obtained in our culture system appear to be T_H1-type NKT cells that have strong antitumor activity through direct and indirect mechanisms.

In the present study, we developed an effective method for $V\alpha24^+$ NKT cell expansion through the use of G-CSF-mobilized peripheral blood. We also featured the possible clinical applications of $V\alpha24^+$ NKT cells in adoptive immunotherapy, both in autologous and allogeneous settings. Further research needed to achieve this goal is underway.

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Evaluation of cytomegalovirus-specific cytotoxic T-lymphocytes in patients with the HLA-A*02 or HLA-A*24 phenotype undergoing hematopoietic stem cell transplantation

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Summary:

Cytomegalovirus-specific cytotoxic T-lymphocytes (CMV-CTL) are essential for the control of CMV reactivation. To monitor the quantity and function of CMV-CTL after hematopoietic stem cell transplantation (HSCT), two CMV epitopes that bind to HLA-A*0201 NLVPMVATV (A*02NLV) and HLA-A*2402 QYDPVAALF (A*24QYD) were evaluated for their immunological potential. Samples from patients with the HLA-A*02 or HLA-A*24 serotype were analyzed by tetramer, intracellular cytokine staining and enzyme-linked immunospot (ELISPOT) assay. There were significantly more A*02NLV-specific CMV-CTL than A*24QYD (23×10^6 *vs* 0.4×10^6/l). The frequency of IFN-γ-producing cells was also higher upon stimulation with A*02NLV than with A*24QYD (2.5 *vs* 0.1%/CD8). Furthermore, the magnitude of CMV-CTL expansion was two- to 50-fold when cells were cultured with A*02NLV, while only an insignificant increase was observed in culture with A*24QYD. Although the number of A*24QYD-specific CMV-CTL was very low in most of the HLA-A*24 patients, the incidence of CMV reactivation did not differ between those with HLA-A*02 and HLA-A*24 serotype alone. These results suggest that an epitope other than A*24QYD plays a major role in patients with HLA-A*24. Our study also showed that A*02NLV may be a useful epitope for monitoring CMV-CTL not only in patients with HLA-A*0201 but also in those with the A*0206 genotype.

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Patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) are at high risk for cytomegalovirus (CMV)-associated disease as a consequence of prolonged T-cell immunodeficiency.[1] Although prophylactic administration of gancyclovir or foscarnet reduces the risk of severe CMV disease, these prophylactic strategies are complicated by significant myelosuppression, nephrotoxicity and even secondary engraftment failure. The development of a reliable method to evaluate CMV-specific immunity will be of critical importance for the prompt initiation of treatment. To address this need, several studies have been conducted with monitoring of tetramer-based CMV-specific cytotoxic-T-lymphocytes (CMV-CTL) in patients with HLA-A*0201 and HLA-B*0702.[2–9] However, the allele frequency of A*0201 is about 26%, with lower frequencies among African-American (16%) and Asian populations (15%).[10] This frequency is much lower in Japanese (11%).[11] Furthermore, the allele frequency of B*0702 is only 5% among Japanese.[11] To make the approach realistic, practical and more widely applicable, the further development of CMV-CTL monitoring among other HLA types is required. HLA-A*2402 is a major alternate candidate, since it is highly represented in Japanese (58%) and other ethnic groups including Caucasian (17%), Hispanic (27%) and Chinese (33%).[12] HLA-A*0206 is also attractive, since its frequency is as high as that of HLA-A*0201 in the Asian population, and is also significant in a certain population of Caucasians in North America.[10,11]

HLA-A*2402-restricted CMV-specific epitope was introduced by Kuzushima *et al* in 2001,[13] and is now widely recognized as the major epitope for HLA-A*2402.[14–17] In this study, we compared the immunological potential of two major CMV pp65 epitope peptides for HLA-A*0201 and HLA-A*2402 for use in monitoring of CMV-CTL by tetramer assay, intracellular cytokine assay, enzyme-linked immunospot (ELISPOT) assay and CTL culture. We also evaluated whether the HLA-A*0201-restricted CMV pp65 epitope could also be used for the A*0206 genotype.

Materials and methods

Subjects

After we obtained written informed consent, peripheral blood samples were obtained from patients with an HLA-

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A*02 or HLA-A*24 serotype who had undergone allogeneic HSCT between 2002 February and 2004 May at National Cancer Center Hospital (Tokyo, Japan). Patients who received non-T-cell-depleted HSCT from a serologically HLA full-matched donor were eligible for this study. Both conventional (CST) and reduced-intensity HSCT (RIST) were included. Those who received a regimen containing antithymocyte globulin (ATG) were excluded since the immunorecovery of T cells is delayed with ATG.

Patients who could be followed up until day 160 after transplantation were eligible for analysis. The characteristics of the 66 eligible patients are shown in Table 1. Of the 34 HLA-A*02-positive patients, 15 were also positive for HLA-A*24 (A*02/A*24 patients). The other 32 patients were only positive for HLA-A*24. Genotyping for HLA-A*02 was performed in 19 patients: nine had HLA-A*0201, nine had HLA-A*0206 and one had both the HLA-A*0201 and HLA-A*0206 genotypes. Since a great majority of the A*24 serotype is A*2402 genotype among Japanese, genotyping for HLA-A*24 was omitted.

In all, 13 patients received bone marrow transplantation (BMT) from an unrelated donor (U-BM), three received BMT from a related donor (R-BM) and the remaining 50 received peripheral blood HSCT from a related donor (R-PB). With regard to the conditioning regimen, 21 patients received a CST regimen that included 120 mg/kg cyclophosphamide (CY) plus 16 mg/kg busulfan (BU) or 120 mg/kg CY plus 12 Gy of total body irradiation (TBI), whereas 45 received an RIST regimen with 0.66 mg/kg cladribine plus 8 mg/kg BU or 180 mg/m^2 fludarabine plus 8 mg/kg BU with or without 4 Gy of TBI.

Diagnostic tests for CMV infection and CMV disease

CMV seropositivity was assessed by the detection of IgG antibodies to CMV late antigen. The presence of CMV-specific IgG antibodies in donors or patients before HSCT was taken as a marker for CMV carrier status. CMV antigenemia was monitored weekly after engraftment to day 90, and at longer intervals thereafter by using the immunocytochemical detection of pp65 antigen in leuko-

Table 1 Patient characteristics

	All patients (n=66)		*Patients with HLA-A*02 (n=34)*		*Patients with HLA-A*24 only (n=32)*	
Sex						
Male	37	56.1%	22	64.7%	15	46.9%
Female	29	43.9%	12	35.3%	17	53.1%
Mean age (range)	46.8	(21–67)	47.8	(21–67)	45.7	(22–64)
Diagnostic indication for HSCT						
AML	16		7		9	
ALL	5		4		1	
CML	3		2		1	
MDS	18		10		8	
NHL	17		9		8	
MF	1		1		0	
ATL	2		0		2	
Solid tumor	4		1		3	
Conditioning regimen						
CST	21	31.8%	11	32.4%	10	31.3%
BU/CY	16		8		8	
CY/TBI	5		3		2	
RIST	45	68.2%	23	67.6%	22	68.8%
Flu/BU	27		11		16	
Flu/BU/TBI	4		4		0	
CdA/BU	12		7		5	
CdA/BU/TBI	2		1		1	
GVHD prophylaxis						
CSP	25		9		16	
CSP+MTX	37		23		14	
Tacrolimus	4		2		2	
Stem cell source						
Related PB	50	75.8%	24	70.6%	26	81.3%
Related BM	3	4.5%	2	5.9%	1	3.1%
Unrelated BM	13	19.7%	8	23.5%	5	15.6%
CMV serology before HSCT						
High risk (recipient+)	59	89.4%	33	97.1%	26	81.3%
Intermediate risk	5	7.6%	0	0%	5	15.6%
Low risk (recipient−, donor−)	2	3.0%	1	2.9%	1	3.1%

cytes. Test results were considered to be positive when more than one leukocyte was positively stained. CMV disease was diagnosed clinically, with confirmation by biopsy of the involved organ.

Peptides and CMV antigen

The following >80% pure peptides, which were assessed as CMV antigen epitope, were obtained using high-performance liquid chromatography (Qiagen, Tokyo): HLA-A*0201 binding peptide NLVPMVATV (AA 495–503, referred to as A*02NLV) or HLA-A*2402 binding peptide QYDPVAALF (AA 341–349, 'A*24QYD') from the CMV pp65 phosphoprotein. HLA-A*0201 binding peptide YLEPGPVTA from glycoprotein 100 (gp100 AA 280–288)[18] and HLA-A*2402 binding peptide AFLPWHRLF from tyrosinase,[19] which is an antigen for melanoma, were used as negative controls.

Tetramer staining

CD8-FITC-, CD4-PC5-, CD19-PC-5-, CD13-PC5- and PE-conjugated tetrameric HLA-A*0201 (A*02NLV-tetramer) or HLA-A*2402 CMV peptide complexes (A*24QYD-tetramer), both purchased form Beckman Coulter Inc. (Fullerton, CA, USA), were added to 200 μl heparinized blood or cultured cell suspension, and incubated for 30 min at room temperature in the dark. When whole-blood samples were used, the red blood cells were lysed after antibody staining. After being washed twice with BSA-containing PBS, the cells were fixed and acquired on a flow cytometer (FACS Calibur, Becton Dickinson). Analysis was performed using Cellquest software. The CD4−, CD19−, CD13− and CD8+ tetramer-positive fraction of the lymphocyte gate was defined as CMV-CTL.

Intracellular cytokine assay

Intracellular cytokine staining was performed asdescribed recently,[20] with the following modifications. Briefly, peripheral whole blood (1 ml) was stimulated for 6 h at 37°C with 10 μg/ml A*02NLV or A*24QYD, in the presence of costimulatory monoclonal antibodies, CD28 and CD49d (Becton Dickinson; 1 μg/ml each). Breferdin A (Sigma; 10 μg/ml) was added for the last 4 h of incubation. Positive and negative controls were obtained by stimulating the cells with 10 μg/ml staphylococcal enterotoxin B or PBS. Samples were lysed, permeabilized and stained with CD69-FITC, IFN-γ-PE, CD3-APC and CD8-PerCP, and analyzed using a FACS Calibur. IFN-γ-positive cells in the CD3+ and CD8+ fraction of the lymphocyte gate were defined as peptide-specific IFN-γ-secreting T cells. CD69 was used as a marker for activated T cells.

ELISPOT assay

Peripheral blood mononuclear cells (PBMCs) were frozen in FBS containing 10% dimethyl sulfoxide (DMSO) at −140°C until use. Frozen PBMCs were thawed and washed in RPMI 1640 (Sigma) supplemented with 10% FBS, gentamicin and streptomycin (hereafter referred to as culture medium), and incubated overnight at 37°C prior to use. PBMCs (0.5×10^5 to 2.0×10^5/well) were stimulated for 24 h at 37°C with A*02NLV or A*24QYD at a concentration of 10 μg/ml. As a negative control, gp100 or tyrosinase was used.

PVDF-bottomed 96-well plates (Millipore) were coated overnight with antibody to human IFN-γ (1-D1K, Mabtech). After the plates were washed and blocked, the cells were transferred to the plate and incubated for 24 h at 37°C After removing the cells, a biotinylated mAb (7-B6-1 Biotin) was applied at 1 μg/ml and the wells were further incubated for 2 h at room temperature. After washing, streptavidin-alkaline phosphatase diluted 1:1000 was added and the wells were incubated for 1 h at room temperature. After washing, the substrate was added and the wells were incubated until spots emerged. The spots were counted using a phase-contrast microscope.

Expansion of CMV-specific CTL

PBMCs were separated from heparinized blood by Ficoll–Hypaque (IBL, Japan) density-gradient centrifugation, and diluted at 1×10^6 cells/ml in culture medium. PBMC (2×10^6 cells) were seeded in a 24-well plate, and A*02NLV or A*24QYD was added to a final concentration of 5 μM on day 0. The peptide was diluted to 10 mg/ml in DMSO prior to use, and the same amount of DMSO as was contained with peptide was used as a negative control. The culture was fed on days 4 and 7 by replacing half of the medium with fresh culture medium containing 20 U/ml IL-2, and 10 μM CMV peptide. Cells were cultured for 14 days.

Statistical analysis

The significance of differences in the frequency of CMV reactivation, high CMV antigenemia and CMV disease was evaluated by the χ^2 test or Fisher's exact test according to the sample size. Values of $P<0.05$ were considered significant.

Results

CMV infection in relation to CMV serology and HLA-A locus

The CMV reactivation status after HSCT is shown in Table 2. Among all 66 patients, 44 (67%) showed reactivation as evidenced by the appearance of pp65+ leukocytes in the blood, and 23 (35%) had an elevated level of antigenemia >10/50 000. When the patient and donor were both CMV seropositive, the CMV reactivation rate was 74%, while only one of seven CMV-seronegative recipients had CMV reactivation with low titers of antigenemia. All of the six patients who developed CMV disease had been CMV seropositive before transplantation and had undergone RIST (five R-PB, one U-BM). The CMV reactivation rate was higher among unrelated HSCT (85%) than among related pairs (62%). All of the seven patients who received U-BM with an RIST regimen developed CMV antigenemia, and six had antigenemia

Table 2 CMV reactivation status after HSCT

	Total	*CMV reactivation*		*Antigenemia >10/50 000*		*CMV disease*	
All patients	66	44 (66.7%)	$P=0.394$	23 (34.8%)	$P=0.898$	6 (9.1%)	$P=1.000$
Donor and patient CMV seropositive	50	37 (74.0%)		18 (36.0%)		5 (10.0%)	
Related donor	53	33 (62.3%)	$P=0.192$	15 (28.3%)	$P=0.048$*	5 (9.4%)	$P=1.000$
Unrelated donor	13	11 (84.6%)		8 (61.5%)		1 (7.7%)	
Patients with HLA-A*02	34	24 (70.6%)	$P=0.486$	13 (38.2%)	$P=0.552$	4 (11.8%)	$P=0.673$
Patients with only HLA-A*24	32	20 (62.5%)		10 (31.3%)		2 (6.3%)	

*Statistically significant.

>10/50 000, including one who developed CMV disease. The rate of CMV reactivation, antigenemia >10/50 000 and CMV disease tended to be higher in HLA-A*02 (71, 38 and 12%, respectively) than in HLA-A*24 patients (63, 31 and 6%, respectively), but these differences were not significant.

Enumeration of CMV-specific CTL by tetramer assay

Peripheral blood samples were taken at least 4 weeks after transplantation with confirmed engraftment. Additional requirements for sampling included complete remission in leukemia patients and PR or stable disease without bone marrow involvement in those with lymphoma or solid tumor. The absolute number of CMV-CTL, and the percentage of CMV-CTL per lymphocyte and per CD8+ lymphocyte were assessed by tetramer assay. A*02NLV- or A*24QYD-tetramer was chosen according to the patient's HLA subtype. For those with A*02/A*24, both A*02NLV- and A*24QYD-tetramer stainings were performed independently (Figure 1a and b). A total of 185 samples from 33 HLA-A*02 patients and 147 samples from 44 HLA-A*24 patients, including 78 samples from 14 A*02/A*24 patients, were obtained. One to 10 samples (median, 5) were obtained from each patient. Samples were not obtained from one A*02/A*24 patient and two HLA-A*24 single-positive patients who had been entered into this study since they did not meet the sampling criteria mentioned above.

For the 185 samples assessed for A*02NLV-tetramer and 147 samples assessed for A*24QYD-tetramer, the median number of days from transplantation at the time of sampling and the mean lymphocyte count in the samples was, respectively, 233 (28–622) and 217 (28–633), and 1.94×10^9/l and 1.93×10^9/l.

The mean numbers of CMV-CTL for A*02NLV- and A*24QYD-tetramer among all of the samples were, respectively, 23×10^6/l and 0.4×10^6/l, and the mean percentage of CMV-CTL was 1.59 and 0.03% per lymphocyte (3.5 and 0.06% per CD8+ T-lymphocyte, respectively). With regard to A*02NLV-tetramer, 176 of the samples (95%) had detectable CMV-CTL and 83 (45%) showed more than 1% positive cells per lymphocyte (Figure 1c), whereas only 80 (54%) had detectable CMV-CTL, and none showed more than 1% for A*24QYD-tetramer (Figure 1d). All of the nine samples that were negative for A*02NLV-tetramer were also negative for A*24QYD-tetramer. Samples that were negative for A*02NLV-tetramer were taken from patients who received transplantation from seronegative donor and did not have CMV reactivation. Otherwise, a detectable level of CMV-CTL was present in all of the HLA-A*02 patients immediately after engraftment. However, 36% of the patients did not achieve detectable levels of CMV-CTL with A*24QYD-tetramer, and only 11% of the HLA-A*24 patients obtained a sufficient level (0.05% or more) of CMV-CTL throughout the course. Similarly, in the 78 samples from A*02/A*24 patients, the mean percentage of CMV-CTL for A*02NLV- and A*24QYD-tetramer was 1.47 and 0.01% per lymphocyte. (Figure 1e and f). While 73 of the samples (94%) were positive for A*02NLV-tetramer and only 16 (21%) were below 0.05%, only 37 (47%) were positive for A*24QYD-tetramer ($P<0.0001$) and 75 (96%) were below 0.05% ($P<0.0001$). While all of the five samples that were negative for A*02NLV-tetramer were also negative for A*24QYD-tetramer, 36 of the 41 A*24QYD-tetramer-negative samples were positive for A*02NLV-tetramer.

The staining of A*02NLV-tetramer was dull in samples from HLA-A*0206 genotype compared to that in HLA-A*0201, and samples with both HLA-A*0201 and HLA-A*0206 showed mixed tetramer bright and dull populations in the tetramer-positive fraction (Figure 2). The frequency of A*02NLV-tetramer-positive CMV-CTL in the samples from HLA-A*0206 patients was as high as that in samples from HLA-A*0201 patients. The mean percentage of A*02NLV-tetramer-positive cells per lymphocyte and the positive rate of A*02NLV-tetramer in 75 samples from 10 HLA-A*0201 patients and 57 samples from 10 HLA-A*0206 patients were, respectively, 1.5 and 2.7, and 99% (74 samples) and 93% (53 samples).

Intracellular cytokine assay

Intracellular cytokine was assessed in 11 samples from 11 A*02/A*24 patients. The mean percentages of intracellular IFN-γ-positive cells per CD8+ lymphocyte when stimulated with A02*NLV, A*24QYD and PBS were, respectively, 2.5% (0.05–7.3%), 0.11% (0.01–0.31%) and 0.16% (0.02–0.55). The percentages of IFN-γ-positive cells in samples from eight HLA-A*0206 patients were as high as those in three samples from HLA-A*0201 patients. All of the 11 samples showed a higher percentage of IFN-γ-positive cells when stimulated with A02*NLV than with

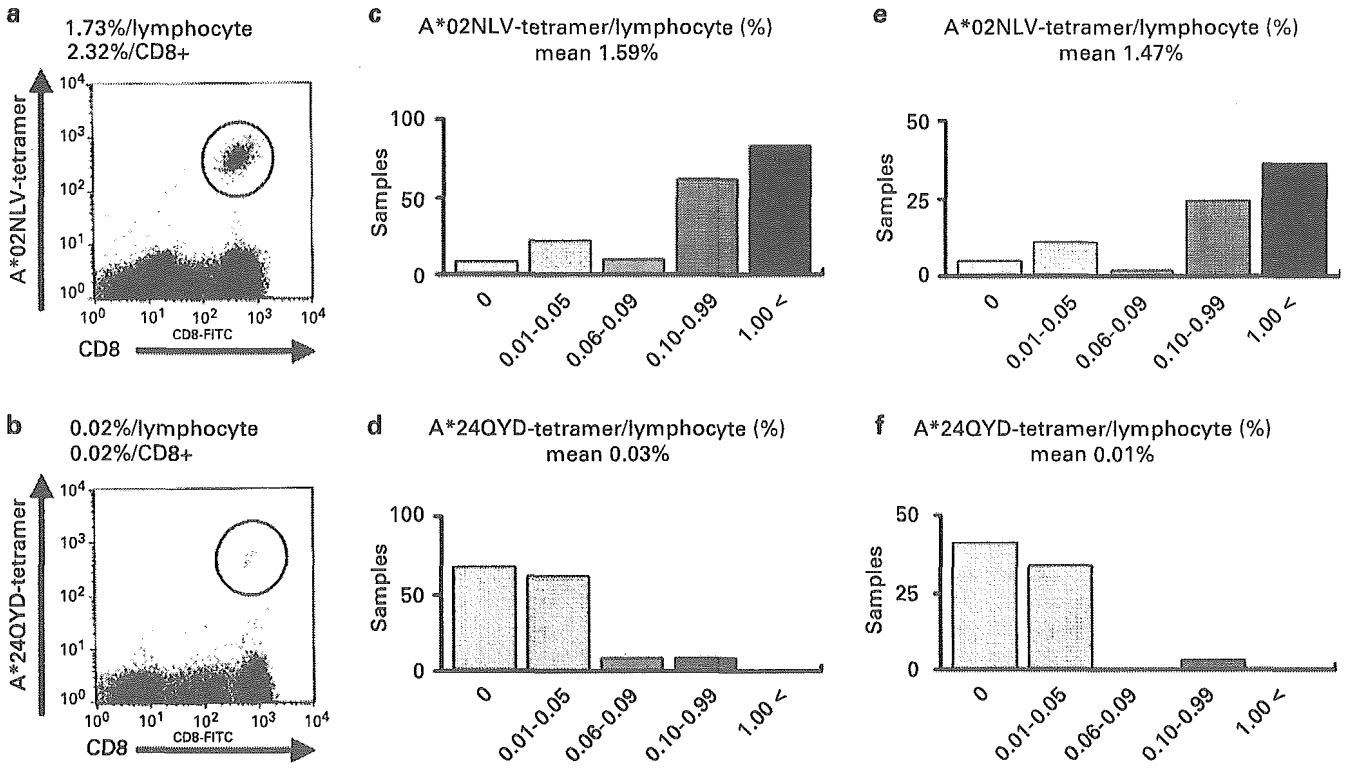

Figure 1 Staining of A*02NLV-tetramer and A*24QYD-tetramer. The percentage of CMV-CTL was higher for A*02NLV-tetramer (1.7%/lymphocyte) (**a**) than with A*24QYD-tetramer (0.02%/lymphocyte) (**b**) in a patient with both the HLA-A*0201 and HLA-A*24 phenotypes. The percentage of CMV-CTL was measured by tetramer assay in 185 samples taken from 33 patients with HLA-A*02 (**c**), 147 samples from 44 patients with HLA-A*24 (**d**) and 78 samples taken from 14 patients with both the HLA-A*02 and HLA-A*24 phenotypes (**e** and **f**). The mean percentage of tetramer-positive cells per lymphocyte was 1.6% for A*02NLV (**c**), 0.02% for A*24QYD (d), 1.5% for A*02NLV (**e**) and 0.01% for A*24QYD (**f**).

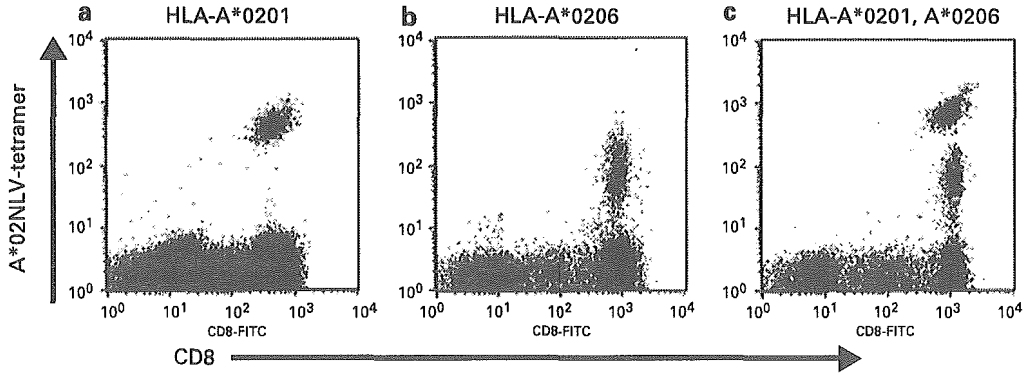

Figure 2 A*02NLV-tetramer staining in patients with different HLA-A genotypes. Tetramer staining is brighter in HLA-A*0201 (**a**) than in HLA-A*0206 (**b**). Patients with both the HLA-A*0201 and HLA-A*0206 genotypes show both tetramer bright- and dull-positive CMV-CTL (**c**).

A*24QYD. When stimulated with A*24QYD, the percentage of IFN-γ-positive cells did not differ from that in the negative control (Figure 3a–c).

A simultaneous tetramer assay also showed a higher CMV-CTL rate in A*02NLV-tetramer (average, 4.3%/CD8) than in A*24QYD-tetramer (0.02%/CD8). The number of IFN-γ-positive cells stimulated with A02*NLV correlated with the number of A*02NLV-tetramer-positive CMV-CTL (Figure 3d).

Intracellular cytokine and tetramer assay were performed in one A*02/A*24 patient with relapsed AML after RIST (not listed in Table 1 since he did not achieve clinical remission after RIST). This patient showed a higher percentage of A*24QYD-tetramer-positive CMV-CTL (1.2%/CD8) than A*02NLV-tetramer-positive CMV-CTL (0%/CD8), and also showed a higher percentage of IFN-γ-positive cells when stimulated with A*24QYD (0.5%/CD8) than with A02*NLV (0.03%/CD8).

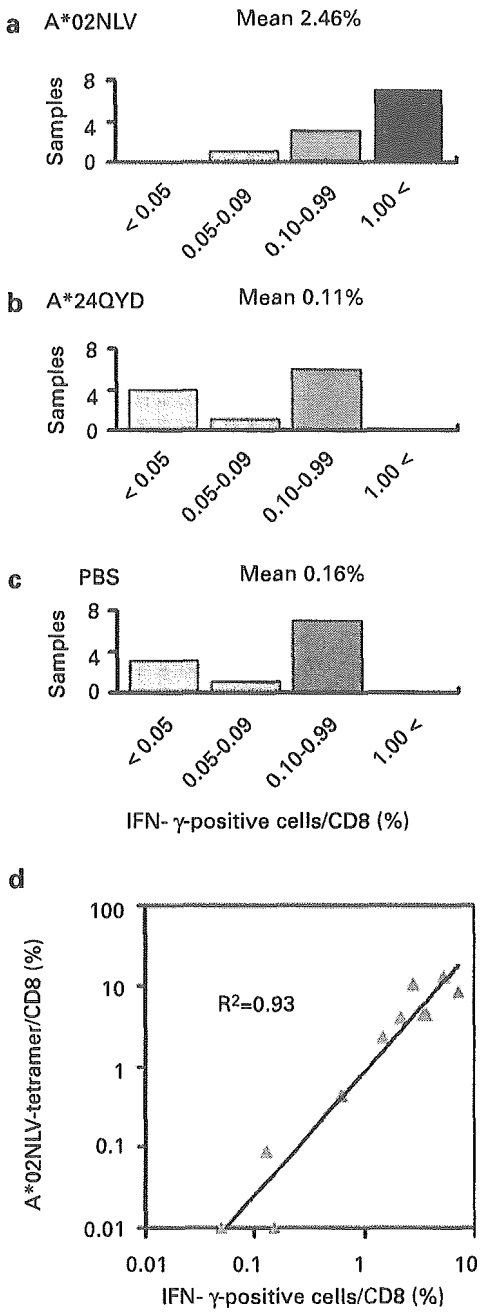

Figure 3 Proportion of IFN-γ-producing cells as assessed by intracellular cytokine staining. The percentage of IFN-γ-positive cells per CD8 lymphocyte was assessed in 11 samples obtained from 11 patients with both the HLA-A*02 and HLA-A*24 phenotypes who were not on steroid treatment. Cells stimulated with A*NLV showed more than 1%/CD8 IFN-γ-positive cells in seven samples (**a**), while cells stimulated with A*24QYD (**b**) were not significantly different from the negative control (**c**). The percentage of A*02NLV-tetramer-positive cells per CD8 correlated with the percentage of IFN-γ-producing cells per CD8 when stimulated with A*02NLV peptide (**d**).

ELISPOT assay

ELISPOT assay was performed in 12 PBMC samples obtained from seven A*02/A*24 patients. The average number of spots was $50/10^5$ PBMC when stimulated with A02*NLV, while this was $4/10^5$ PBMC for A*24QYD, $1/10^5$ PBMC for gp100 and $2/10^5$ PBMC for tyrosinase.

Expansion of CMV-specific CTL

Seven PBMC samples obtained from seven A*02/A*24 patients were cultured with or without CMV peptide, and CMV-CTL was analyzed by tetramer assay on day 14 of culture. A*02NLV-tetramer-positive CMV-CTL increased significantly in all seven samples cultured with A*02NLV, while only a slight increase in A*24QYD-tetramer-positive CMV-CTL was observed in two samples cultured with A*24QYD. The mean percentage of A*02NLV-tetramer-positive CMV-CTL per lymphocyte was 2% (0.09–8) before culture, and this increased to 19% (0.4–63) after culture with A*02NLV, but decreased to 0.4% (0.01–0.9) after culture without peptide. The mean A*24QYD-tetramer-positive CMV-CTL per lymphocyte was 0.004% (0–0.01) before culture and 0.04% (0–0.2) after culture with A*24QYD peptide, with only one sample showing a significant increase. The expansion rate in samples from five HLA-A*0206 patients was as high as that in two samples from HLA-A*0201 patients (Figure 4). The percentage of CMV-CTL per lymphocyte correlated with CMV-CTL per CD8 lymphocyte, and also with the absolute number of CMV-CTL after culture.

Discussion

Our study showed that the frequency of A*24QYD-tetramer-positive CMV-CTL was extremely low compared to that of A*02NLV. We also found that A*24QYD is not a potential epitope for monitoring CMV-specific CTL, since more than 1% A*02NLV-tetramer-positive CMV-CTL was detected in 45% of the samples, while the frequency of A*24QYD-tetramer-positive CMV-CTL was below 0.05 in 88% of the samples: 46% had an undetectable level. Both an intracellular cytokine assay and ELISPOT assay showed that the stimulation potential of A*24QYD for the secretion of IFN-γ was weak, and the results of the intracellular cytokine assay for A*24QYD were similar to those in the negative control. Moreover, the efficiency at expanding CMV-CTL was also significantly lower in A*24QYD. These results support the notion that the low frequency of A*24QYD-tetramer-positive CMV-CTL is not a false-negative result due to the quality of the analytical procedures, and the presence of one tetramer-positive patient served as a functioning internal control to prove the quality of the A*24QYD-tetramer.

In our study, the frequency and severity of CMV reactivation did not differ between HLA-A*02 and HLA-A*24 patients. A recent study with renal transplant recipients also showed that neither HLA-A*02 nor HLA-A*24 was related to the risk of CMV infection.[21] These results suggest that epitopes other than A*24QYD may play a major role in HLA-A*24 patients. Although we cannot rule out the possibility that a CMV-specific response is not restricted to HLA-A*24, we could at least show that the frequencies of other HLA alleles including HLA-B*07, which is known to have a strong CMV epitope, were not significantly different between HLA-A*02 and HLA-A*24 individuals. Furthermore, the frequency of CMV reactivation was not higher among HLA-A*24

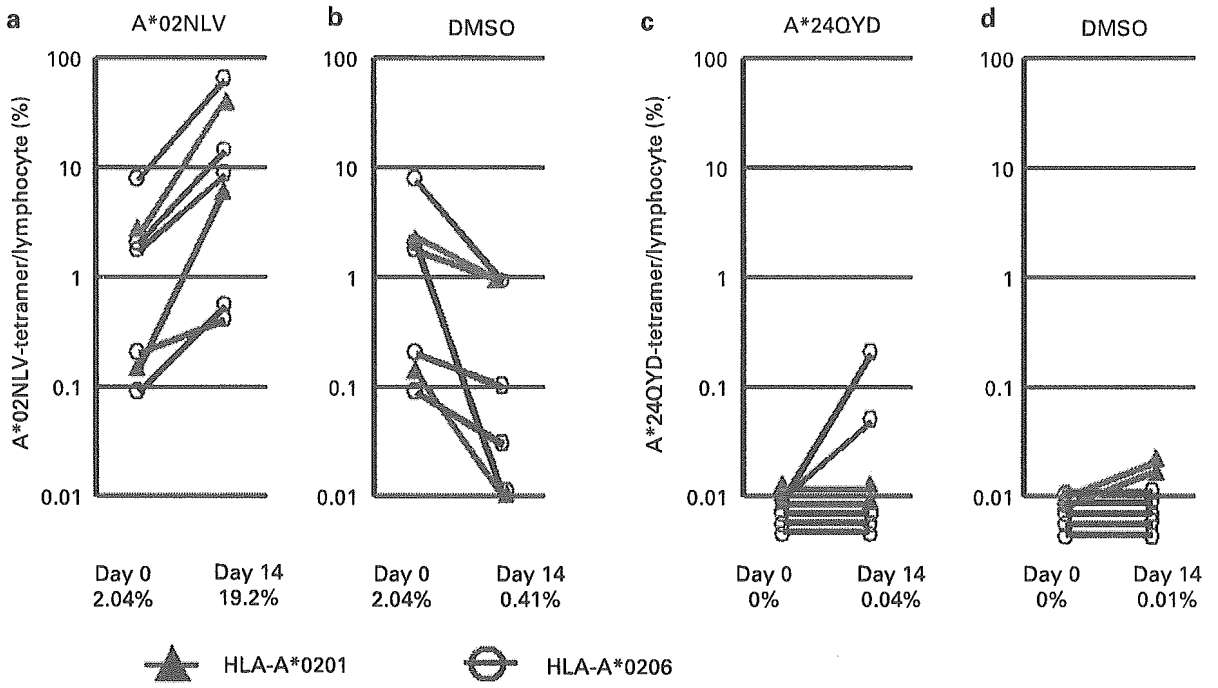

Figure 4 CMV-CTL culture in patients with both the HLA-A*02 and HLA-A*24 phenotypes. CMV-CTL was cultured with A*02NLV (**a**), A*24 QYD (**c**) or DMSO as a negative control (**b**, **d**) in seven samples from seven patients with the HLA-A*02 and HLA-A*24 phenotypes. A*02NLV-tetramer-positive cells were significantly increased in both the HLA-A*0201 and HLA-A*0206 genotypes when cells were cultured with A*02NLV (**a**), and they decreased when cells were cultured without peptide in all seven samples (**b**). Only two samples showed an increase in A*24QYD-tetramer-positive cells when cultured with A*24QYD (**c**).

homologous patients. These facts suggest the presence of a potent CMV epitope that is restricted to HLA-A*24. The contributions of other HLA-A*24 epitopes, such as VYALPLKML (VYA),[13,16,22] AYAQKIFKIL (AYA)[23] and FTSQYRIQGKL (FTS),[10] have been reported, but tetramer-based analysis has only been performed in A*24QYD.[5] Furthermore, it has been reported that IFN-γ secretion after stimulation with VYA was significantly lower than that with A*24QYD,[16] which does not have the capacity to sensitize T cells even at a higher concentration of 10 μM.[13] It has also been reported that no CTL response was detected by IFN-γ ELISPOT assay using AYA peptide.[23] To confirm this, we performed an ELISPOT assay and intracellular IFN-γ assay with A*24QYD, VYA, AYA and FTS in HLA-A*24 samples. As a result, none of the peptides except for A*24QYD showed a positive result, which demonstrates that A*24*QYD is among the strongest epitopes currently known.

One A*02/A*24 patient (not included in the analysis of this study because of relapsed AML), who had a high titer of A*24QYD-tetramer-positive CMV-CTL and no A*02NLV-tetramer-positive CMV-CTL, showed the HLA-A*0207 and HLA-A*2402 genotypes. The reason that A*24QYD was the dominant epitope in this patient may be that A*02NLV cannot be presented in the A*0207 genotype. To support this, Lacey *et al*[24] suggested that a higher frequency of tetramer-positive cells of less-favored epitopes was observed in patients lacking an immunodominant allele. Since there seems to be significant interpatient variation in the corresponding epitope, negative data with a single epitope may simply imply that the target is out of focus. Several groups have measured the T-cell response to CMV by flow cytometry using overlapping CMV peptide mixtures.[25–28] It has also been reported that with the use of such peptides of various lengths, both CD4+ and CD8+ cells could be stimulated. Since CD4+ and CD8+ CTL to multiple epitopes are both important for CMV immunity, we recognize that it would be more ideal to pulse PBMC with a CMV peptide mixture, instead of single peptides to monitor CMV immunity after HSCT.

Intracellular cytokine staining showed that not only CD3+ and CD8+ lymphocytes but also CD8-negative cells produced IFN-γ when stimulated by CMV peptide. It is assumed that cells other than CMV-CTL were influenced by the cytokines secreted from stimulated cells to non-specifically produce IFN-γ. Although ELISPOT is widely used for detecting antigen-specific cells,[29–32] it is impossible to distinguish whether the spots are specific from CTL or nonspecific from other functioning mononuclear cells, including CD4+ lymphocytes, NK or NK-T cells, since no other information to specify the cytokine-secreting cells can be obtained. In our study, the sensitivity of the ELISPOT assay did not differ from that of the intracellular cytokine assay, with a lower frequency of positive cells compared to the tetramer assay, which was compatible with the previous report.[33] Based on these results, the intracellular cytokine assay seems to be suitable for evaluating the function of CTL.

Our study proved that A*02NLV may be used for monitoring and in the expansion of CMV-CTL not only in HLA-A*0201 but also in the HLA-A*0206 genotype, which suggests that A*02NLV is crosspresented by HLA-

A*0201 and A*0206. This is very important, since the HLA-A*0206 genotype accounts for a considerable population of the HLA-A*02 serotype in certain ethnic groups.[10,11] The crosspresentation of epitopes by related alleles of the HLA-A*02 supertype with different binding affinities has been reported,[34] and the possibility of A*02NLV being presented by five different allelic variants of HLA-A*02 has also been reported.[10] The relatively dull staining of A*02NLV-tetramer for HLA-A*0206 may be due to the lower binding affinity of the epitope peptide in the HLA-A*0206 genotype. However, this should be confirmed by experiments using HLA-A*0206-tetramer, since we also used HLA-A*0201-tetramer in the assay for HLA-A*0206 samples. In either case, the fact that the percentage of tetramer-positive cells and the stimulation potential of A*02NLV for producing IFN-γ and expanding CTL did not differ significantly between HLA-A*0201 and A*0206 confirms that A*02NLV is a potential epitope for monitoring CMV-CTL not only in patients with the HLA-A*0201 genotype but also in those with the A*0206 genotype. It is important to know the HLA restriction and dominance of CMV epitopes for immunotherapeutic strategies. There have been successful studies on CMV-specific T-cell expansion that have involved sorting IFN-γ-producing cells on stimulation with CMV epitope peptides,[20] or the collection of CMV-CTL using tetramer.[35,36] These procedures have the advantage that CTL could be expanded from a small amount of PBMC obtained from a single blood draw. Tetramer-based cell expansion has thus far been performed only on HLA-A*0201 patients, but our results suggests that it may also be possible to apply this to HLA-A*0206 patients. Furthermore, these procedures could be applied to more than 90% of the population if a dominant HLA-A*24 epitope were identified. More effort should be focused on the investigation of CMV epitope for HLA-A*24 serotype.

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