after digestion, 55.7%). Five plasma samples from each group of patients were tested. After deoxyribonuclease digestion, the percentages of reduction of EBV DNA were 100%, 92.8%, 96.8%, 97.3%, and 100% in the samples from the patients with T cell–type infection and 99.8%, 98.3%, 100%, 100%, and 100% in the samples from the patients with NK cell–type infection. Thus, plasma from both groups of patients was sensitive to deoxyribonuclease, indicating that most of the EBV DNA in plasma, rather than consisting of enveloped virions, consisted of free EBV DNA molecules, which were likely derived from dead or damaged cells.

The clonality of EBV was analyzed by use of Southern blotting. The majority of both T cell–type and NK cell–type infections were monoclonal (table 2). There was a trend in that those patients with polyclonal or oligoclonal proliferation had lower viral loads, although there was no difference between the 2 types of CAEBV infection.

Cytokine profiles. Differences in the symptoms or immunologic responses between the 2 types of CAEBV infection might be due to the differences in the cytokine production profiles of either EBV-infected cells themselves or inflammatory cells. The plasma concentrations of cytokines (IL-1β, IL-4, IL-10, IL-12, IL-13, and IFN-γ) were estimated and compared between the 2 types of CAEBV infection. IL-1β, IL-10, and IFN-γ, none of which are detected in healthy individuals, were detected in plasma from many patients with CAEBV infection (figure 2); there were no significant differences between the T cell–type and NK cell–type infections. IL-13, which also is not detected in healthy individuals, was frequently detected in the patients with NK cell–type infection, and the concentration was higher than that in the patients with T cell–type infection. The patients who developed

Table 3. Summary of Epstein-Barr virus (EBV) gene expression in peripheral-blood mononuclear cells.

Type of chronic active EBV infection	*EBNA1*[a]			*EBNA2*	*LMP1*	*LMP2A*	*BZLF1*	gp350/220	*GAPDH*
	Common	Qp	Cp/Wp						
T cell (n = 11)	4	1	0	0	4	3	0	0	11
NK cell (n = 8)	4	3	0	0	4	4	0	0	8

NOTE. Data are no. of samples positive for the indicated gene. EBNA, EBV nuclear antigens; GAPDH, human glyceraldehyde-3-phosphate dehydrogenase; LMP, latent membrane protein.

[a] For *EBNA1*, 3 different primer sets were used to determine promoter usage.

hemophagocytic syndrome had high concentrations of inflammatory cytokines, such as IL-1β and IFN-γ.

Next, the transcription of cytokine genes in PBMCs was investigated in the 19 patients from whom fresh samples were available. Assays were performed for IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p35, IL-13, IL-15, IFN-γ, and TNF-α. Transcription of the genes for IL-1α, IL-1β, IL-10, IL-13, IL-12p35, IL-15, TNF-α, and IFN-γ was high in the patients with CAEBV infection, whereas transcription of the genes for IL-4 and IL-5 was undetectable in most patients. There were no statistical differences in the transcription of these cytokine genes between the 2 types of CAEBV infection, including for IL-13.

DISCUSSION

It has been shown that infections of T and NK cells play a central role in the pathogenesis of CAEBV infection. A recent national survey in Japan showed that the majority of patients with CAEBV infection had infections that belonged to either the T cell type or the NK cell type; only 2 of 82 patients had an infection that mainly involved B cells [12]. EBV-infected T cell or NK cell lines have been established from patients with CAEBV infection [20, 21]. In the present study, we identified 20 patients with CAEBV infection in whom mainly T cells were infected and 19 patients with CAEBV infection in whom mainly NK cells were infected. Recently, a small-scale study reported that, during acute EBV-associated hemophagocytic lymphohistiocytosis, EBV infection was predominant in $CD8^+$ cells, but that, in patients with CAEBV infection, EBV infection was predominant in non-$CD8^+$ cells [30]. However, the results of the present study indicated that, in some of our patients with CAEBV infection, EBV infection was predominant in $CD8^+$ T cells.

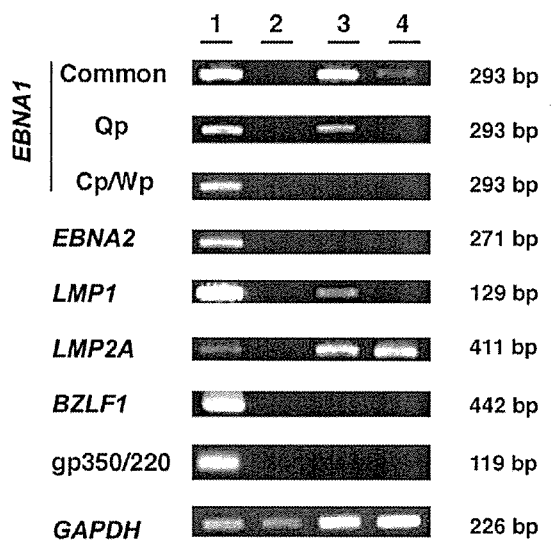

Figure 1. Expression of Epstein-Barr virus (EBV) genes in peripheral blood. *Lane 1:* EBV-positive lymphoblastoid cell line. *Lane 2:* BJAB, an EBV-negative B cell line. *Lane 3:* Peripheral blood from a patient with T cell–type chronic active EBV (CAEBV) infection. *Lane 4:* Peripheral blood from a patient with NK cell–type CAEBV infection. EBNA, EBV nuclear antigens; GAPDH, human glyceraldehyde-3-phosphate dehydrogenase; LMP, latent membrane protein.

To determine which cells were infected with EBV, we used immunomagnetic cell isolation to fractionate PBMCs, followed by quantitative PCR. This method is very rapid and convenient, but its disadvantage is the relatively poor purity of the selected cells. EBV-infected cells can contaminate uninfected cell fractions. Furthermore, the real-time PCR assay is so sensitive that the contaminating EBV genome can be detected; therefore, this method can determine only the cell population that is mainly infected. The low levels of EBV DNA seen in other cell populations do not always mean that they are infected with EBV; however, the results of the present study showed that, in some of our patients, both NK cells and B cells were infected with EBV (table 2). Perhaps >1 cell lineage harbors EBV in some patients with CAEBV infection. Electric cell sorting followed by EBER-1 in situ hybridization is a more accurate method for determining EBV-infected cell lineages. Using this method, Kasahara et al. found that, in some patients with CAEBV infection, different cell lineages were infected [30].

Originally, CAEBV infection was characterized by an unusual pattern of EBV-related antibodies, such as high titers of anti-VCA and anti–EA-DR IgG or the absence of anti-EBNA antibody [3, 4, 6, 31], although recent observations indicate that high titers of these EBV-related antibodies are not necessary for CAEBV infection to be diagnosed [5]. In patients with CAEBV infection—and especially in patients with T cell–type infection who have high titers of EBV-related antibody—high

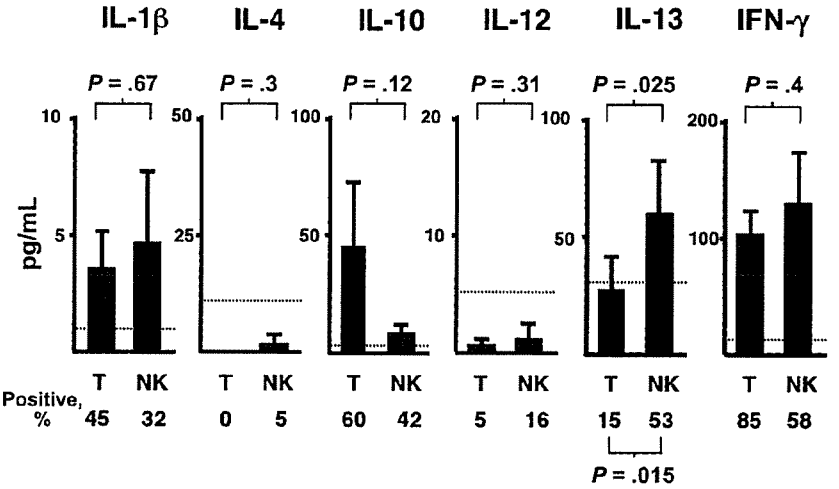

Figure 2. Comparison of plasma cytokine concentrations. Concentrations were estimated by use of an immunoassay and compared between patients with T cell–type ($n = 20$) and NK cell–type ($n = 19$) chronic active Epstein-Barr virus infection. Boxes and error bars indicate means and SEs, respectively; the dotted lines indicate the upper limits of healthy individuals. The Mann-Whitney U test was used to compare plasma cytokine concentrations, and Fisher's exact test was used to compare positivity rates. IFN, interferon; IL, interleukin.

titers of antibodies against the early and late EBV antigens and the existence of cell-free EBV DNA in plasma suggest the possibility of lytic cycle infection [6, 32, 33]. In the present study, a deoxyribonuclease-digestion experiment showed that the presence of EBV DNA in plasma was attributable to free nucleic acids that were likely released from dead or damaged cells. Furthermore, the pattern of EBV gene expression in PBMCs was latency type II, which supports the absence of lytic cycle replication in the PBMCs, at least, of patients with CAEBV infection. Lytic cycle infection may occur in tissue, although our results for tissue samples, while limited, showed no sign of a lytic cycle. It is also possible that we did not detect the occurrence of lytic cycle replication if lytic cycle infection occurred in <1% of EBV-infected cells. There is no definite proof of lytic cycle replication in tissues from patients with CAEBV infection. Some investigators have reported the expression of early or late EBV gene transcripts, such as those for BZLF1 or viral IL-10 [30, 34, 35], whereas other investigators have reported the absence of expression of these transcripts in tissue samples [36].

Our observation here of the absence of lytic cycle infection is particularly important with regard to selection of the treatment strategy for patients with CAEBV infection. Antiviral drugs that suppress viral DNA polymerase and lytic cycle replication may not be suitable for the treatment of CAEBV infection; however, therapies that reduce or eliminate EBV-infected T cells or NK cells may be suitable choices. Chemotherapy or hematopoietic stem-cell transplantation are suitable in this regard—the successful treatment of CAEBV infection by hematopoietic stem-cell transplantation has been reported [37, 38]. Alternatively, EBV-related antigens expressed in T cells or NK cells may be the targets of treatment. Cytotoxic T cells that were generated from LCL and targeted to latency type III antigens have been administered to patients with CAEBV infection [39, 40]. On the basis of the present result that the pattern of EBV gene expression was latency type II, cytotoxic T cells specific for latency type II antigens, such as LMP1 or LMP2A, would be more favorable for the control and eradication EBV-infected cells if they are inducible [41, 42].

In the present study, patients with CAEBV infection had high concentrations of proinflammatory (IL-1β), Th1-type (IFN-γ), and anti-inflammatory (IL-10) cytokines. Transcription of the genes for these cytokines was also high in PBMCs. The up-regulation of various cytokine genes has also been reported in patients with CAEBV infection in other studies [43–45]. These cytokines are thought to be produced either by EBV-infected T cells or NK cells or by reacting inflammatory cells. On the one hand, it has been shown that EBV-infected T cells produce proinflammatory (IL-6 and TNF-α), Th1-type (IL-2 and IFN-γ), and anti-inflammatory (transforming growth factor β1) cytokines [14, 46]. Shen et al. reported that, during EBV-infected nasal NK/T cell lymphoma, human IL-10, an anti-inflammatory cytokine that suppresses cytotoxicity against EBV-infected cells, was expressed [47]. On the other hand, reacting inflammatory cells, such as macrophages, can produce most of the cytokines seen in the present study. Unfortunately, because of our study design, it is impossible to determine whether EBV-infected or reacting cells were the main sources of these cytokines. However, the high concentrations of and the elevated

transcription of genes for various cytokines must contribute to the diverse symptoms seen in patients with CAEBV infection.

One of the purposes of the present study was to find virologic differences between the T cell–type and the NK cell–type infection. One main difference was that we found frequent detection and a high concentration of IL-13 in the patients with NK cell–type infection. IL-13 is a Th2-type cytokine that induces the differentiation of B cells, the production of antigen-specific antibody, and a class switch to IgE and that also suppresses the cytotoxic functions of monocytes and macrophages [48]. IL-13 is primarily produced by activated T cells and is not usually detected in plasma from healthy individuals [49]. The cytokine is produced by Reed-Sternberg cells during Hodgkin disease, which is associated with EBV infection [50]. Although the reason why IL-13 was produced in the patients with NK cell–type infection is unclear, the high concentration of IL-13 may explain the high serum IgE levels and the hypersensitivity to mosquito bites, both of which are frequently seen in patients with NK cell–type infection [5].

The other difference between the 2 types of CAEBV infection is that the patients with NK cell–type infection had a higher viral load in PBMCs. This is particularly interesting, because NK cell–type infection is usually milder and progresses slowly [5, 12]. In contrast, the viral load in plasma was similar between the 2 types of CAEBV infection. These results suggest that sources of EBV DNA other than PBMCs exist in patients with T cell–type disease, the more severe, rapid type of CAEBV infection. In patients with T cell–type infection, the cell-free EBV DNA may come from tissue, such as lymph nodes or the spleen, where EBV-infected T cells infiltrate and proliferate. Indeed, patients with T cell–type infection have a higher incidence of hepatomegaly and lymphadenopathy, as was shown in the present study. The higher viral load in plasma could also be explained by the naturally high rate of apoptosis in activated T cells. It is still unclear why T cell–type infection is severe and progresses rapidly. The distribution of infected cells, determined by the differences in homing receptors among cells, may determine the symptoms and prognosis. A recent animal model showed that activated T cells are selectively trapped in the liver, primarily by intracellular adhesion molecule 1, which is constitutively expressed on sinusoidal endothelial cells and Kupffer cells [51]. We previously reported a patient with primary EBV infection who had severe hepatitis and whose liver was infiltrated with EBV-infected $CD8^+$ cells. In patients with T cell–type CAEBV infection, EBV-infected, presumably activated T cells might accumulate in the liver and cause hepatitis. Although further studies are necessary, our findings should help to clarify the pathogenesis of CAEBV infection and facilitate the development of more-effective treatments.

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RESEARCH ARTICLE

Retroviral vector backbone immunogenicity: identification of cytotoxic T-cell epitopes in retroviral vector-packaging sequences

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*Retroviral vectors are the frequently applied gene delivery vehicles for clinical gene therapy, but specificity of the immunogenicity to the protein encoded by the inserted gene of interest is a problem which needs to be overcome. Here, we describe human cytotoxic T-lymphocyte (CTL) clones recognizing epitopes derived from the protein encoded by the retroviral vector backbone, which were established during the course of our attempts to generate CTLs against cytomegalovirus (CMV) or human papilloma virus (HPV) in vitro. In the case of healthy CMV-seronegative donors, CTL lines specific for retrovirally transduced cells were generated in four out of eight donors by stimulating CD8 T cells with CD40-activated B (CD40-B) cells retrovirally transduced with CMV-pp65. Two CTL clones derived from one of the CTL lines were found to recognize epitopes from gag in the context of HLA-B*4403 and -B*4601, respectively. Similarly, an HLA-B*3501-restricted CTL clone from a cervical cancer patient recognized an epitope located in the junctional regions of the gag and pol sequences. These results show that polypeptides encoded by components of the retroviral vector backbone are in fact immunogenic, generating CTLs in vitro in human cells. Thus, potential CTL responses to retroviral products should also be considered in clinical settings.*

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Keywords: *cytotoxic T lymphocyte; retroviral vector; epitope*

Introduction

Murine leukemia virus (MLV)-based vector is one of the frequently used gene delivery vehicle, being employed in approximately a quarter of the approved clinical protocols worldwide (http://www.wiley.co.uk/genmed/clinical). However, there are still problems with the retroviral vectors used currently in the clinical setting. In particular, the issue of safety has often been raised, mainly because of the possibility of generation of replication-competent retroviruses and unexpected activation of genes adjacent to the viral integral sites. In this regard, it should be noted that development of leukemia manifesting clonal integration of the retrovirus into a proto-oncogene *LMO2* was observed in two SCID-X1 patients in a French trial.[1] Another important problem to overcome is the immunogenicity of the retroviral vectors themselves, that could induce humoral and cellular immune responses in the hosts that eventually diminish effective gene transfer.[2–10] Most retroviral vectors so far reported contain retroviral coding sequences, such as the 5′ end of the *gag* gene to increase their packaging efficiency.[11–14] Because a number of potential start codons, such as ATG or CTG, remain intact in the extended packaging sequences in most of the retroviral vectors,[12] polypeptides translated from them may induce immune responses, in addition to those against transgene products such as α-L-iduronidase[6] or selectable markers (eg, neomycin,[15] hygromycin and/or herpes virus thymidine kinase[16,17]). However, it still needs to be determined whether or not the products encoded by the retroviral vector backbone sequences are immunogenic in humans.

The LZRSpBMN,[18] Molony MLV-derived retroviral vector used in this study, contains full-length long terminal repeats (LTRs) and an extended packaging sequence with a portion of the *pol* gene derived from MFG retroviral vectors,[19] which have been used in a number of clinical trials, including a French trial for SCID-X1.[1] The extended packaging sequence is made by a combination of the original packaging signal and 426 bp of the 5′ end of the *gag* gene, followed by 375 bp of the 3′ end of the *pol* gene which harbors the splicing acceptor sites.[14] In wild-type MLV, Gag and Pol proteins are translated from the unspliced mRNA. The MFG vector still expresses a significant amount of the unspliced messages.[20] There are many potential start

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codons within the extended packaging sequence, such as CTG of gPr80^{gag} and ATG of Pr65^{gag}. Moreover, some (cryptic) polypeptides may be translated from genomic (unspliced) mRNA. Thus, it is possible that these products may give rise to host immune responses which could lead to rapid clearance of retrovirally transduced cells after *in vivo* infusion.

In this study, we demonstrated that human cytotoxic T-lymphocyte (CTL) clones recognizing epitopes derived from the protein encoded by the retroviral vector backbone. Two identified CTL epitopes were located in the *gag* gene, and the other epitope was in the artificial polypeptide joining the *gag* and *pol* sequences.

Results

In a previous study, we achieved efficient generation of cytomegalovirus (CMV)-pp65-specific CTL lines from CMV-seropositive donors using CD40-activated B (CD40-B) cells retrovirally transduced with the CMV-pp65 gene as antigen-presenting cells (APC).[21,22] With all of eight CMV-seronegative donors, however, we failed to generate pp65-specific CTL lines. Thus, to enhance the efficiency of induction of pp65-specific CTLs, we cultured CD8 T cells in the presence of interleukin-12, which is known to be an immunostimulatory cytokine. In fact, CTL lines were then induced in seven out of the eight seronegative donors. Unexpectedly, however, four of them showed cytotoxicity against not only pp65-transduced EBV-transformed lymphoblastic cell lines (LCLs) but also LCLs transduced with an irrelevant antigen, EGFP, whereas untransduced LCLs were not lysed (Figure 1a). These results suggest that the CTL lines were specific for viral-related antigens generated in the retrovirally transduced cells. These interesting findings prompted us to identify the antigens recognized. By limiting dilution, we established two CTL clones, 8C7 and 9C10, from a CMV-seronegative donor and their specificity was evaluated. IFN-γ ELISPOT assays revealed that clones 8C7 and 9C10 recognized 293T cells transfected with the retroviral vector, LZRSpBMN, in combination with HLA-B*4403 and -B*4601, respectively (Figure 1b), indicating that the antigens recognized by the CTL clones were indeed derived from the retroviral vector.

Because, in the retroviral vector we used, 5′LTR is active as an RNA polymerase II promoter, and the start codons of gPr80^{gag} and Pr65^{gag} in the extended packaging signal are not mutated, it is possible that several different polypeptides may be translated from the genomic mRNA.[20] Thus, we subcloned the region spanning the start codon of gPr80^{gag} through a unique *Bam*HI site of the vector into a mammalian expression vector, termed pcDNA3-gPr80, to determine which polypeptides translated from the extended packaging signal are antigenic. Both CTL clones could recognize 293T cells transfected with both pcDNA3-gPr80 and restricting HLA alleles, indicating that the region contains the CTL epitopes.

For their identification, linear expression fragments encoding serial C-terminal truncations of the region were constructed and expressed in 293T cells (Table 1), as previously reported.[22] The HLA-B*4403-restricted CTL clone 8C7 recognized transfectants expressing aa 1–169 or longer, but not 1–138, indicating that the epitope fully or partially lies between aa 139 and aa 169. With the aid of a computer algorithm,[23] peptides presented by HLA-B*4403 within the localized region were predicted (Table 2). Because a fragment encoding a decamer peptide,

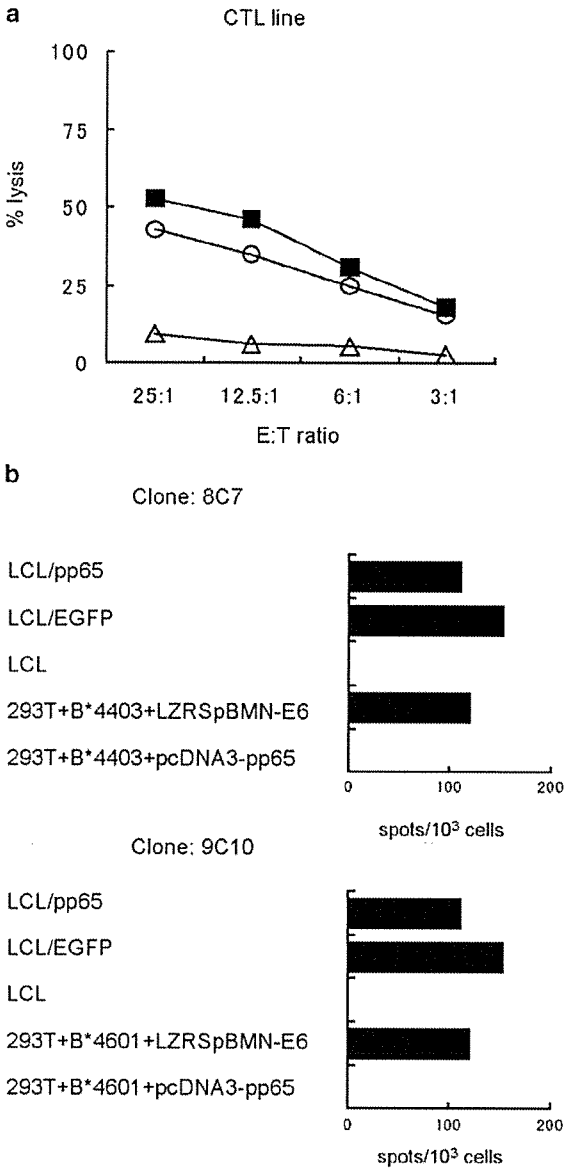

Figure 1 *Effector cell activity of the CTL line and clones generated from a CMV-seronegative donor. To generate CTL lines, CD8$^+$ T cells from a CMV-seronegative donor were stimulated three times with autologous CD40-B cells transduced with CMV-pp65 gene retrovirally, and tested. (a) Cytolytic activity of the CTL lines was assessed against autologous LCL retrovirally transduced with the CMV-pp65 (LCL/pp65; closed squares) or EGFP genes (LCL/EGFP; open circles), or untransduced LCL (open triangles) over a range of E/T ratios. Cytolytic activity assessed by ^{51}Cr release is shown as percentage specific lysis. (b) By limiting dilution, two CTL clones (8C7, 9C10) were obtained from the CTL line shown in (a). To determine HLA restriction and specificity of the CTL clones, IFN-γ ELISPOT assays were conducted using autologous LCL/pp65, LCL/EGFP, LCL or 293T cells transfected with the genes indicated. Each bar represents the number of spots per 10^3 cells.*

AEWPTFNVGW (aa 141–150), was well recognized by the CTL clone 8C7, we defined this decamer as the CTL epitope presented by HLA-B*4403 (Figure 2a).

In the case of CTL clone 9C10, the same region was recognized (Table 1). The binding motif for HLA-B*4601 has been reported as Met at position 2 and Tyr or Phe at the C-terminus,[24] but peptide binding prediction by the computer algorithm for HLA-B*4601 is not currently available. Because there were four Phe, but no Met and Tyr within the region, we generated linear expression fragments encoding four kinds of decamer peptides ending at each Phe. A fragment encoding TFCSAEWPTF (aa 137–146) was well recognized by the CTL clone 9C10. As shown in Figure 2b, C-terminal deletion affected the recognition by the CTL clone, whereas N-terminal deletions did not. By titration assay with synthetic peptides (data not shown), a nonamer peptide, FCSAEWPTF (aa 138–146), was identified as the minimal epitope presented by HLA-B*4601.

Similarly, CTL clones specific for retrovirally transduced cells were obtained by stimulation of CD8 T cells from a cervical cancer patient using HPV16-E6 and E7 transduced CD40-B cells as APC. One of the CTL clones, 3B4, was restricted by HLA-B*3501 and recognized 293T cells transfected with pcDNA3-gPr80 and HLA-B*3501 (Figure 3). With the linear expression fragments of serial C-terminal truncations, a region containing the CTL epitope was localized within aa 237–331 (Table 1). Several HLA-B*3501-restricting peptides within this region were predicted by the computer algorithm (Table 2). Among them, the CTL clone 3B4 well recognized a decamer, APIWPYEILY (aa 240–249, Figure 3b), which interestingly was composed of the artificial junctional region of *gag* and *pol* (Figure 4).[18]

Table 1 Identification of the regions recognized by the CTL clones using deletion mutants in ELISPOT assays

	CTL clone	*8C7*	*9C10*	*3B4*
Length of fragments	*HLA restricted*	*B*4403*	*B*4601*	*B*3501*
1–66		–	–	–
1–138		–	–	–
1–169		+	+	–
1–191		+	+	–
1–248		+	+	–
1–331		+	+	+
1–370		+	+	+

'+' indicates the presence of specific spots by each CTL clone, and '–' indicates the absence of spots.

Discussion

In the present study, we demonstrated that human CTLs specific for HLA class I-bound peptides encoded by retroviral vector backbone sequences can readily be induced *in vitro*. To the best of our knowledge, this is the first identification of epitopes derived from residual retroviral coding sequences rather than inserted cDNAs recognized by human cells. In mice, it has been already shown that Moloney MLV- or other retrovirus-induced tumor cell lines, such as RMA and FBL-3, are lysed by CTL responses, and that the immunodominant epitope of Moloney MLV restricted by H-2D^b is located within the gag leader protein encoded in the extended packaging sequence of the retroviral vector.[25] With regard to humoral immune responses, induction of anti-MLV antibodies has been reported in mice and nonhuman primates[5] as well as in patients treated for brain tumors with murine retroviral vector producer cells.[26] Thus, it is likely that the immune responses against not only inserted gene products but also retroviral coding sequence products can similarly be induced *in vivo*, with vector-based treatment of patients.

The immunogenicity of transferred gene products also remains one obstacle in the development of efficient clinical gene therapy protocols, particularly when introducing artificial or xenogenic sequences. Various attempts have been made to overcome this problem, including asanguineous perfusion after infection of retroviral vectors in liver transplant cases,[4] transplantation of transduced hematopoietic stem cells[7,27,28] or infusion of antigen-specific regulatory T cells,[29] with the aim of establishing immune tolerance to transgene products. In the French clinical trial for the X-linked form of SCID,[1] which is the first recorded case of a therapeutic effect of retroviral gene therapy in humans, the transferred gene is expressed in the patients long term. Immune responses against the transferred common γ-chain gene or MFG-based retroviral vector coding sequences have not been reported so far, which may be due to relative immunodeficiency in these X-SCID patients.

Table 2 Results for HLA-binding motif prediction by computer algorithm

CTL clone	*HLA restricted*	*Range submitted*	*Rank*	*Position*	*Sequence*	*Score*
8C7	B*4403	129–169	1	141–150	AEWPTFNVGW	48
			2	161–169	ITQVKIKVF	2.3
			3	148–156	VGWPRDGTF	2
			4	141–149	AEWPTFNVG	1.6
			5	138–146	FCSAEWPTF	1
3B4	B*3501	237–331	1	240–249	APIWPYEILY	40
			2	240–248	APIWPYEIL	20
			3	305–314	HPYRVGDTVW	10
			4	300–309	RPVVPHPYRV	8
			5	268–276	NSPSLQAHL	5

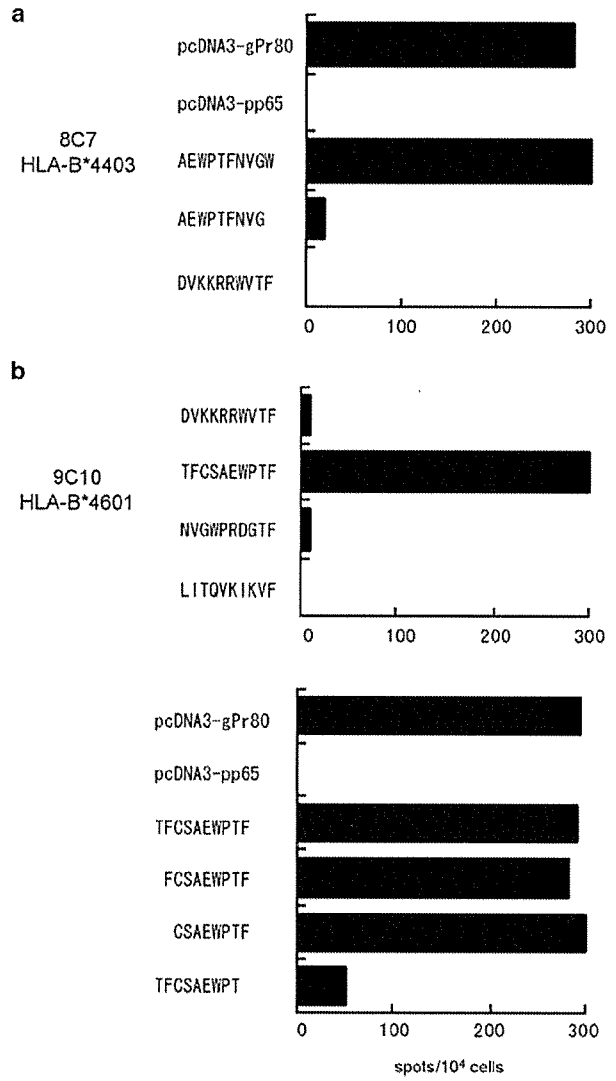

Figure 2 Identification of the CTL epitopes recognized by the CTL clones 8C7 and 9C10. Linear expression fragments encoding various peptides, including the predicted epitopes by computer algorithms, were transfected to 293T cells, together with restricting HLA cDNA. Recognition by the pp65-specific CTL clones was evaluated 48 h later by ELISPOT assay. Each bar represents the number of spots per 10^4 cells. (a) Recognition of the linear expression fragment encoding the predicted peptide, AEWPTFNVGW, or other peptides by the CTL clone 8C7. (b) Recognition of the linear expression fragments encoding the candidate peptides by the CTL clone 9C10 is shown in the upper columns. Effects of N- or C-terminal deletion are shown in the lower panel.

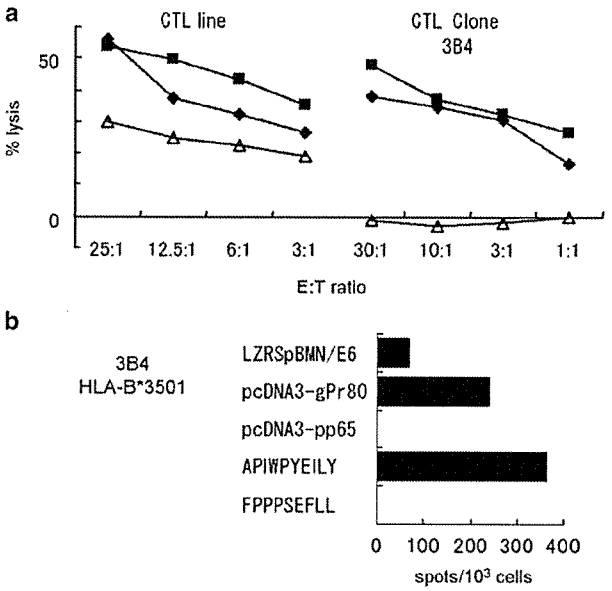

*Figure 3 Effector cell activity of the CTL line and clones generated from a cervical cancer patient. A CTL line was generated by stimulating $CD8^+$ T cells from a cervical cancer patient three times with autologous HPV16-E6 and -E7 transduced CD40-B cells. By limiting dilution, one CTL clone 3B4 was then obtained. (a) Cytolytic activity of the CTL line (left) and the CTL clone (right) was assessed against autologous LCL/E6 (closed squares), LCL/E7 (closed diamonds), or untransduced LCL (open triangles) over a range of E/T ratios. (b) Determination of the epitope for the CTL clone 3B4. ELISPOT assays were conducted using 293T cells transfected with plasmids encoding the indicated gene or with linear expression fragments encoding the indicated peptides, together with HLA-B*3501 cDNA. Each bar represents the number of spots per 10^3 cells.*

Retention of retroviral sequences may also result in generation of replication-competent retroviruses as a result of homologous recombination.[11] Substitution of the packaging sequence of MLV-based vectors by sequences derived from other viruses has been successfully employed without loss of the transduction efficiency.[30] Indeed, attempts have been made to remove all ATG start codons without losing efficient viral packaging.[12,14] These approaches may be able to provide more effective ways to avoid immunogenicity of retroviral vectors and also generation of replication-competent retroviruses. Finally, the present identification of an immunogenic HLA-B35-restricted peptide derived from the *gag–pol* junctional region may be of clinical significance because vectors encoding various chimeric molecules are currently often used.

In conclusion, peptides encoded by the retroviral vector backbone sequence show potent immunogenicity *in vitro* so as to induce CTL responses. Thus, it is likely that they may also stimulate CTL responses *in vivo*, leading to rapid clearance of retrovirally transduced cells and resulting in adverse effects. We should take into consideration this problem by actively monitoring immune responses in retroviral vector-mediated gene therapies.

Materials and methods

Donors and cells

Peripheral blood samples were obtained from CMV-seronegative healthy donors or cervical cancer patients after we obtained informed consent under a protocol approved by the Institutional Review Board of Aichi Cancer Center according to the Declaration of Helsinki. HLA typing was carried out at The HLA Laboratory (Kyoto, Japan). Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by centrifugation on a Ficoll (Amersham Biosciences, Uppsala, Sweden) density gradient, and CD8-positive and -negative fractions were separated using CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cryopreserved until use.

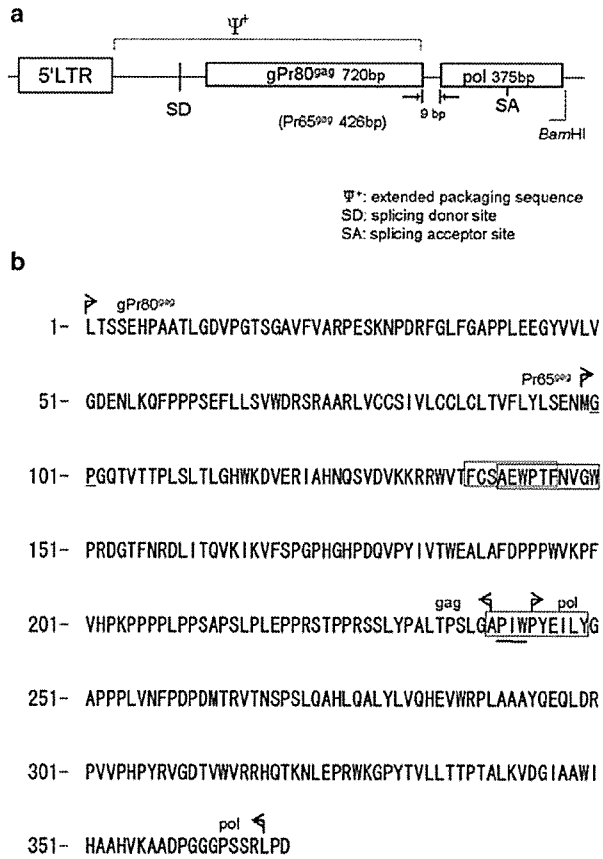

Figure 4 Leader sequence of the retroviral vector, LZRSpBMN. (a) Schematic representation of the leader sequence in LZRSpBMN. This region is identical with that of the MFG vector. (b) The amino-acid sequence translated from the gag–pol fusion region starting at gPr80gag start codon (CTG). The start codon of Pr65gag is Met99 and the junctional region of gag and pol is underlined. The CTL epitopes identified in this study are boxed (see Figures 2 and 3).

Plasmids and synthetic peptides

Plasmids, pcDNA3-pp65, pcDNA3-EGFP, pcDNA3.1. (Invitrogen, Tokyo, Japan) encoding HLA-class I cDNA, LZRSpBMN-pp65 (the backbone plasmid, LZRSpBMN-Z was kindly provided by Dr G Nolan, Stanford University, Stanford, CA, USA), pLBPC-pp65 and pLBPC-EGFP were constructed as previously described.[21,31] All peptides were purchased from Toray Research Center (Tokyo, Japan). HPV16-E6 or -E7 gene (kindly provided by Dr T Kiyono, National Cancer Center Research Institute, Tokyo, Japan) was inserted into the LZRSpBMN vector (LZRSpBMN-E6 or LZRSpBMN-E7, respectively) and the pLBPC vector (pLBPC-E6 or pLBPC-E7, respectively). To generate pcDNA3-gPr80, the region spanning the start codon of gPr80gag through a unique *Bam*HI site of the LZRSpBMN-Z vector (Figure 4a) was inserted into multiple cloning site of the pcDNA3.1 vector.

Generation of CD40-activated B cells and LCLs

CD40-B cells were generated as previously described.[21,32,33] In brief, a thawed CD8-negative fraction of PBMCs was cultured on a γ-irradiated (96 Gy) human CD40L-transfected NIH3T3 cell line[34] (t-CD40L; kindly provided by Dr Gordon Freeman, Dana-Farber Cancer Institute, Boston, MA, USA) in the presence of IL-4 (4 ng/ml; Ono Pharmaceutical, Osaka, Japan) and cyclosporin A (CsA, 0.7 μg/ml; Sandoz, Basel, Switzerland) in 2 ml of Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% pooled human serum. The expanding cells were transferred onto freshly prepared t-CD40L cells and fed with cytokine-replenished medium without CsA every 3–4 days. LCLs were established from the CD40-B cells with supernatant of an EBV producing cell line (B95-8, ATCC, Manassas, VA, USA) in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum (FCS; IBL, Takasaki, Japan), referred to as RPMI-10.

Retroviral transduction of CD40-B cells and LCLs

Retroviral transduction was conducted as previously described.[21] In brief, the retroviral construct, for example, LZRSpBMN-pp65, was packaged in the Phoenix GALV cell line[35] (a gift from H-P Kiem, Fred Hutchinson Cancer Research Center, and from G Nolan, Stanford University, Stanford, CA, USA) using FuGENE 6 (Roche Diagnostics, Mannheim, Germany). CD40-B cells and LCLs were infected with the retroviral supernatant in the presence of 10 μg/ml polybrene (Sigma, Chicago, IL, USA), spun at 1000 *g* for 1 h at 32°C, and incubated. Two days after, LCLs transduced with CMV-pp65 (LCL/pp65), HPV16-E6 (LCL/E6), HPV16-E7 (LCL/E7), EGFP (LCL/EGFP) were selected in the presence of puromycin (0.7 μg/ml; Edge Biosystems, Gaithersburg, MD, USA). Transduction efficiency were assessed as previously described.[21]

Generation of antigen-specific CTL lines using retrovirally transduced CD40-B cells

Thawed CD8-positive cells (1×10^6) were cocultured with γ-irradiated (33 Gy) autologous retrovirus-transduced CD40-B (CD40-B/pp65) cells (1×10^6) in 2 ml RPMI 1640 supplemented with 10% pooled human serum, recombinant human IL-7 (50 U/ml; Genzyme, Cambridge, MA, USA) and IL-12 (5 ng/ml; R&D systems, Minneapolis, MN, USA) at 37°C in 5% CO_2. On days 7 and 14, $CD8^+$ cells were restimulated, and 1 day after each stimulation, recombinant human IL-2 (Chiron, Emeryville, CA, USA) was added to the cultures at the final concentration of 20 U/ml. If necessary, rapidly growing cells were split into 2–3 wells and fed with fresh media containing 20 U/ml IL-2.

Epitope selection and construction of linear expression fragments

Linear expression fragments encoding various C-terminus truncated *gag–pol* gene of LZRSpBMN (Table 1) or various peptides (Table 2, Figure 2) were generated using an overlapping PCR method.[22] Targeted region-specific 5′ and 3′ primers incorporating additional sequences (single- and double-underlined, see below) were designed, for example, 5′primer, TCGGATCCACCATGCTGA CGAGTTCGGAAC (30 bp) and 3′primer, GACTCGAGC GCTATAAGATCTCATATGGCC (30 bp) for the fragment encoding aa 1–248, and used for PCR (KOD Plus; Toyobo, Osaka, Japan) with a template retroviral vector, LZRSpBMN. The CMV promoter (P_{CMV}) and BGH

polyadenylation signal (pA) were independently amplified from pcDNA3.1 by PCR using the following primers: 5′ P_{CMV}, CTTAGGGTTAGGCGTTTTGC; 3′ P_{CMV}, NNCATGGTGGATCCGAGCTCGGTA; 5 ′pA, NNTAGCGCTCGAGTCTAGAGGG; 3′ pA, GGTTCTTTCCGCCTCAGAAG; 'N' means a mixture of A/C/T/G). The 3′ P_{CMV} and 5′ pA primers contained overlapping sequences (underlined) with the 5′primer and 3′primer, respectively, of the targeted region. The three PCR products, P_{CMV}, the targeted region, and pA, were conjugated by second PCR using primers, 5′ P_{CMV} and 3′ pA. Each linear expression fragment was termed '1-XXX' (C-terminus truncated *gag–pol* gene; 'XXX' indicates the amino-acid positions of the C-terminus) or peptide sequence.

ELISPOT assays

ELISPOT assays were performed as described earlier.[21,22] In brief, a MultiScreen-HA plate (MAHA S4510, Millipore, Bedford, MA, USA) was coated with anti-human IFN-γ mAb (M700A; Endogen, Woburn, MA, USA) and used as an ELISPOT plate. 293T cells were cotransfected with plasmids encoding each of the individual donor's HLA-class I alleles and either antigen encoding plasmids or PCR products of the linear expression fragment by TransIT-293 (Mirus, Madison, WI, USA), and used as stimulator cells after 2 days. The transfected 293T cells, LCLs or retrovirally transduced LCLs were mixed with 10^3 or more effector cells from the CTL lines or clones generated. After cells had been incubated in 200 μl RPMI-10 in a round-bottom 96-well plate (Costar Corning, Cambridge, MA, USA) for 4 h, all the aliquots were transferred into an ELISPOT plate and incubated for an additional 16 h. To visualize spots, a biotin-labeled anti-human IFN-γ antibody (M701B; 1 μg/ml, Endogen), streptavidin–alkaline phosphatase (Biosource International, Camarillo, CA, USA), and substrate were used. Spots were counted after computerized visualization using a scanner (Canon, Tokyo, Japan).

Chromium release assays

LCLs or retrovirally transduced LCLs were labeled in 100 μl RPMI-10 with 3.7 MBq ^{51}Cr for 1 h at 37°C. After 4 h incubation with effector cells, supernatants were counted in a gamma counter. The percentage of specific lysis was calculated as follows: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

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INTERFERON-γ DIFFERENTIALLY REGULATES SUSCEPTIBILITY OF LUNG CANCER CELLS TO TELOMERASE-SPECIFIC CYTOTOXIC T LYMPHOCYTES

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There is accumulating evidence that peptides derived from the catalytic subunit of human telomerase reverse transcriptase (hTERT) are specifically recognized by CD8$^+$ cytotoxic T lymphocytes. We investigated the cytotoxicity of a human leukocyte antigen (HLA)-A*2402-restricted hTERT-derived peptide 461–469 (hTERT$_{461}$)-specific CD8$^+$ T-cell clone, designated as K3-1, established from a healthy donor by repetitive peptide stimulation. This clone exhibited cytotoxicity against 4 out of 6 HLA-A24-positive lung cancer cell lines with positive telomerase activity but not 4 HLA-A24-negative examples. When the target cells were pretreated with 100 U/ml of interferon (IFN)-γ for 48 hr, the susceptibility to K3-1 increased with PC9 cells but unexpectedly decreased with LU99 cells. However, in both cell lines, the expression of molecules associated with epitope presentation such as HLA-A24, transporters associated with antigen processing, low molecular weight polypeptide 7 and proteasome activator 28 was similarly increased after IFN-γ treatment. Results of CTL assays using acid-extracted peptides indicated that the epitope increased on PC9 cells but not on LU99 cells after IFN-γ treatment. Semi-quantitative reverse transcriptase polymerase chain reaction disclosed that the expression of hTERT was attenuated in LU99 but not in PC9 cells, accounting for the decreased cytotoxicity mediated by K3-1. The attenuation of the hTERT expression and K3-1-mediated cell lysis after IFN-γ treatment was also observed in primary adenocarcinoma cells obtained from pulmonary fluid of a lung cancer patient. Our data underline the utility of peptide hTERT$_{461}$ in immunotherapy for lung cancer, as with other malignancies reported earlier, and suggest that modulation of hTERT expression by IFN-γ needs to be taken into account in therapeutic approach.

Key words: *telomerase; hTERT; immunotherapy; lung cancer; Interferon-γ*

Human chromosomes terminate with 5–15 kilobases of repetitive telomeric DNA $(TTAGGG)_n$,[1] which protect against DNA degradation, end-to-end fusion, rearrangements and chromosome loss.[2] In normal cells, such as cultured skin fibroblasts, telomeric DNA becomes shortened with every round of replication,[3] ultimately leading to replicative senescence. In contrast, with permanently established cell lines from malignant tumors, telomeres are believed to be elongated by a unique ribonucleoprotein enzyme, called telomerase, which adds telomeric sequences *de novo*.[1] Indeed, there is clear evidence that telomerase activity is involved in tumorigenesis.[4,5] Normal tissues display little or no telomerase activity, and activation of the enzyme may therefore play a critical role in cell immortalization.

Human telomerase complexes are composed of telomerase RNA component,[6] telomerase protein 1[7,8] and hTERT.[9] Messenger RNA expression of hTERT is essential for telomerase activation during cellular immortalization and tumor progression,[9] and ectopic expression of the hTERT gene in telomerase-negative cells can induce telomerase activity to levels comparable to those in immortal telomerase-positive cells.[10] The expression of hTERT has been frequently demonstrated in telomerase-positive primary tumors and cancer cell lines but found to be low or undetectable in normal tissues.[9–13] Thus, hTERT could be a candidate universal tumor antigen for immunotherapy and vaccine approaches.

Several studies have been conducted to test the possibility that hTERT could serve as a tumor antigen recognized by specific CTL. Indeed, hTERT peptide-specific CTL have proved cytotoxic to cell lines derived from various malignancies including leukemias,[14,15] osteosarcoma, ovarian carcinoma, non-Hodgkin's lymphoma,[15] multiple myeloma,[15,16] melanoma[15,17] and cancers of breast, colon, lung,[17] prostate[17,18] or kidney.[18] Recent studies revealed that hTERT is expressed in 89%[13] to 93.9%[19] of primary lung cancers.

In our study, we first asked the question whether hTERT-specific CTL recognize and kill lung cancer cells applying an HLA-A*2402-restricted hTERT-derived peptide (hTERT$_{461}$)-specific CD8$^+$ T-cell clone, generated from a healthy donor, and a panel of lung cancer cell lines with positive telomerase activity as targets. The findings confirm and extend previous results, supporting the feasibility of developing CTL-based immunotherapy targeting hTERT in some, if not all, lung cancer patients. In addition, interesting evidence was obtained to demonstrate that IFN-γ treatment of the target cells did not always enhance CTL recognition.

Abbreviations: CD40-B, CD40-activated B; CTL, cytotoxic T lymphocyte; FITC, fluorescein isothiocyanate; hTERT, human telomerase reverse transcriptase; IRF, interferon regulatory factor; HLA, human leukocyte antigen; IL, interleukin; LCL, lymphoblastoid B-cell line; LMP, low molecular weight polypeptide; MAb, monoclonal antibody; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; RT-PCR, reverse transcription polymerase chain reaction; TAP, transporters associated with antigen processing.

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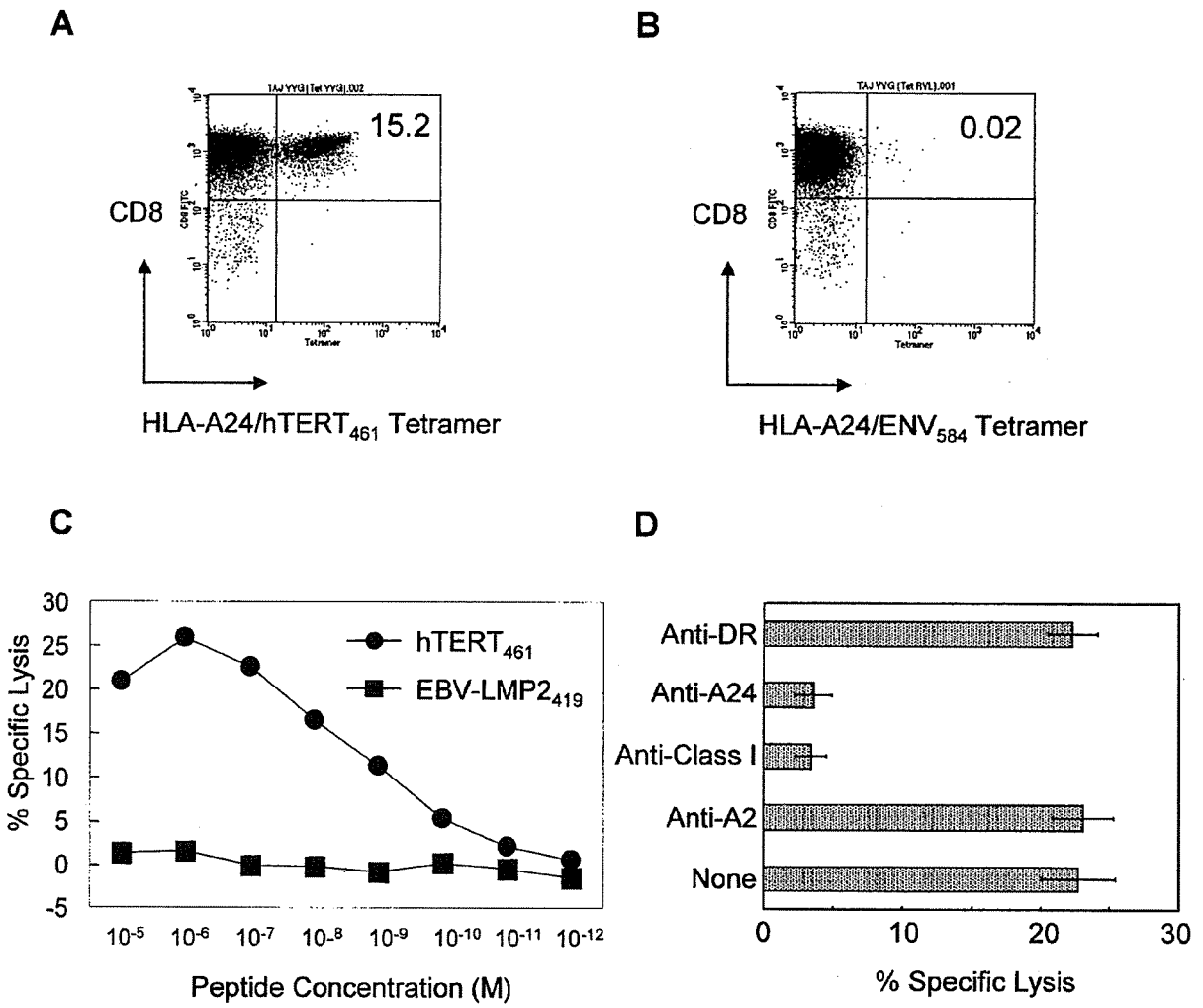

FIGURE 1 – Characterization of polyclonal CTL specific to $hTERT_{461}$. Polyclonal $CD8^+$ T cells after stimulation 4 times were stained with FITC-labeled anti-CD8 antibodies and PE-labeled HLA-A24-tetramers incorporating $hTERT_{461}$ (*a*) or a control peptide, ENV_{584} (*b*), and analyzed by flow cytometry. The percentages of tetramer-positive cells in total $CD8^+$ T cells are shown. (*c*) Results of CTL assays using serial dilutions of $hTERT_{461}$ (closed circle) and an EBV-derived control peptide, $EBV\text{-}LMP2_{419}$ (closed square). Cytotoxicity of polyclonal CTL to T2-A24 cells in the presence of indicated concentrations of each peptide was determined by ^{51}Cr release assays at an effector-target ratio of 1. (*d*) Inhibitory effect of an anti-HLA class I monoclonal antibody or an anti-HLA-A24 monoclonal antibody on cytotoxicity of a CTL clone K3-1 against a HLA-A24-positive cell line, PC9. Chromium-labeled target cells were incubated with either monoclonal antibodies specific to HLA class I, HLA-A24, HLA-A2 or HLA-DR molecules, before addition of K3-1 cells. The cytotoxic assays were done at an effector-target ratio of 10.

MATERIAL AND METHODS

Donors and cell lines

Peripheral blood mononuclear cells (PBMC) were isolated from 4 HLA-A24-positive healthy donors by centrifugation on a Ficoll density gradient. Epstein-Barr virus (EBV)-transformed LCL were established as previously described[20] and cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (Life Technologies Limited, Auckland, NZ), 2×10^{-3} M L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin and 5×10^{-5} M β-mercaptoethanol (referred to as complete medium). CD40-activated B (CD40-B) cells were generated using NIH-3T3-hCD40 ligand cells (kindly provided by Dr. G. Freeman, Dana-Farber Cancer Institute, Boston, MA) as previously described.[21,22] Pulmonary fluid was obtained from an HLA-A24-positive patient with lung adenocarcinoma for primary culture of the cancer cells. The study design and purpose, which had been approved by the institutional review board of Aichi Cancer Center, were explained fully to all donors. Samples were obtained after informed consent was confirmed.

Human lung cancer cell lines, LC-1/sq and LU99 cells, were purchased from the Japanese Collection of Research Bioresources (Tokyo, Japan) and RIKEN Cell Bank (Tsukuba, Japan), respectively. LC-1/sq cells were maintained in 45% RPMI1640 medium and 45% Ham's F12 (Sigma Chemical Co.) supplemented with 10% FCS, L-glutamine, penicillin, streptomycin and kanamycin. All other lung cancer cell lines (LU99, PC9, 11-18, LC99A, LC65A, LK79, A549, QG56 and RERF-LC-MT) and a chronic megakaryoblastic leukemia cell line, MEG-01, were maintained in the complete medium. K562 cells were maintained in IMDM (Sigma Chemical Co.) supplemented with 10% FCS, L-glutamine, penicillin, streptomycin and kanamycin. HLA-A*2402-trans-

TABLE I – TELOMERASE ACTIVITY AND HLA-A24 EXPRESSION OF TARGET CELL LINES USED IN THIS STUDY

Cells	Tumor origin	Telomerase activity[1]	Surface HLA-A24 expression[2]
Lung cancer cell lines			
PC9	Adenocarcinoma	+	+
11–18	Adenocarcinoma	+	+
LC-1/sq	Squamous cell carcinoma	+	+
LU99	Giant cell carcinoma	+	+
LK79	Small cell carcinoma	+	+
LC99A	Large cell carcinoma	+	+
LC65A	Small cell carcinoma	+	–
RERF-LC-MT	Adenocarcinoma	+	–
A549	Adenocarcinoma	+	–
QG56	Squamous cell carcinoma	+	–
Hematopoietic cell lines[3]			
MEG-01	Leukemia	+	+
K562	Leukemia	+	–
T2-A24	—	Not done	+

[1]Telomerase activity was detected as described in the Material and Methods.–[2] To detect surface expression of HLA-A24 molecules, cells were stained with an anti-HLA-A24 antibody and subsequently with FITC-labeled anti-mouse IgG $F(ab')_2$ fragments and analyzed by flow cytometry.–[3]MEG-01, a control cell line expressing telomerase and HLA-A24 molecules; K562, a representative cell line susceptible to natural killer-like cytotoxicity; T2-A24, a TAP-deficient cell line expressing HLA-A24 molecules.

fected, TAP-negative T2-A24 cells[23] were cultured in complete medium containing 0.8 mg/ml of G418 (Gibco, Grand Island, NY). Pulmonary fluid containing adenocarcinoma cells was diluted with the complete medium and cultured in the presence or absence of IFN-γ for 48 hr. After the incubation, adherent cells were used for RT-PCR analysis and as target cells for hTERT-specific CTL.

A retrovirus encoding HLA-A*2402 was infected into the HLA-A24-negative cell lines, QG56 and A549, as previously described.[24] The infected cells were maintained in complete medium with puromycin at the final concentration of 0.6 (for QG56) or 0.9 (for A549) μg/ml for selection and designated as QG56–A24 and A549–A24, respectively.

Peptides

Two HLA-A24-restricted CTL epitope peptides derived from hTERT,[14] VYAETKHFL (residues 324–332, designated as $hTERT_{324}$) and VYGFVRACL (residues 461–469, designated as $hTERT_{461}$), a human immunodeficiency virus-1 (HIV-1) envelop peptide RYLRDQQLL[25] (residues 584–592, designated as ENV_{584}) and an EBV latent membrane protein 2 peptide TYGPVFMCL[20] (residues 419–427, designated as EBV-$LMP2_{419}$) were synthesized by Toray Research Center (Kamakura, Japan).

Cell staining and flow cytometric analysis

Surface expression of HLA-A24 molecules was examined by indirect immunofluorescence using an HLA-A24 MAb (One Lambda, Inc. Canoga Park, CA) and FITC-labeled anti-mouse IgG $F(ab')_2$ fragments (IMMUNOTECH, Marseilles, France). MHC-tetramers were produced as previously described.[23,26] $CD8^+$ T cell lines were stained with PE-labeled HLA-A*2402-tetramers incorporating $hTERT_{324}$, $hTERT_{461}$ or ENV_{584}. Flow cytometric analysis of the stained cells was performed using a FACSCalibur (Becton Dickinson, San Jose, CA) and the data were analyzed using CellQuest software (Becton Dickinson).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured cell lines. Gene-specific oligonucleotide primers were synthesized at Proligo (Kyoto, Japan) and used to evaluate the mRNA expression pattern of hTERT,[13] TAP-1, TAP-2[27] and IRF-1.[28] RT-PCR was performed using a thermal cycler (Perkin-Elmer, Wellesley, MA) and the products were analyzed by 1.5% gel electrophoresis and ethidium bromide visualization.

Western blot analysis

Western blot analysis was performed as described previously[29] with slight modifications. Briefly, cells were lysed in lysis buffer (50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 0.5% Triton X-100, 10 μM leupeptin, 2.8 μM pepstatin and 0.85 mM phenylmethanesulfonyl fluoride) for 30 min at 4°C. The post-nuclear supernatant was quantified by absorbance at 280/260 nm for protein concentrations, and aliquots of 130 μg protein were applied to 12% SDS-PAGE. The proteins were blotted onto Immobilon-P membranes (Millipore Corporation, Bedford, MA), blocked with PBS containing 10% low fat dry milk and 0.1% Tween-20 overnight at 4°C and probed with rabbit polyclonal Abs specific to low molecular weight polypeptide 7 (LMP7) and proteasome activator 28 (PA28) α subunits (Affinity, Mamhead, U.K.) followed by peroxidase-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA). Proteins were visualized using an ECL Western blot detection system (Amersham Biosciences, Buckinghamshire, UK).

Generation of hTERT-specific polyclonal and clonal CTL using peptide-pulsed CD40-B cells as antigen presenting cells

CD40-B cells (2.5×10^5) were pulsed with $hTERT_{324}$ or $hTERT_{461}$ at 1×10^{-5} M for 1 hr and irradiated at 33 Gy. $CD8^+$ T cells (1×10^6) were isolated from donated PBMC with the aid of CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cocultured with autologous peptide-pulsed CD40-B cells in 2 ml culture medium in the presence of 25 ng/ml IL-7 (Genzyme, Cambridge, MA) and 5 ng/ml IL-12 (R&D Systems, Minneapolis, MN) at 37°C in a 5% CO_2 incubator. On days 7, 14, 21 and 28, $CD8^+$ T cells were restimulated with peptide-pulsed and γ-irradiated CD40-B cells. One day after each restimulation, human recombinant IL-2 (Takeda Chemical Industries, Osaka, Japan) was added to a final concentration of 20 U/ml. If necessary, rapidly growing cells were split into 2 to 3 wells and fed with fresh culture medium containing 20 U/ml of IL-2. Specificity of the T cells was examined with tetramer staining and cytotoxic assays. To establish T-cell clones, limiting dilution of the polyclonal CTL was performed.[23] Briefly, polyclonal $CD8^+$ T cells were seeded at 1 or 3 cells/well in round-bottomed 96-well plates containing the culture medium (0.2 ml) with anti-CD3 MAb (30 ng/ml, Ortho Diagnostics, Raritan, NZ), IL-2 (30 U/ml), γ-irradiated (33 Gy) 1×10^5 PBMC and γ-irradiated (55 Gy) 2×10^4 LCL. After 2 weeks of culture, growing cells positively stained for the HLA-A*2402/$hTERT_{461}$-tetramer were transferred into flasks and expanded as above.

CTL assay

Target cells were labeled with chromium (^{51}Cr) in 100 μl culture medium for 1 h at 37°C. In some experiments, predetermined amounts of blocking antibodies, W6/32 (anti-HLA class I), MA2.1 (anti-HLA-A2), A11.1 (anti-HLA-A24) and HDR-1 (anti-HLA class II) were added to the wells 30 min before adding

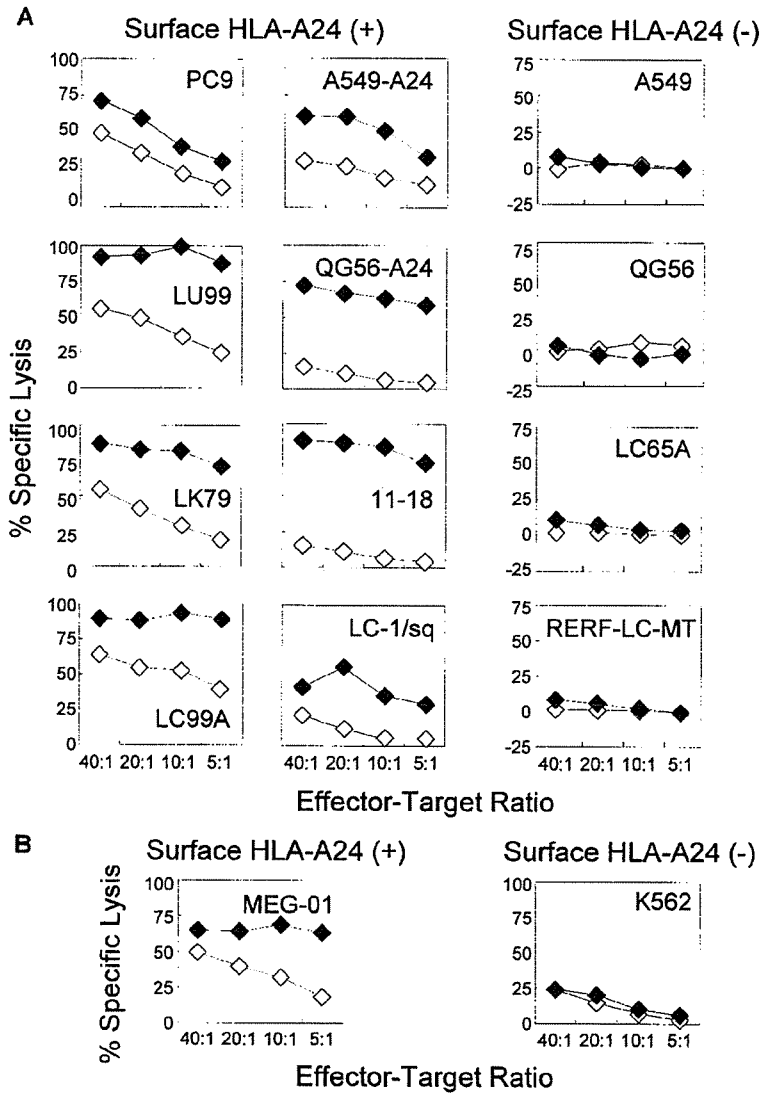

FIGURE 2 – Cytotoxicity of $hTERT_{461}$-specific CTL clone, K3-1 against cancer cell lines. (a) Cytotoxicity of $hTERT_{461}$-specific CTL clone K3-1 for 8 lung cancer cell lines with positive surface HLA-A24 expression and 4 with negative surface HLA-A24 expression as target cells (see Table I). The HLA-A*2402 gene was retrovirally transfected into A549 and QG56 cells, and the resultant transfectants designated as A549-A24 and QG56-A24, respectively. Assays were performed in the presence (closed diamond) or absence (open diamond) of 1×10^{-7} M $hTERT_{461}$ at the indicated effector-target ratios. (*b*) Cytotoxicity of $hTERT_{461}$-specific CTL clone K3-1 against MEG-01, a control hematopoietic cell line expressing telomerase and HLA-A24 molecules, and K562, a representative cell line susceptible to natural killer cytotoxicity, as target cells. Assays were performed in the presence (closed diamond) or absence (open diamond) of 1×10^{-7} M $hTERT_{461}$ at the indicated effector-target ratios.

effector cells to determine the HLA restriction. In others, target cells were treated with 100 U/ml of IFN-γ for 48 hr before chromium labeling. The plates were incubated for 5 hr at 37°C, and the supernatants were counted in a gamma counter. The percentage specific ^{51}Cr release was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

Extraction of naturally processed peptides from cell lines

Isolation of peptides from cell cultures was performed as previously described[30] with slight modifications. Briefly, confluent PC9 or LU99 cells (either treated or untreated with IFN-γ for 48 hr) in T225 flasks (Costar, Cambridge, MA) were washed 3 times with PBS and incubated with 5 ml of citrate-phosphate buffer (pH 3.3) for 1 min. The buffer containing eluted peptides was harvested and stored at −80°C until use. Peptides were repetitively stripped for 4 consecutive days.

The acid-extracted peptides were filtered and concentrated on SepPak Light C18 Cartridges (Waters Corporation, Milford, MA) according to the manufacturer's instructions. Bound peptides were eluted with 80% acetonitrile and 0.1 % trifluoroacetic acid, concentrated in a Speed-Vac (Savant Instruments, Inc., Hicksville, NY) and pulsed on ^{51}Cr-labeled T2-A24 cells. K3-1-mediated target cell lysis was assessed as described above.

Measurement of telomerase activity

Telomerase activity was measured by the telomeric repeat amplification protocol using Telo TAGGG Telomerase PCR ELISA^{PLUS} (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's instruction. Samples were considered as telomerase-positive if the difference in absorbance (absorbance of sample − absorbance of heat-treated sample) was more than 2-fold background activity, according to the protocol supplied with the reagents.

RESULTS

Generation of hTERT peptide-specific $CD8^+$ CTLs

To generate hTERT-specific $CD8^+$ T cell lines, $CD8^+$ T cells of 4 HLA-A24-positive healthy donors were stimulated weekly with autologous CD40-B cells pulsed with either of the HLA-A*2402-restricted hTERT-derived peptides, $hTERT_{324}$ or $hTERT_{461}$. After

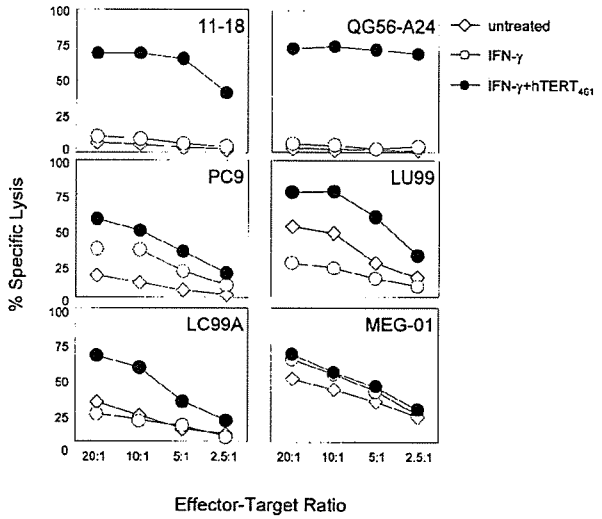

FIGURE 3 – K3-1-mediated lung cancer cell lysis induced by IFN-γ treatment. Cytotoxicity of the $hTERT_{461}$-specific CTL clone K3-1 against HLA-A24-positive lung cancer cells determined with (open circle) or without (open diamond) IFN-γ pre-treatment (100 U/ml for 48 hr). Cytolysis of IFN-γ treated cells was also tested in the presence of 1×10^{-7} M $hTERT_{461}$ (closed circle). Assays were performed at the indicated effector-target ratios.

the fourth stimulation, the T-cell lines were stained with HLA-A24-tetramers incorporating $hTERT_{324}$, $hTERT_{461}$ or ENV_{584}. A T cell line from a donor stimulated with $hTERT_{461}$ was specifically stained with HLA-A24-tetramers incorporating $hTERT_{461}$ but not ENV_{584} (15.2% *vs.* 0.02% in total CD8+ cells, Fig. 1*a,b*). This line showed cytotoxicity to T2-A24 cells pulsed with $hTERT_{461}$ dose-dependently but not with control peptide $EBV\text{-}LMP2_{419}$ (Fig. 1*c*). None of the other polyclonal T-cell lines were stained with HLA-A24-tetramers incorporating peptides used for individual stimulation, even after a fifth stimulation (data not shown).

A $CD8^+$ CTL clone was established by limiting dilution of the polyclonal T-cell line and designated as K3-1. The integrity of K3-1 was assessed with the HLA-A24/$hTERT_{461}$-tetramer (data not shown).

Lysis of lung cancer cell lines by the $hTERT_{461}$ peptide-specific CTL clone, K3-1

We next examined K3-1-mediated cytotoxicity against a panel of lung cancer cell lines (Table I). Among the 10 lung cancer cell lines examined, 6 were positive for HLA-A24 expression, and all cell lines featured telomerase activity (Table I). Results for cytotoxicity are summarized in Figure 2*a*, only HLA-A24-positive lung cancer cell lines (PC9, LU99, LK79 and LC99A) being affected. The degree of cell lysis was comparable to that observed for a leukemia cell line, MEG-01 cells (Fig. 2*b*), previously reported to be well recognized by HLA-A24-restricted hTERT-specific CTL.[14] The cytotoxicity of K3-1 against PC9 cells was blocked by an anti-HLA-A24 MAb, but not anti-HLA-A2 or HLA-DR MAbs showing HLA-A24 restriction (Fig. 1*d*). The K3-1-mediated cytotoxicity against PC9 cells was specifically inhibited by the presence of T2-A24 cells pre-pulsed with the cognate but not an irrelevant peptide (data not shown), indicating K3-1 could recognize $hTERT_{461}$ naturally processed and presented on the surfaces of the target cells. As shown in Figure 2*a* (center column), some HLA-A24-positive and HLA-A24-transfected lung cancer cell lines were not effectively lysed by K3-1 despite confirmation of surface expression of HLA-A24 by indirect immunofluorescence and flow cytometry. However, the cytotoxicity against these cell lines was enhanced in the presence of $hTERT_{461}$ in the medium, suggesting insufficient epitope density on these cells.

HLA-A24-negative cell lines, A549, QG56, LC65A and RERF-LC-MT cells were not lysed at all by K3-1 either in the presence or absence of the cognate peptide (Fig. 2*a*, right column). K562 cells were included to assess the degree of NK-like cytotoxicity of K3-1 cells, which turned out to be negligible (Fig. 2*b*).

K3-1-mediated lung cancer cell lysis after IFN-γ treatment

Pretreatment of target cells with IFN-γ is well known to augment epitope processing and presentation.[30] Thus, we asked the question whether IFN-γ treatment augments CTL-mediated cell lysis of the cytolysis-negative cell lines (Fig. 2*a*, center column) by improved epitope processing and presentation.[30] The cytolysis-positive cell lines were also tested. As demonstrated in Figure 3, there was no augmentation of K3-1-mediated lysis in the 11–18 and QG56-A24 cases. Lysis of LC-1/sq and A549-A24 cells was also not augmented by IFN-γ treatment (data not shown). Of note, K3-1-mediated lysis of PC9, LU99, LC99A and MEG-01 cells was differentially affected by IFN-γ pretreatment (Fig. 3). Thus the lysis of PC9 and MEG-01 cells was increased by the treatment, but with LC99A cells, it was unchanged or slightly decreased. Most interestingly, the lysis of LU99 cells was clearly reduced by the IFN-γ treatment.

IFN-γ induces gene expression of components involved in antigen processing and presentation in the lung cancer cells

Unexpectedly, IFN-γ affected K3-1-mediated lysis differently on PC9, LU99, LC99A and MEG-01 cells (Fig. 3). Therefore, we examined whether there is any difference of expression pattern of molecules important for class I antigen presentation. First, HLA-A24 expression was studied and found to be increased after IFN-γ treatment in all the cell lines (Fig. 4*a*). Second, expression of TAP-1 and TAP-2 was studied using semi-quantitative RT-PCR, mRNAs of both being also consistently increased after the treatment (Fig. 4*b*). Third, the expression of the LMP7, 1 of the 3 catalytic subunits of immunoproteasomes, and PA28, a regulator of the immunoproteasome, was studied using Western blotting with specific MAbs. In all the cell lines but QG56-A24, where the expression did not change, both proteins were increased after the treatment (Fig. 4*c*). In summary, we could not detect any difference in expression patterns of these molecules to account for the differential influence of IFN-γ.

Differential susceptibility of lung cancer cell lines to cytotoxicity of the CD8+ CTL clone, K3-1

To disclose the differential susceptibility to K3-1 in more detail, we compared cytotoxicity against 2 lung cancer cell lines, PC9 cells whose lysis was increased by IFN-γ and LU99 cells whose lysis was decreased, in the presence of a wide range of cognate peptide concentrations. After IFN-γ treatment, PC9 cells were efficiently lysed by K3-1 with any concentration of the peptide (Fig. 5*a*). In contrast, PC9 cells without IFN-γ treatment and LU99 cells, irrespective of IFN-γ treatment, demonstrated exogeneous peptide dose-dependent K3-1-mediated cell lysis, which was similar to the results using T2-A24 cells as target cells (compare Fig. 5*a* and Fig. 1*c*). These observations strongly suggest that the epitope density is saturated on the PC9 cells after IFN-γ treatment but not on the PC9 cells without the treatment and LU99 cells either with or without IFN-γ treatment.

In addition, to confirm that the epitope density was increased in PC9 but not LU99 cells after IFN-γ treatment, naturally processed peptides were acid-eluted from the cells, concentrated and tested by K3-1 after pulsing on T2-A24 cells applying ^{51}Cr-release assays. The results demonstrated in Figure 5*b* indicate elevation in the epitope peptides on the surfaces of PC9 but not LU99 cells after the IFN-γ treatment.

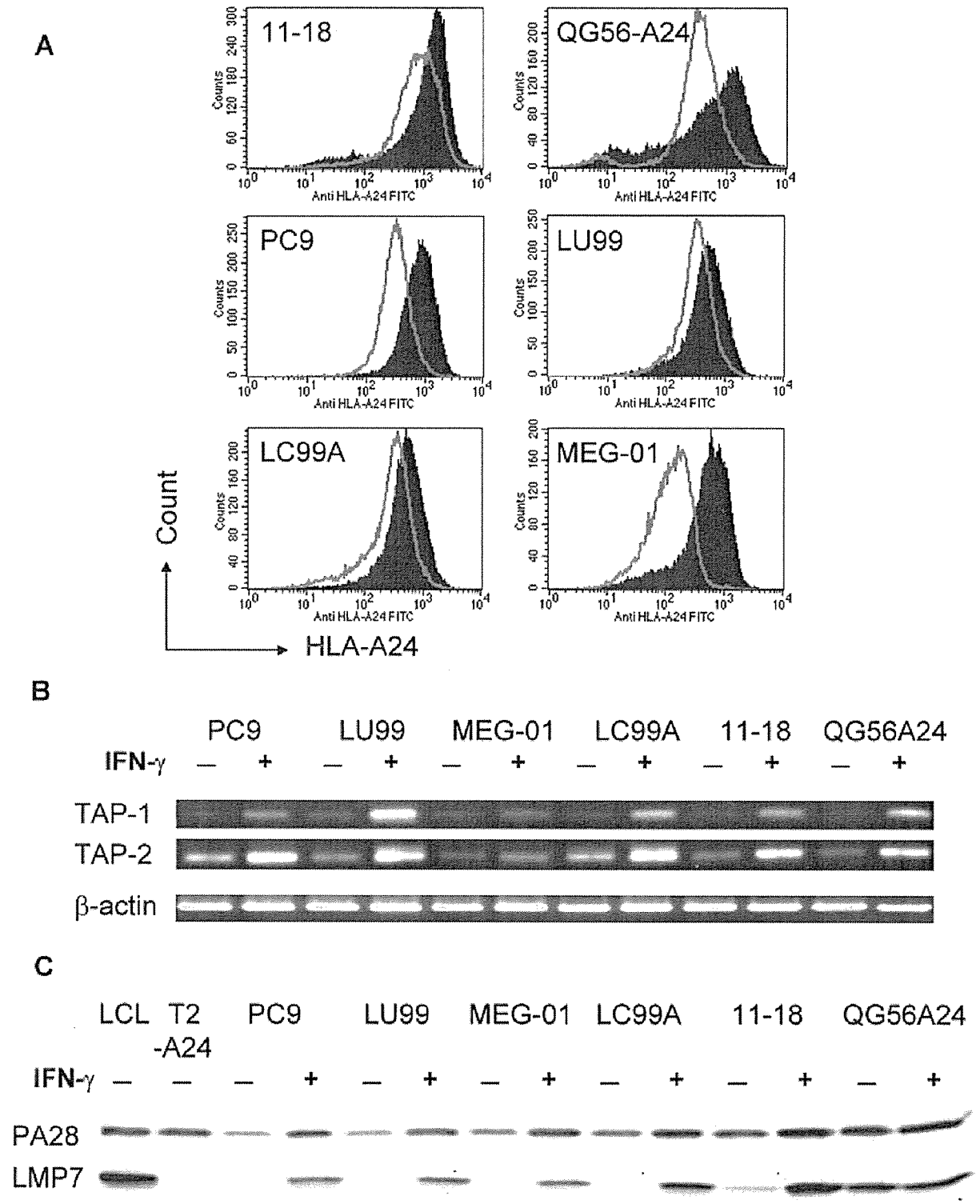

FIGURE 4 – Effects of IFN-γ on the regulation of molecules which play roles in antigen processing and presentation. (*a*) Results for surface HLA-A24 expression with (black shadowed) and without (lined) IFN-γ treatment analyzed by flow cytometry. Surface expression of HLA-A24 molecule was examined by indirect immunofluorescence using an HLA-A24 MAb and FITC-labeled anti-mouse IgG $F(ab')_2$ fragments. (*b*) Results of semi-quantitative RT-PCR analysis of TAP-1, and -2. Primers specific for TAP-1, and -2, as well as β-actin as a control were used for amplification of mRNA from cancer cell lines either treated or untreated with IFN-γ. (*c*) Results of Western blot analysis of PA28 and LMP7 molecules. Samples were obtained before and after treatment of cancer cells with IFN-γ.