presenting molecules.

Faster development of NBU-induced lymphomas is observed in TL transgenic mice. We administered NBU orally to B6 and C3H as well as to their $T3^b$-TL gene-transduced counterparts, B6.Tg.Con.3-1 and Tg.Con.3-1, respectively, to study the possibility that anti-TL immunity affects lymphoma development. As shown in Fig. 2, the survival times of B6 and C3H were prolonged, compared with their TL transgenic counterparts, in which TL-specific T cells are assumed to be eliminated and/or anergized, suggesting that anti-TL immunity protects against lymphoma growth *in vivo*. In addition, the survival time of C3H was found to be slightly longer than that of B6, reflecting slower development of lymphomas in C3H than in B6, although the difference is not statistically significant.

Grafted skins from B6.Tg.Con.3-1 on B6 are not rejected. To compare anti-TL immunity in B6 with that in C3H, skin-graft experiments were carried out with various combinations of transgenic, congenic and inbred strains of mice. As previously reported, skin grafts from Tg.Con.3-1 were rejected efficiently by recipient C3H mice, while $(B6\times C3H)F_1$ mice were less potent at rejecting Tg.Con.3-1-derived skin, i.e., rejection was observed in 7 out of 9 grafts in this study, and 22 out of 29 grafts in the previous study.[15] In contrast to C3H, B6 could not reject skin grafts from B6.Tg.Con.3-1 (Table 1), although the expression level of TL in the skin of B6.Tg.Con.3-1 was comparable to that of Tg.Con.3-1 (data not shown). We also analyzed $CD11c^+$ Langerhans cells of both strains, but no significant difference in the cell number or TL expression was observed (data not shown). One possible explanation for the failure of B6 mice to reject B6.Tg.Con.3-1 skin grafts might be $H\text{-}2^b$-restricted active suppression by regulatory T cells. *In vivo* administration of anti-CD4 and/or CD25 mAbs, however, did not induce rejection of B6.Tg.Con.3-1 skin (data not shown), suggesting that at least $CD4^+CD25^+$ regulatory T cells (Treg)[21,22] and type 3 helper T cells (Th3)[23,24] are not responsible for the weak or absent responsiveness. When $(B6\times C3H)F_1$ mice received skin from B6.Tg.Con3-1, graft crisis was evident in 4/10. The results also demonstrated that $(B6\times C3H)F_1$ were less potent at rejecting B6.Tg.Con.3-1 than Tg.Con.3-1 skin grafts.

The number of TL tetramer$^+$ cells does not always correlate with skin graft rejection. Attempts were made to generate TL-specific CTLs from B6 mice receiving skin grafts from B6.Tg.Con.3-1, in the same way as employed for CTL induction in the C3H system,[9,15,19] but were unsuccessful, in concordance with the results of the skin-graft experiments described above. As shown in Fig. 3, less than 5% of $CD8^+$ cells were found to be positive for TL tetramer staining in primary MLC derived from B6.Tg.Con-1 skin-grafted B6 spleen, while 25–30% of those from Tg.Con.3-1 skin-grafted C3H were positive. In addition, only a TL tetramer-weakly positive population, considered to express low avidity TCR for TL,[6,19] was induced from B6.

Tetramer analysis was also conducted with $(B6\times C3H)F_1$ mice grafted with Tg.Con.3-1 or B6.Tg.Con.3-1 skins, and the results are summarized in Table 2. We found that 7.5 to 15% of $CD8^+$ cells in primary MLC with the spleens from Tg.Con.3-1 skin-grafted F_1 mice were TL tetramer-positive, being lower than that with Tg.Con.3-1 skin-grafted C3H mice (25–30%) as shown in Fig. 3, in accordance with the results of skin grafting, which showed that immunity against TL in F_1 mice is generally weaker than that of C3H (Table 1). The proportion of TL tetramer$^+$ cells in $CD8^+$ T cells in primary MLC with the spleen cells from B6.Tg.Con.3-1 skin-grafted F_1 mice was lower than in the Tg.Con.3-1 skin-grafted F_1, being in general agreement with the results of skin grafting. These results together suggest that the precursor frequencies of TL-specific CTL in B6 are lower than in C3H with intermediate values for $(B6\times C3H)F_1$. When examined at the level of individual mice, however, the proportion of TL tetramer$^+$ cells does not always correlate with the fate of the grafted skins.

TL-specific CTL is negatively selected and/or anergized to a certain extent by reacting with H-2K^b. In our previous study, it was of in-

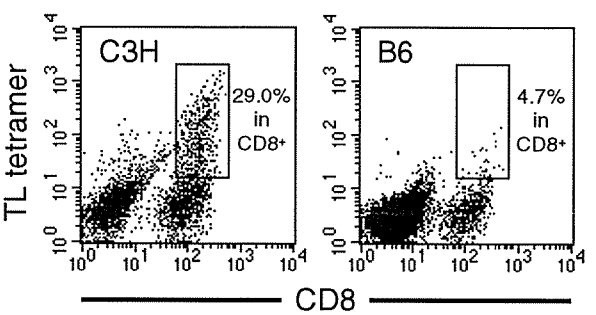

Fig. 3. Tetramer analysis of primary MLC. MLC was performed with spleen cells from skin-grafted mice 8 weeks after transplantation as described previously.[9,15,19] Spleen cells from C3H and B6 that had received skin grafts from Tg.Con.3-1 and B6.Tg.Con.3-1, respectively, were stimulated once with γ-irradiated spleen cells from the respective TL transgenic strains. Cells from MLC were incubated with the PE-labeled $T3^b$-TL tetramer (1 μg/ml) at room temperature for 30 min and then with an FITC-labeled anti-CD8α mAb at 4°C for 30 min.

Table 2. Frequency of CTL precursors against TL in skin-grafted $(B6\times C3H)F_1$ mice

Mouse	Skin grafting		MLC/TL tetramer staining[1] (%)	
	Skin graft	Result	Stimulator	TL tetramer$^+$/$CD8^+$ cells
1	Tg.Con.3-1	Rejected	Tg.Con.3-1	15.4
			B6.Tg.Con.3-1	14.0
2		Rejected	Tg.Con.3-1	7.4
			B6.Tg.Con.3-1	7.5
3		Crisis	Tg.Con.3-1	15.0
			B6.Tg.Con.3-1	14.3
4	B6.Tg.Con.3-1	Crisis	Tg.Con.3-1	8.5
			B6.Tg.Con.3-1	7.9
5		Accepted	Tg.Con.3-1	6.5
			B6.Tg.Con.3-1	8.7
6		Accepted	Tg.Con.3-1	9.0
			B6.Tg.Con.3-1	7.5

1) Spleen cells were obtained from the F_1 mice at 8 weeks after skin grafting, and stimulated with spleen cells from TL transgenic mice from 5 days. Cells from such primary MLC were stained with PE-TL tetramers and FITC-labeled anti-CD8α mAb (see "Materials and Methods" and also the legend of Fig.3).

terest that the immune responses against TL$^+$ skin grafts were weaker in H-2K^b transgenic mice (C3H background) than in C3H mice (rejection of Tg.Con.3-1 grafts, 51/52 in C3H versus 6/10 in Tg.H-2K^b-1),[15] suggesting that H-2K^b expression in the thymus and/or periphery might result in negative selection and/or tolerance induction of a certain population of TL-specific T cells, especially CTL. To explore this possibility, we employed two unique TL$^+$ B lymphomas (110501 and 110201b) established from Tg.Con.3-1 mice by oral administration of NBU in the same way as described previously.[12, 16] As shown in Fig. 4, these lymphomas were defined as of B-cell origin, since they expressed B220 but not Thy-1. A higher level of TL (5–10 times more as compared with TL$^+$ T lymphomas, such as C3NB1 (C3H origin)[12] and ERLD (B6 origin)) and MHC class I were expressed, but little or no expression of MHC class II (I-A^k and I-E^k), CD40, CD80 (data not shown) or CD86 was observed. When these TL$^+$ B lymphomas were transplanted into C3H nude mice or Tg.Con.3-1 mice, they grew progressively, as with TL$^+$ T lymphomas, but when they were transplanted into C3H mice, they grew to a certain extent, but were finally rejected in most mice (Figs. 5 and 6), suggesting that T lymphocytes were involved in the rejection. Unexpectedly, when the tumor cell numbers were increased, they were rejected more readily. *In vivo* antibody-depletion experiments showed that CD8 cells are predominantly involved in TL$^+$ B lymphoma rejection (data not shown). When TL$^+$ B lymphomas were transplanted into (B6×C3H)F$_1$ or Tg.H-2K^b-2 mice (C3H background) expressing H-2K^b ubiquitously, they were not rejected as expected from our previous study on skin grafting,[15] as described above, supporting the possibility that TL-specific effector T cells are negatively selected and/or anergized to a certain extent in these mice by interacting with H-2^b molecules in the thymus and/or periphery. Of those, H-2K^b molecules seem to be more important than H-2D^b, since the frequency of TL-specific T lymphocytes in Tg.H-2K^b-2 mice determined by TL tetramer analysis was 7.5–15%, being comparable to that of (B6×C3H)F$_1$ (data not shown).

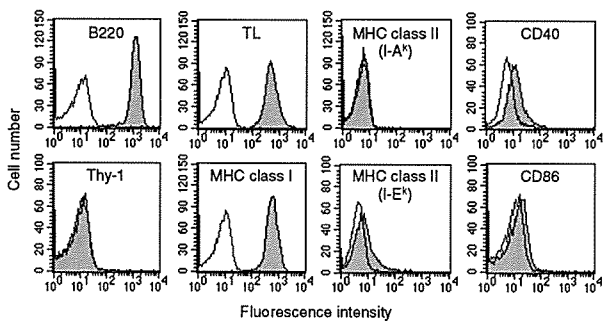

Fig. 4. Surface phenotype of NBU-induced B lymphomas derived from Tg.Con.3-1. NBU-induced B lymphoma cells (110501) were stained and analyzed on a FACSCalibur. Similar results were also obtained with another NBU-induced B lymphoma 110201b from Tg.Con.3-1.

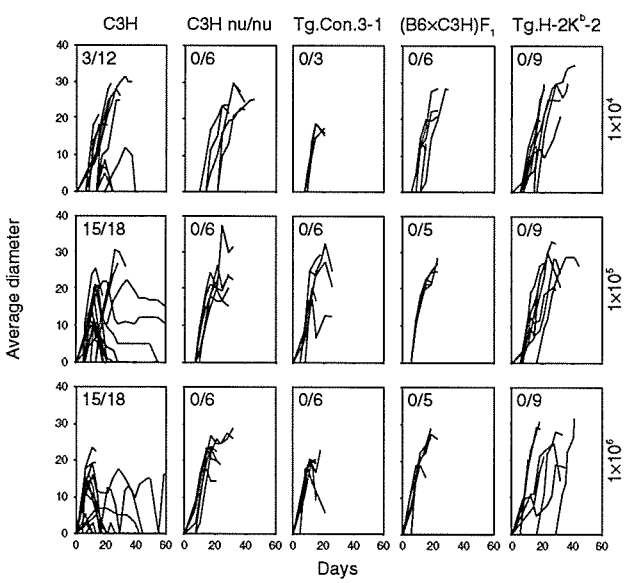

Fig. 5. Growth of NBU-induced B lymphomas *in vivo*. Mice were injected subcutaneously (s.c.) with NBU-induced B lymphoma cells (110501). The number of surviving mice in each group at day 60 is shown (surviving/total mice), and cell numbers injected are given on the right. Tumor size values are the averages of the longest and the shortest diameters. Similar results were also obtained with another NBU-induced B lymphoma 110201b from Tg.Con.3-1.

Anti-TL antibody activity is higher in C3H than B6 mice receiving TL$^+$ skin grafts. We previously demonstrated by antibody-depletion experiments that CD4$^+$ T cells are indispensable for rejection of TL$^+$ skin from Tg.Con.3-1.[25] Such observations suggest the possibility that the weaker immune responsiveness against TL in B6 than in C3H is due to not only negative selection and/or anergy of CD8$^+$ CTLs, but also the lack of Th activity. We evaluated helper T cell (Th) activity in anti-TL immunity by measuring anti-TL antibodies in the sera from skin-grafted mice in both B6 and C3H strains, due to the lack of a direct assessment system of Th function for CTL induction. As shown in Fig. 7A, anti-TL antibody activity was much higher in sera

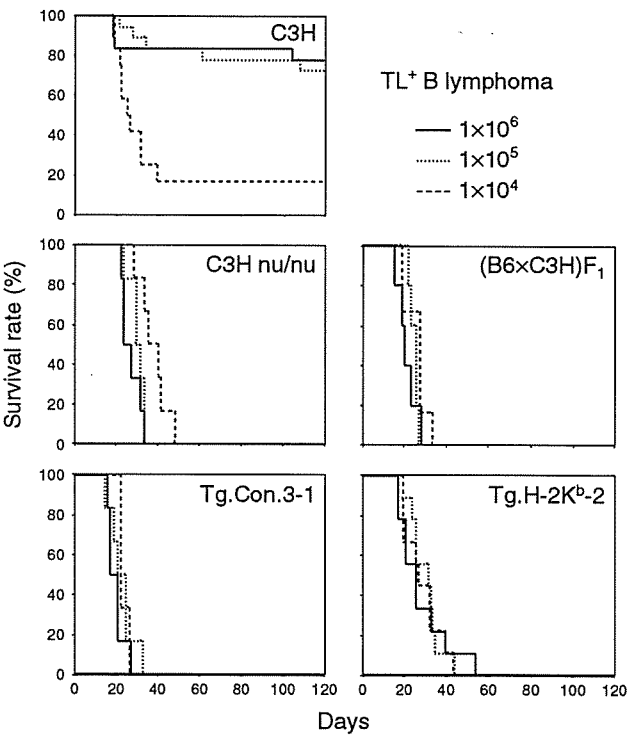

Fig. 6. Survival time of mice transplanted with NBU-induced B lymphomas. Mice were injected s.c. with NBU-induced B lymphoma cells (110501), and the survival time was determined. The cell numbers injected were 1×10^4 (broken line), 1×10^5 (dotted line), and 1×10^6 (unbroken line). Similar results were also obtained with another NBU-induced B lymphoma 110201b from Tg.Con.3-1.

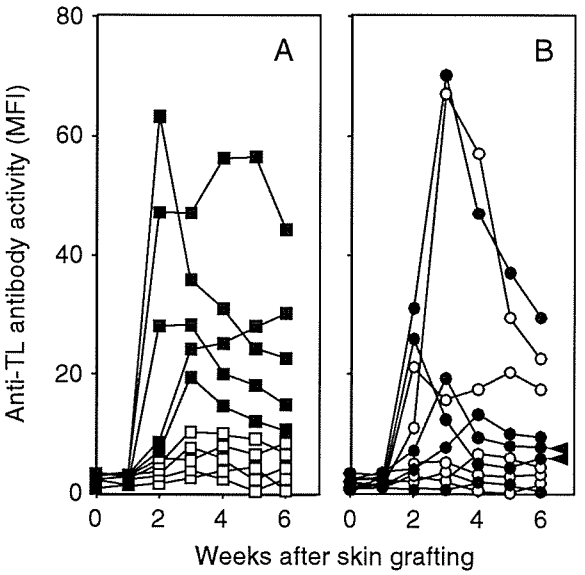

Fig. 7. Antibody activity against TL in the sera from skin-grafted mice. $T3^b$-TL transfected and untransfected RMA-S cells were indirectly stained with sera plus FITC anti-mouse immunoglobulins and analyzed on a FACSCalibur. Relative antibody activity against TL was calculated by use of the following equation: mean fluorescence intensity (MFI) of stained $T3^b$-TL transfected RMA-S cells−MFI of stained RMA-S cells. (A) C3H and B6 mice were transplanted with skins from Tg.Con.3-1 (n=5, closed squares, all mice rejected the grafts) and B6.Tg.Con.3-1 (n=5, open square, all mice accepted the grafts), respectively. (B) $(B6\times C3H)F_1$ mice were transplanted with skins from Tg.Con.3-1 (n=5, closed circles) or B6.Tg.Con.3-1 skin (n=5, open circles). All B6.Tg.Con.3-1 skin-transplanted F_1 mice accepted the grafts. Three F_1 mice rejected Tg.Con.3-1 skin, while 2 showed crisis (as indicted by arrowheads).

from C3H than B6 animals, suggesting the possibility that the Th activity in generation of anti-TL CTLs is also higher in the former. In general, anti-TL antibody activity in $(B6\times C3H)F_1$ mice was slightly weaker than in the C3H strain, although there were some exceptional individuals (Fig. 7B). No apparent differences were observed between B6.Tg.Con.3-1 skin- and Tg.Con.3-1 skin-grafted mice, implying that the skins from both strains have similar antigenicity in inducing antibody production against TL.

Discussion

Recent findings that susceptibility to chemically induced and spontaneous tumorigenesis is enhanced in immunodeficient mice strongly support the concept of cancer immune surveillance (reviewed in Ref. 26). In the present study, we observed that NBU-induced lymphoma developed more slowly in C3H than the TL transgenic counterpart strain, Tg.Con3-1, in which TL-reactive lymphocytes cannot be detected by conventional assays such as TL tetramer and cytoplasmic IFN-γ staining (data not shown), and we suggested that TL-specific immunity plays a certain role in protection against lymphoma development *in vivo*, at least when NBU is used as a carcinogenic agent. In addition, we recognized that the development of lymphomas was slightly slower in C3H than in B6. In the previous study, we found that the T/B ratio and TL expression of NBU-induced lymphomas were somewhat different between C3H and B6.[12] A half of C3H-derived lymphomas (8/16) expressed TL (all of them were of T-cell origin), while the rest (3 T lymphomas and 5 B lymphomas) were TL-negative. On the other hand, all B6-derived lymphomas (17/17) were of T-cell origin, and almost all (16/17) expressed TL. The relatively high frequency of TL^- lymphoma development in C3H may reflect cancer immunoediting[26,27] due to a tumor escape mechanism from immunity against TL. Moreover, it is of interest that primary T lymphomas derived from B6 strains tend to show heterogeneous TL expression profiles when studied by flow cytometry, suggesting multi-clonal T cell origin (data not shown). These findings together favor the possibility that the immune responses against TL in C3H play a more pivotal role than in B6, conferring protection against TL^+ lymphoma development; this view seems to be supported by the results of the skin graft experiments, in which C3H rejected TL^+ skin from Tg.Con3-1, whereas B6 could not reject B6.Tg.Con3-1 skin, although differences in host factors other than immunity between these two strains may also be involved.[28–30] It should also be noted in this regard that lymphoma development in B6 was significantly slower than in the TL transgenic counterpart strain, B6.Tg.Con.3-1, suggesting that immune surveillance against TL, albeit weaker than in C3H, is also working in the B6 strain. To our knowledge, these experimental results are the first to suggest that tumor antigen-specific immune surveillance is actually working *in vivo*.

In this and previous skin-graft experiments, we showed that the immune responses against TL are stronger in C3H than in B6, $(B6\times C3H)F_1$ or $H\text{-}2K^b$ transgenic mice. The stronger response against TL in C3H mice is not due to the allogenicity of $T3^b$-TL, since the amino acid sequences of $T3^b$-TL (B6) and $T3^k$-TL (C3H) are identical. When $(B6\times C3H)F_1$ mice were grafted with TL transgenic skins, they rejected the grafts less efficiently than C3H, suggesting that the frequency of TL-specific CTL precursors is lower in F_1 mice than in C3H, which is supported in part by the results of TL tetramer experiments (Fig. 3 and Table 2). In this study, we showed that even naïve C3H mice rejected MHC class II negative B lymphomas expressing a high level of TL, but most of $(B6\times C3H)F_1$ and $Tg.H\text{-}2K^b\text{-}2$ could not (Figs. 5 and 6). These results together suggest that TL-specific CTL are deleted and/or tolerized to a certain extent in these strains expressing $H\text{-}2^b$, especially $H\text{-}2K^b$. We previously demonstrated that TL-specific CTL recognize the framework of the α1/α2 domains of TL.[18] We therefore searched for specific amino acid sequences common between $T3^{b\,(or\,k)}$-TL and $H\text{-}2K^b$, but not $H\text{-}2K^k$ or D^k, which might be responsible for the negative selection and/or tolerance induction, but none could be elucidated. These results suggest that $H\text{-}2^b$ molecules, especially $H\text{-}2K^b$, presenting antigenic peptides may form antigenic determinants which resemble TL (free of peptides) and work as restriction elements for negative selection and/or tolerance induction. In addition, there may be a difference in TL-specific Th activity between B6 and C3H contributing to the difference in CTL induction of these strains, since $CD4^+$ T cells have been shown to be necessary for graft rejection of TL^+ skin.[25] In this regard, we obtained the interesting result in this study that $(B6\times C3H)F_1$ mice rejected Tg.Con.3-1 skin more efficiently than B6.Tg.Con.3-1 skin (Table 1), suggesting that activation of $H\text{-}2^k$-restricted Th cells may be necessary for graft rejection. The results that humoral immune response against TL was weaker in B6 and $(B6\times C3H)F_1$ than in C3H may also support this possibility, although there were some exceptional individuals among $(B6\times C3H)F_1$ mice (Fig. 7).

Another interesting finding obtained in this study is that even naïve C3H mice were able to reject large numbers of two TL^+ B lymphomas expressing a very high level of TL (derived from Tg.Con.3-1), while conventional TL^+ T lymphomas were not rejected,[12] suggesting that a higher expression of TL plus expression of certain co-stimulatory molecules on B lymphomas (not on T lymphomas) may be important to activate anti-TL immunity, although the TL^+ B lymphomas are not strong expressers of CD40, CD80 and CD86 (Fig. 4 and not shown). This

observation supports the report by Schultze *et al.* that human B lymphocytes are a very good source of antigen-presenting cells (APC), at least *in vitro*.[31] When B lymphocytes are activated with CD40 ligand (or anti-CD40 mAb) plus IL-4, they proliferate vigorously, whereas mature dendritic cells (DC), which are known as the best APC population, do not. Using activated B lymphocytes as APC, we have also established CTL clones and succeeded in defining many new epitopes of cytomegalovirus pp65.[32,33] Prophylactic tumor suppression experiments against TL^+ conventional T lymphoma by immunization with TL^+ B cells activated with anti-CD40 mAb plus IL-4 are now under way in an attempt to establish more effective and convenient immunization protocols than those employed earlier using the skin or DC.[12]

A further interesting finding is that the number of TL $tetramer^+$ cells generated in primary MLC does not always correlate with skin-graft rejection at the level of individual mice. In melanoma patients, it is also reported that immunological responses monitored *in vitro* against certain tumor antigens do not always correlate with clinical outcomes.[34,35] Furthermore, anti-TL antibody activity does not always correlate with skin-graft rejection either, as shown in Fig. 7B. These findings altogether suggest that *in vitro* monitoring methods so far established are not sufficient to determine exactly the functional anti-tumor activity *in vivo*. In this regard, more reliable and sensitive methods need to be developed to establish more effective immunization protocols.

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Clinical relevance of a newly identified HLA-A24-restricted minor histocompatibility antigen epitope derived from BCL2A1, ACC-1, in patients receiving HLA genotypically matched unrelated bone marrow transplant

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Summary

Minor histocompatibility antigens (mHAs) are major histocompatibility complex (MHC)-associated peptides, which trigger T-cell responses that mediate graft *versus* host disease (GVHD) and graft *versus* leukaemia effects. We recently identified a new mHA epitope, termed ACC-1, which is presented by HLA-A*2402 and encoded by *BCL2A1*, whose expression is restricted to haematopoietic cells including leukaemic cells. HLA-A24/ACC-1 tetramer detected the presence of ACC-1-specific $CD8^+$ cells in the peripheral blood of a patient up to 7 months following transplantation, and these tetramer-positive cells were expandable *in vitro* by ACC-1 peptide stimulation. A retrospective analysis of 320 patients with HLA-A*2402 who had received a human leucocyte antigen (HLA) genotypically matched unrelated donor through the Japan Marrow Donor Programme was conducted to determine whether ACC-1 disparity is associated with adverse clinical outcomes such as GVHD. Among these patients, ACC-1 disparity was detected in 55 (17·2%) donor/recipient pairs. After adjusting for known risk factors, the hazard ratios or odds ratios of acute and chronic GVHD, relapse and disease-free survival were not statistically different between patients receiving ACC-1 compatible and incompatible transplantation. These data suggest that disparity of haematopoietic cell-specific mHA, ACC-1, is unlikely at least to augment GVHD, and that T cells specific for ACC-1 may also be used for immunotherapy of recurring leukaemia without GVHD.

Keywords: minor histocompatibility antigen, ACC-1, graft *versus* leukaemia effect, graft *versus* host disease.

Allogeneic haematopoietic cell transplantation (allo-HCT) is an effective treatment for various types of haematological malignancies. The curative graft *versus* leukaemia (GVL) effect of allo-HCT is mainly mediated by donor-derived T cells that are specific for minor histocompatibility antigens (mHAs) (Goulmy, 1997; Warren *et al*, 1998), which are major histocompatibility complex (MHC)-associated peptides that originate from polymorphisms in the genome. However, relapse of the original disease has still been one of the major causes of treatment failure even with the beneficial GVL effect following allo-HCT (Weiden *et al*, 1981; Horowitz *et al*, 1990). Donor lymphocyte infusion (DLI) has been developed for patients with relapsing leukaemia (Kolb *et al*, 1990) and shown to induce long-lasting complete remissions in chronic myeloid leukaemia (CML) in chronic phase (Mackinnon *et al*, 1995; Dazzi *et al*, 2000). The powerful immunotherapeutic effect of mHA-specific T cells eradicating relapsing leukaemia by DLI has been shown using human leucocyte antigen (HLA)-tetramer technologies (Marijt *et al*, 2003). It is generally believed that mHAs, exclusively expressed on recipient haematopoietic cells including leukaemic cells, such as HA-1 and HA-2 (Mutis *et al*, 1999) or those on lineage-specific

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haematopoietic cells such as HB-1 (Dolstra *et al*, 1999) may result in a GVL effect in the absence of severe graft *versus* host disease (GVHD). In line with the same concept, we have sought and identified two novel haematopoietic lineage-specific mHA epitopes using linkage analysis; one is restricted by HLA-A24 (designated as ACC-1^Y, corresponding to adenine at nucleotide position 56) and another is by HLA-B44 (designated as ACC-2^D, corresponding to adenine at nucleotide position 245), both spanning two of the three polymorphic amino acids on the *BCL2A1* gene (Akatsuka *et al*, 2003). The ACC-1^Y- and ACC-2^D-specific $CD8^+$ cytotoxic T-lymphocyte (CTL) clones were originally derived from the peripheral blood mononuclear cells (PBMC) of patients receiving a HLA-identical sibling transplant for acute myeloid leukaemia in partial remission and CML in accelerated phase respectively (Akatsuka *et al*, 2002). These patients did not develop acute GVHD and have not suffered a leukaemia relapse for more than 2 years.

Before proceeding with clinical trials, there would be several ways to test whether a newly identified mHA is safe for immunotherapeutic intervention (e.g. it will not cause severe GVHD). One such approach is an *in situ* skin model recently reported by Dickinson *et al* (2002), in which CTLs specific for mHAs encoded by ubiquitously expressed genes, but not those expressed only on haematopoietic tissues, damaged cultured dermal epithelia. Another approach is a statistical analysis to examine whether donor/recipient disparity of a mHA of interest will increase severe GVHD or decrease relapse, as applied for HA-1, the most extensively studied mHA, whose expression is limited to haematopoietic cells (Goulmy *et al*, 1996; Tseng *et al*, 1999; Gallardo *et al*, 2001; Lin *et al*, 2001; Socie *et al*, 2001).

In this study, we showed that T cells reactive with a synthetic HLA-A24 tetramer incorporating the ACC-1^Y peptide were detectable in the recipient PBMC up to 200 d following bone marrow transplantation (BMT) and that these T cells could be easily expanded *in vitro*. Secondly, we questioned whether donor/recipient disparity of ACC-1 was associated with an increased risk of GVHD in recipients of HLA-identical unrelated BMT through the Japan Marrow Donor Programme (JMDP) by genotyping the ACC-1 locus in DNA samples from 320 donor/recipient pairs.

Materials and methods

Patient selection

This study was approved by the Institutional Review Boards of the Aichi Cancer Centre and the JMDP. Between March 1993 and April 1998, 444 patients with haematological malignancies received non-T cell-depleted BMT from an HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 genotypically matched unrelated donor identified through the JMDP (Morishima *et al*, 2002). Of these, we selected 320 patient/donor pairs on the basis of the presence of HLA-A24, which is the restriction HLA molecule for ACC-1. GVHD prophylaxes used were short-term methotrexate and either cyclosporine ($n = 283$) or tacrolimus ($n = 37$); antithymocyte globulin was not used. Patients were assessed for acute and chronic GVHD according to previously published criteria (Glucksberg *et al*, 1974; Shulman *et al*, 1980). The patient characteristics are summarized in Table I.

Detection of ACC-1^Y-specific T cells in post-HCT PBMC was applied to those patients who received an HLA-identical, HLA-A24 positive, ACC-1 disparate transplant in a separate study approved by the Institutional Review Boards of the Aichi Cancer Centre. An Epstein–Barr virus transformed B lymphoid cell line (LCL) was established by infecting an aliquot of PBMC with B95-8 supernatant (ATCC, Rockville, MD, USA).

Genotyping of ACC-1 polymorphism

Genotyping was performed blind to the recipient's clinical information. We performed allelic discrimination to detect the *BCL2A1* G56A polymorphism using fluorogenic 3′-minor groove binding (MGB) probes in a polymerase chain reaction (PCR) assay. Primers flanking the +56 polymorphic region were: 5′-ATTTACAGGCTGGCTCAGGACTA-3′ (forward) and 5′-GGACCTGATCCAGGTTGTGGTAT-3′ (reverse), and MGB probes complementary to the polymorphic region were: 5′-FAM-CTGCAGTGCGTCCT-MGB-3′ for the 'G' allele and 5′-VIC-TCTGCAGTACGTCCTA-MGB-3′ for the 'A' allele (Applied Biosystems, Tokyo, Japan). The PCR was conducted

Table I. Patient characteristics.

	ACC-1		
	Compatible	Incompatible	*P*-value
No. of pairs	264	55	
Median patient age, years (range)	25 (1–50)	26 (7–50)	0·40
Median donor age, years (range)	34 (20–50)	33 (21–49)	0·43
Sex (donor/recipient), *n* (%)			0·99
Male/male	102 (39)	22 (40)	
Male/female	66 (25)	14 (25)	
Female/male	59 (22)	11 (20)	
Female/female	38 (14)	8 (15)	
Disease, *n* (%)			0·83
Standard risk leukaemia*	118 (45)	22 (40)	
High risk leukaemia†	115 (43)	26 (47)	
Others	32 (12)	7 (13)	
Preconditioning, *n* (%)			0·86
TBI regimen	224 (85)	47 (86)	
Non-TBI regimen	41 (15)	8 (14)	
GVHD prophylaxis, *n* (%)			0·27
Cyclosporine based	232 (88)	51 (93)	
Tacrolimus based	33 (12)	4 (7)	

TBI, total body irradiation; GVHD, graft *versus* host disease.
*Acute leukaemia in first complete remission and chronic myeloid leukaemia in first chronic phase.
†More advanced stage than standard risk leukaemia.

in 10 μl reaction containing 200 nmol/l G-probe, 100 nmol/l A-probe, 500 nmol/l each of the primers, 1X TaqMan Universal PCR Master Mix (Applied Biosystems) and 1 μl DNA (corresponding to 50–100 ng DNA) in a MicroAmp optical 96-well plate. PCR cycling conditions were: predenature, 95°C for 10 min followed by 35 cycles of 92°C for 15 s and 60°C for 1 min in an GeneAmp PCR System 9700 (Applied Biosystems). The PCR products were analysed on an ABI 7900HT with the aid of SDS 2.0 software (Applied Biosystems). ACC-1 was considered incompatible with GVHD/GVL if the donor was ACC-1^C homozygous and the patient was either ACC-1^Y homozygous or heterozygous (i.e. ACC-1^Y/ACC-1^Y or ACC-1^Y/ACC-1^C).

Tetramer construction and flow cytometric analysis

The MHC-peptide tetramers were produced as described previously (Altman *et al*, 1996; Kuzushima *et al*, 2001). In brief, HLA-A*2402 heavy chain and β2-microglobulin were produced in BL21(DE3) pLysS (Novagen, Madison, WI, USA). The C-terminus of the heavy chain was modified by the addition of a substrate sequence for the biotinylating enzyme BirA. Monomeric HLA/β2-microglobulin/peptide complexes were folded *in vitro* in the presence of the 9-mer ACC-1^Y peptide, DYLQYVLQI. The MHC was biotinylated by using recombinant BirA enzyme (Avidity, Denver, CO, USA) and then converted into tetramers with phycoerythrin-labelled streptavidin (Molecular Probes, Eugene, OR, USA). For staining, PBMC or T-cell lines were incubated with the tetramer at a concentration of 20 μg/ml at room temperature for 15 min followed by incubation with fluorescein isothiocyanate-conjugated anti-CD3 and Tricolor anti-CD8 monoclonal antibody (Caltag, Burlingame, CA, USA) on ice for 15 min. Cells were fixed with 0·1% formaldehyde before analysis with a FACSCalibur flow cytometer and CellQuest software (Becton-Dickinson, Mountain View, CA, USA).

Induction of ACC-1^Y-specific T-cell lines using peptide-pulsed CD40-activated B cells

CD40-activated B (CD40-B) cells were generated by incubating donor PBMC on a γ-irradiated (96 Gy) human CD40L-transfected NIH3T3 cell line (t-CD40L; kindly provided by Dr Gordon Freeman, Dana-Farber Cancer Institute, Boston, MA, USA) in the presence of human interleukin (IL)-4 (4 ng/ml; Ono Pharmaceutical, Osaka, Japan) and cyclosporin A (0·7 mg/ml; Sandoz, Basel, Switzerland) in 2 ml of Iscove's modified Dulbecco's medium (Invitrogen, San Diego, CA, USA) supplemented with 10% pooled human serum. The expanding cells were transferred onto freshly prepared t-CD40L cells and fed with cytokine-replenished medium every 3–4 d. Thawed PBMC collected at various time points following BMT (1×10^6) were co-cultured with γ-irradiated (33 Gy) peptide-pulsed (1 μmol/l) autologous CD40-B cells (1×10^6) in 2 ml Roswell Park Memorial Institute 1640 medium supplemented with 10% pooled human serum at 37°C in 5% CO_2. On days 7 and 15, cells were restimulated, and 1 d after each stimulation, recombinant human IL-2 (Chiron, Emeryville, CA, USA) was added to the cultures at the final concentration of 20 U/ml.

Cytotoxic assays

Target cells were labelled with 3·7 MBq of ^{51}Cr for 2 h, and 1×10^3 target cells/well were mixed with T-cell lines at various effector:target (E:T) ratios in a standard 4-h cytotoxicity assay using 96-well round-bottom plates. All assays were performed at least in duplicate. The percentage of specific lysis was calculated as follows:

$$\frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Maximum cpm} - \text{Spontaneous cpm}} \times 100.$$

Data collection and statistical analysis

Clinical data were collected using standardized forms provided by the JMDP. A final clinical survey of patients was carried out on 1 July 2001. Standard risk for leukaemia relapse was defined as acute leukaemia in first complete remission and CML in first chronic phase, whereas high risk was defined as a more advanced stage than standard risk. Homogeneity between ACC-1^Y incompatible and compatible patients was evaluated with the chi-squared test for qualitative variables and Mann–Whitney *U*-test was used for continuous variables. The probability of acute GVHD, chronic GVHD, leukaemia relapse and disease-free survival were calculated by the Kaplan–Meier method, and assessed by the log-rank test. The Cox proportional hazard model was applied for multivariate analysis. The following variables were evaluated: ACC-1 disparity, patient age (linear), donor age (linear), sex (donor/patient pair), risk of leukaemia relapse (standard *versus* high), preconditioning [total body irradiation (TBI) regimen *versus* non-TBI regimen] and GVHD prophylaxis (cyclosporine *versus* tacrolimus). Statistical significance was set at $P < 0.05$.

Results

Detection of ACC-1^Y-specific T cells in post-BMT PBMC and their in vitro expansion

Of 14 HLA-A24 positive patients enrolled in our study, only one patient had received an ACC-1 GVHD/GVL-direction incompatible transplant, and from whom the CTL clone used for defining ACC-1^Y epitope was established. The percentage of tetramer-binding cells in the peripheral $CD8^+$ cells collected on days 43 and 212 were 0·2% and 0·07% respectively (Fig 1A). After two cycles of *in vitro* stimulation with peptide-pulsed donor CD40-B cells, the percentage of tetramer-binding cells among $CD8^+$ cells in the T-cell lines increased dramatically (Fig 1B; 96% for day 43, 69% for day 75 and 7·2% for day 212). Figure 1C shows the growing

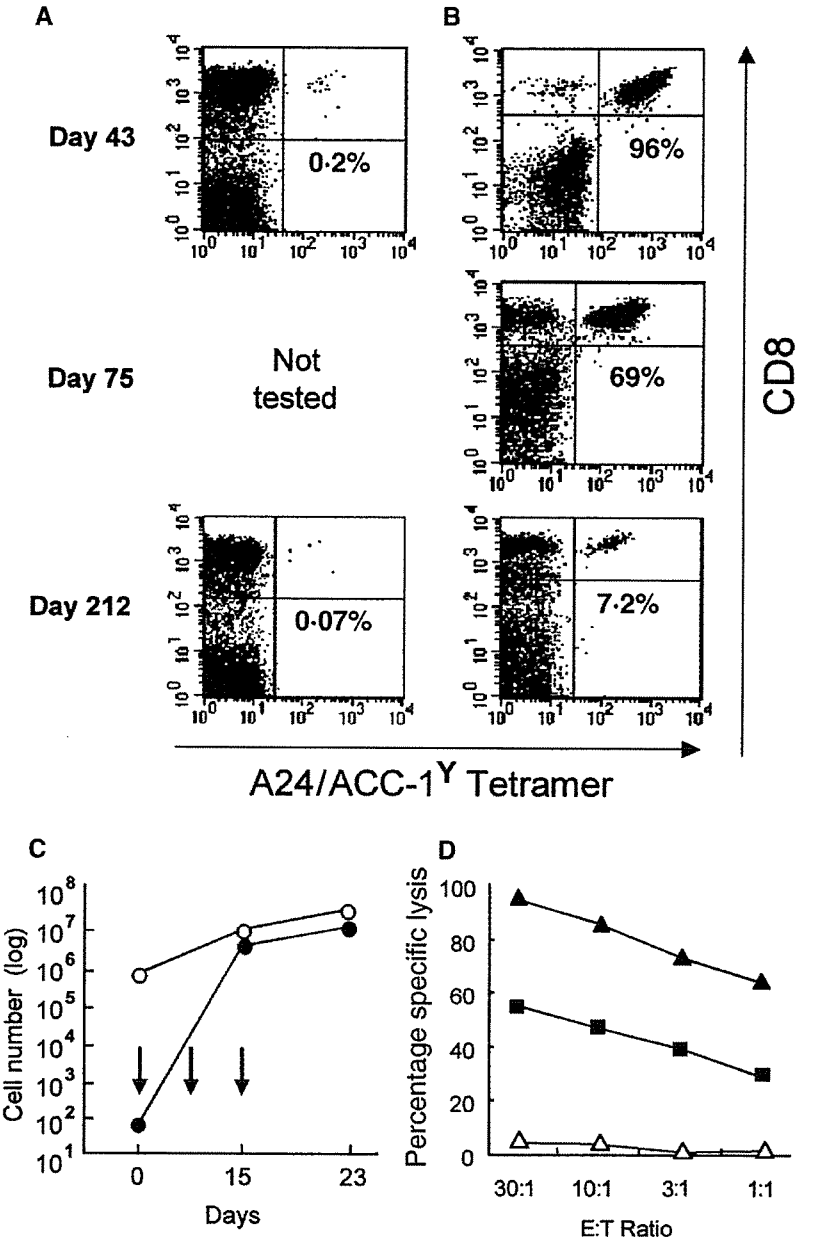

Fig 1. Detection of ACC-1^Y-specific T cells after bone marrow transplantation, and their expandability and function. (A) HLA-A24/ACC-1^Y tetramer was used to detect the presence of ACC-1^Y-specific $CD8^+$ cells in post-transplant PBMC collected on days 43 and 212 after transplant. The patient received marrow from his HLA identical, HLA-A24 positive and ACC-1 disparate brother (i.e. ACC-1^C homozygous). (B) The remaining PBMC used above and also PBMC collected on day 75 were stimulated twice with ACC-1^Y peptide-pulsed (1 μmol/l) activated donor B cells (see 'Materials and Methods') and assayed on day 15 for HLA-A24/ACC-1^Y tetramer reactivity. (C) The T-cell line induced from PBMC collected on day 43 as shown in (B) was further stimulated on day 15. The absolute number of growing cells (open circles) and HLA-A24/ACC-1^Y tetramer-positive cells (closed circles) were examined on days 7, 15, 23 after the first stimulation. The arrows indicate stimulation with peptide-pulsed activated donor B cells. (D) Cytotoxicity of the above T-cell lines tested on day 15 against donor LCL (open triangles), ACC-1^Y peptide pulsed (10 μmol/l) donor LCL (closed triangles) and patient LCL at the E:T ratio shown. Data shown are representative of two independent experiments performed in triplicate.

absolute number of tetramer-binding $CD8^+$ T cells following stimulations for PBMC obtained on day 43. The T-cell line lysed ACC-1^Y peptide pulsed donor LCL and patient LCL but not donor LCL pulsed with antigenicity negative ACC-1^C peptide, suggesting the *in vitro* expanded T-cell line retained ACC-1^Y-specific cytotoxic function. These results indicate that ACC-1^Y-specific T cells persisted in the patient peripheral blood for at least 7 months following BMT and they were expandable *in vitro* with ACC-1^Y peptide-pulsed antigen presenting cells.

Table II. Multivariate analysis for factors affecting clinical outcome following bone marrow transplantation.

Outcome and factor	Odds/hazard ratio (95% CI)	*P*-value
Acute GVHD (grades II–IV)		
ACC-1 disparity	0·91 (0·52–1·58)	0·73
Patient age	0·98 (0·97–1·00)	0·04
Chronic GVHD		
ACC-1 disparity	1·01 (0·59–1·73)	0·96
Donor age	1·04 (1·01–1·07)	0·007
Relapse		
ACC-1 disparity	1·04 (0·51–2·14)	0·91
Risk of leukaemia	3·17 (1·78–5·62)	<0·0001
Disease-free survival		
ACC-1 disparity	1·00 (0·65–1·55)	0·99
Risk of leukaemia	2·12 (1·51–3·00)	<0·0001
Patient age	1·02 (1·01–1·03)	0·006
Donor age	1·03 (1·01–1·05)	0·02

GVHD, graft *versus* host disease; CI, confidence interval.

Statistical analysis

Among the selected 320 donor/patient pairs, genotyping detected 55 ACC-1 incompatible GVHD/GVL cases (17·2%). The characteristics of ACC-1 compatible and incompatible groups are listed in Table I. There were no statistical differences between these two groups for previously identified risk factors for GVHD (Weisdorf *et al*, 1991; Nash *et al*, 1992) such as patient age, donor age, sex (donor/patient pair), disease status, preconditioning regimen and GVHD prophylaxis.

There was no statistical difference in the occurrence of grades II–IV or III–IV acute GVHD between ACC-1 incompatible and compatible patients. Risk factors for acute and chronic GVHD, leukaemia relapse and disease-free survival were analysed by multivariate analysis (Table II). Patient age only correlated with acute GVHD grades II–IV. Other factors, including ACC-1 disparity [hazard risk, 0·9; 95% confidence interval (CI), 0·5–1·6; $P = 0·73$], were not significant. The ACC-1 disparity was not identified as a significant risk factor in terms of chronic GVHD (hazard risk, 1·0; 95% CI 0·6–1·7; $P = 0·96$), relapse (hazard risk, 1·0; 95% CI 0·5–2·1; $P = 0·91$) or disease-free survival (hazard risk, 1·0; 95% CI 0·6–1·5; $P = 0·99$) (Table II).

Discussion

Graft *versus* host disease still remains the most life-threatening complication following allo-HCT or subsequent DLI, although new immunosuppressive drugs or regimens, such as non-myeloablative conditioning, have been introduced. Various strategies to separate GVHD from GVL effects have been explored (reviewed in Farag *et al*, 2003; Kolb *et al*, 2004). Among these, immunotherapy against leukaemia-specific, or recipient haematopoietic cell-specific, antigens (i.e. mHAs) is expected to eradicate leukaemic cells without causing GVHD. ACC-1 encoded by a polymorphic region in *BCL2A1* is one of the promising candidate epitopes for such immunotherapy (Akatsuka *et al*, 2003). In this study, we first showed that ACC-1^Y-specific $CD8^+$ T cells persisted in peripheral blood for at least 7 months following BMT and that they were expandable by stimulating with peptide-pulsed antigen presenting cells without losing its specific cytotoxic activity. Unfortunately, we were unable to find more than a single case eligible for this analysis because ACC-1^Y disparate transplants are estimated to occur at the frequency of <10% between HLA-identical sibling pairs; searches for more cases to verify the reproducibility are underway.

HA-1, one of the mHAs currently being tested in adoptive immunotherapy (Mutis *et al*, 1999), was originally identified as a target for severe acute GVHD (Goulmy *et al*, 1996). Controversial results have been reported in subsequent studies (Tseng *et al*, 1999; Gallardo *et al*, 2001; Lin *et al*, 2001; Socie *et al*, 2001) in terms of its contribution to acute GVHD. Our study did not detect any association between ACC-1 disparity and the occurrence of severe acute GVHD while we found that ACC-1^Y-specific T cells were expandable at least in one patient. Although the ACC-1^C peptide is considered not to be presented (Akatsuka *et al*, 2003), in order to exclude potential reciprocal immunogenicity of the ACC-1^C allele, we performed another statistical analysis by removing donor/patient pairs in which the donor was ACC-1^Y homozygous and the patient was either ACC-1^C homozygous or heterozygous. This attempt did not change the statistical results (T. Nishida and Y. Akatsuka, unpublished observations). We also searched for cases that received HLA-B44 positive and ACC-2-disparate HCT; however, we found only 83 pairs of which 22 received ACC-2-disparate HCT. A preliminary analysis could not detect any clinical relevance, either.

A potential shortcoming of the current statistical study is the use of the recipients of unrelated BMT but not those of sibling BMT. However, it is noted that there has been at least one report that analysed unrelated umbilical cord HCT cases, in which HLA mismatching often occurs, for determining the effects of both cytokine/cytokine receptor polymorphisms and mHAs (Kögler *et al*, 2002). In our study, the donor and recipient were genotypically matched for HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 to minimize HLA effects. Nevertheless, gene polymorphisms between the donor and patient are still more diverse in unrelated HCT cases than in HLA-identical sibling HCT cases, which may limit the statistical power to detect a significant association. Thus, it may be necessary to include other factors, such as polymorphisms in genes encoding cytokines or cytokine receptors (Cavet *et al*, 1999; Socie *et al*, 2001; Nordlander *et al*, 2002), or killer inhibitory receptors (Cook *et al*, 2003), that can affect clinical outcome for determining the impact of mHA disparity in future analyses.

We did not observe any favourable effect of ACC-1 disparity in terms of leukaemia relapse or disease-free survival. In a recent large-scale study, HA-1 disparity was not associated

with these outcomes, suggesting that the naturally occurring immunogenicity following HCT may not be satisfactory for inducing GVL effect (Lin *et al*, 2001). We thus believe that passive immunization by the adoptive transfer of haematopoietic cell-restricted mHA-specific cytotoxic T cells may be necessary to eradicate residual leukaemic cells efficiently following HCT. An attempt to induce ACC-1^Y-specific CTL from ACC-1^Y-negative donors by using ACC-1^Y peptide-pulsed antigen presenting cells is under way for therapeutic use as reported for HA-1 (Mutis *et al*, 1999).

In conclusion, our current results indicate that ACC-1 disparity seems less likely to be associated with the development of severe GVHD and suggest that adoptive immunotherapy targeting ACC-1 may be carried out without the risk of GVHD. However, because haematopoietic cell-specific expression of mHAs may not necessarily exclude the possibility of acute GVHD (Goulmy *et al*, 1996; Gallardo *et al*, 2001; Socie *et al*, 2001), probably due to residual recipient-derived antigen presenting cells remaining in tissues susceptible for GVHD early after HCT (Billiau *et al*, 2002), the timing after HCT and donor-chimaeric status should be carefully considered when infusing mHA-specific T cells for immunotherapy.

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Identification of novel CTL epitopes of CMV-pp65 presented by a variety of HLA alleles

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Cytomegalovirus (CMV)–specific T-cell immunity plays an important role in protection from CMV disease in immunocompromised patients. Identification of cytotoxic T-lymphocyte (CTL) epitopes is essential for monitoring T-cell immunity and also for immunotherapy. In this and previous studies, CMV-pp65–specific CTL lines were successfully generated from all of 11 CMV-seropositive healthy donors, using pp65-transduced CD40-activated B (CD40-B) cells as antigen-presenting cells. By use of enzyme-linked immunospot (ELISPOT) assays, individual CTL epitopes could be mapped with truncated forms of the pp65 gene. For human leukocyte antigen (HLA) alleles with a known binding motif, CTL epitopes within the defined regions were predicted by computer algorithm. For HLA alleles without a known binding motif (HLA-Cw*0801, -Cw*1202, and -Cw*1502), the epitopes were alternatively identified by step-by-step truncations of the pp65 gene. Through this study, a total of 14 novel CTL epitopes of CMV-pp65 were identified. Interestingly, 3 peptides were found to be presented by 2 different HLA class I alleles or subtypes. Moreover, use of CD40-B cells pulsed with a mixture of synthetic peptides led to generation of pp65-specific CTL lines from some of seronegative donors. The study thus demonstrated an efficient strategy for identifying CTL epitopes presented by a variety of HLA alleles. (Blood. 2004;103:630-638)

Introduction

Late-onset cytomegalovirus (CMV) disease in hematopoietic stem cell transplant recipients (later than day 100 after transplantation) remains a major cause of morbidity and mortality, despite the introduction of new antiviral agents; indeed, recent reports have rather indicated an increase in disease.[1-5] Absence of reconstitution of CMV-specific T-cell response at 3 to 4 months after transplantation or the use of immunosuppressive drugs is strongly associated with reactivation of CMV and the subsequent CMV disease.[2-9] Immunologic treatments, such as adoptive transfer of CMV-specific cytotoxic T-lymphocyte (CTL) clones[6,10] or CMV-specific T-cell lines,[11] have successfully protected patients at risk from CMV disease, indicating that T-cell immunity plays an important role in controlling CMV infection. Thus, immunologic monitoring of T-cell immunity against CMV is crucial to evaluate the status of immunocompromised patients.

Identification of the CTL epitopes derived from CMV is very valuable not only for monitoring antiviral immunity but also for ex vivo generation of antiviral CTLs for possible application in adoptive immunotherapy. Immunodominance of pp65 protein among CMV antigens has been reported,[12-14] but previously identified CTL epitopes derived from the pp65 protein were limited to frequent human leukocyte antigen (HLA) class I alleles. Moreover, the pp65 CTL epitope presented by HLA-A*2402, which is the most frequent allele in Japanese individuals, may not be immunodominant because the percentage of $CD8^+$ T cells detectable with the HLA-A*2402/pp65 tetramer in healthy seropositive individuals is relatively low (A*2402, 0.1%[15]; versus A*0201, 0.75%; and B*0702, 1.85%[16]), whereas A*0201- or B*0702-restricted pp65 epitopes are considered immunodominant in white individuals. Therefore, additional pp65 epitopes of clinical significance need to be identified.

We previously reported an efficient strategy for in vitro CTL generation starting with as little as 10 mL of blood.[17] By use of retroviral transduced CD40-activated B (CD40-B) cells as antigen-presenting cells (APCs), pp65-specific CTL lines could be generated from all of 4 CMV-seropositive healthy donors and found to be restricted by multiple HLA class I alleles, suggesting utility for epitope identification. In the present study, using a total of 11 pp65-specific CTL lines, including 7 newly generated ones, we attempted to identify novel CTL epitopes by enzyme-linked immunospot (ELISPOT) assay using stimulator cells transfected with truncated forms of the pp65 gene or linear expression fragments encoding various regions of the gene, with or without the help of computer algorithm–based epitope prediction. This approach was sufficiently sensitive to identify even the subdominant epitopes recognized by the CTL lines. Through this study, a total of

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14 novel CTL epitopes were identified. Immunogenicity of a part of the newly identified epitopes was validated by successful generation of pp65-specific CTL lines from CMV-seropositive and also from some CMV-seronegative donors.

Materials and methods

Donors and cells

Peripheral blood samples were obtained from 11 CMV-seropositive and 8 CMV-seronegative healthy donors after we obtained informed consent. The study was approved by the institutional review board of the Aichi Cancer Center. Informed consent was provided according to the Declaration of Helsinki. CMV seropositivity was analyzed with regard to the presence of CMV-specific immunoglobulin G (IgG) using an enzyme-linked immunosorbent assay, and HLA typing was carried out at The HLA Laboratory (Kyoto, Japan; Table 1). Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by centrifugation on a Ficoll (Amersham Biosciences, Uppsala, Sweden) density gradient, and $CD8^+$ and $CD8^-$ fractions were separated using CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cryopreserved until use.

Plasmids and synthetic peptides

Plasmids, pcDNA3-pp65, pcDNA3–enhanced green fluorescent protein (EGFP), and pcDNA3.1 (Invitrogen, Tokyo, Japan) encoding HLA class I cDNA were constructed as previously described.[17,18] To generate pEAK10-pp65, a portion of pp65 DNA was transferred from pcDNA3-pp65 into the pEAK10 vector (Edge Biosystems, Gaithersburg, MD). A plasmid containing a mutant *pp65* gene defective for the immunodominant HLA-A*0201–restricted epitope (NLVPMVATV to NLVPMVAAATV) was constructed by polymerase chain reaction (PCR)–based mutagenesis using the following primers: sense, ATGGTGGCTGCAGCTACGGTTCAGGGTCAG; antisense, AACCGTAGCTGCAGCCACCATGGGCACCAG (the inserted nucleotides are underlined). This plasmid was termed pcDNA3-pp65$^{\Delta NLV}$. All peptides were purchased from Toray Research Center (Tokyo, Japan).

Table 1. Characteristics of HLA class I and CMV serostatus of donors

Blood donor	HLA-A	HLA-B	HLA-C	CMV serostatus
P01*	1101/2603	1501/-	0401/1502	+
P02*	2402/-	4001/4002	0304/1502	+
P03*	0207/-	4001/4601	0102/0401	+
P04	2402/-	3901/5201	0702/1202	+
P05	2402/-	3501/5101	0303/1402	+
P06*	0201/2402	1501/4002	0303/0702	+
P07	0201/0207	4006/4601	0102/0801	+
P08	2402/3303	4006/4403	0801/1403	+
P09	2402/-	4002/5101	0801/1202	+
P10	1101/2402	1501/5101	0303/1202	+
P11	2402/-	4601/5901	0102/-	+
N01*	2402/3303	4403/5401	0102/1403	-
N02*	0201/0206	3501/4006	0303/0801	-
N03	2402/3101	4002/4006	0304/0801	-
N04	0206/2402	3501/5502	0102/0401	-
N05	0207/3303	4403/4601	0103/1403	-
N06	2402/-	4001/5401	0102/0304	-
N07	2402/2420	5201/5502	0102/1202	-
N08	1101/2402	3501/5201	0303/1202	-

+ indicates CMV seropositive; and -, CMV seronegative.

*Donors participated in the previous study. P01, P02, P03, N01, N02, and P06 correspond to donors 1, 2, 3, 4, 5, and 6, respectively.[17]

Generation of CD40-activated B cells and EBV-transformed lymphoblastic cell lines (LCLs)

CD40-B cells were generated as previously described.[17,19] In brief, a thawed $CD8^-$ fraction of PBMCs was cultured on a γ-irradiated (96 Gy) human CD40L-transfected NIH3T3 cell line[20] (t-CD40L; kindly provided by Dr Gordon Freeman, Dana-Farber Cancer Institute, Boston, MA) in the presence of interleukin 4 (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 modified Dulbecco 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 to 4 days. LCLs were established from the CD40-B cells with supernatant of an Epstein-Barr virus (EBV)–producing cell line (B95-8; American Type Culture Collection, Manassas, VA) in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum (FCS; IBL, Takasaki, Japan), referred to as "complete medium."

Retroviral transduction of CD40-B cells and LCLs

Retroviral transduction was conducted as previously described.[17] In brief, the retroviral construct, LZRSpBMN/pp65 (the backbone plasmid, LZRSpBMN-Z was kindly provided by Dr G. Nolan, Stanford University, Stanford, CA), pLBPC/pp65, or pLBPC/EGFP, was packaged in the Phoenix gibbon ape leukemia virus (GALV) cell line[21] (a gift from H.-P. Kiem, Fred Hutchinson Cancer Research Center; and from G. Nolan, Stanford University, Stanford, CA) 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), spun at 1000*g* for 1 hour at 32°C, and incubated. Two days after, LCLs transduced with pp65 (LCL/pp65) or EGFP (LCL/EGFP) were selected in the presence of puromycin (0.7 μg/mL; Edge Biosystems). Transduction efficiency were assessed as previously described.[17]

Generation of pp65-specific CTL lines using retrovirally transduced CD40-B cells

Thawed $CD8^+$ cells (1×10^6) were cocultured with γ-irradiated (33 Gy) autologous pp65-transduced CD40-B (CD40-B/pp65) cells (1×10^6) in 2 mL RPMI 1640 supplemented with 10% pooled human serum and recombinant human IL-7 (50 U/mL; Genzyme, Cambridge, MA) at 37°C in 5% CO_2. On days 7 and 14, $CD8^+$ cells were restimulated and one day after each stimulation recombinant human IL-2 (Chiron, Emeryville, CA) was added to the cultures at the final concentration of 20 U/mL. If necessary, rapidly growing cells were split into 2 to 3 wells and fed with fresh media containing 20 U/mL IL-2. Peptide-pulsed CD40-B cells were also prepared by incubation with 10 μM peptides derived from pp65 and used as APCs. For generation of CTL lines from seronegative donors, IL-12 (5 ng/mL; R&D systems, Minneapolis, MN) was added on day 0.

Construction of deletion mutants

To construct deletion mutants of the *pp65* gene, the plasmid, pcDNA3-pp65, was opened with *Apa*I and *Bam*HI, and progressive 3′ deletions were produced by exonuclease III treatment using the Erase-a-Base System (Promega, Madison, WI). After ligation each clone was sequenced. A total of 22 clones were selected and termed "Δpp65(1-XXX)"; XXX indicates the amino acid position of the C-terminus of each clone.

Epitope selection and construction of linear expression fragments

The epitopes within the defined regions of the pp65 protein from human CMV strain AD169 were predicted by "HLA Peptide Binding Predictions" on the BioInformatics & Molecular Analysis Section (BIMAS) website[22,23] and also by "HLA Epitope binding prediction" (beta testing version) in the HLA Ligand/Motif Database.[24]

Linear expression fragments encoding various regions of the pp65 gene were constructed by 2-step overlapping PCR. Targeted region-specific 5′ and 3′ primers incorporating additional sequences (single- and double-underlined, see

below) were designed (eg, 5′primer, TCGGATCCACCATGCAGTACGATC-CCGTGG [30 bp] and 3′ primer, GACTCGAGCGCTAGAAGAGCGCAGC-CACGG [30 bp] for QYDPVAALF [amino acids (aa's) 341-349]) and used for PCR (KOD Plus; Toyobo, Osaka, Japan) with a template plasmid, pEAK10-pp65. CMV promoter (P_{CMV}) and bovine growth hormone (BGH) polyadenylation signal (pA) were independently amplified from pcDNA3.1 by PCR using the following primers: 5′ P_{CMV}, CTTAGGGTTAGGCGTTTTGC; 3′ P_{CMV}, NNCAT-GGTGGATCCGAGCTCGGTA; 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 5′ primer and 3′ primer, respectively, of the targeted region. The 3 PCR products, P_{CMV}, the targeted region, and pA, were conjugated by second PCR using primers, 5′ P_{CMV} and 3′ pA, and termed "P_{CMV}XXX-XXX" (each "XXX" indicates the amino acid positions of the N- or C-terminus).

ELISPOT assays

ELISPOT assays were performed as described earlier.[17,25] In brief, a MultiScreen-HA plate (MAHA S4510; Millipore, Bedford, MA) was coated with antihuman interferon γ (IFN-γ) monoclonal antibody (M700A; Endogen, Woburn, MA) and used as an ELISPOT plate. The 293T cells were cotransfected with plasmids encoding each of the individual donor's HLA class I alleles and either pcDNA3-pp65, deletion mutants, or PCR products of the linear expression fragment by TransIT-293 (Mirus, Madison, WI) and were used as stimulator cells after 2 days. The transfected 293T, LCL/pp65, or LCL/EGFP cells (with or without peptide pulsing) were mixed with 10^3 or more effector cells from the CTL lines generated. After cells had been incubated in 200 μL complete medium in a 96-well plate (3790; Costar Corning, Cambridge, MA) for 4 hours, all the aliquots were transferred into an ELISPOT plate and incubated for an additional 16 hours. To visualize spots, a biotin-labeled antihuman IFN-γ antibody (M701B; 1 μg/mL, Endogen), streptavidin-alkaline phosphatase (Biosource International, Camarillo, CA), and substrate were used. Spots were counted after computerized visualization using a scanner (Canon, Tokyo, Japan). For peptide titration assays, autologous LCL/EGFP cells were pulsed with various concentrations of synthetic peptides and then used as stimulator cells to see the differences in avidity of the effector cells from CMV-seronegative and CMV-seropositive donors.

Chromium release assay

LCL/pp65 or LCL/EGFP cells were labeled in 100 μL complete media with 3.7 MBq ^{51}Cr for 1 hour at 37°C. Dermal fibroblasts were infected overnight with CMV (strain AD169) supernatant in the presence of 3.7 MBq ^{51}Cr following 500 U/mL IFN-γ pretreatment for 24 hours. For peptide reconstitution assays, 1 μM of synthetic peptide was added 1 hour before introducing effector cells. After 4 hours 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.

Results

Generation and characterization of pp65-specific CTL lines from CMV-seropositive donors using pp65-transduced CD40-B cells as APCs

We previously reported successful generation of pp65-specific CTL lines from all enrolled CMV-seropositive donors using retrovirally transduced CD40-B cells as APCs. In this study, we extended the findings by including additional 7 seropositive donors (Table 1). All CTL lines, with $CD8^+$ phenotype, lysed pp65-transduced autologous LCLs efficiently, but not untransduced autologous LCLs, and the activities were inhibited by anti–HLA class I antibody (data not shown).

To determine the HLA restriction of these CTL lines we conducted ELISPOT assays using 293T cells transfected with the

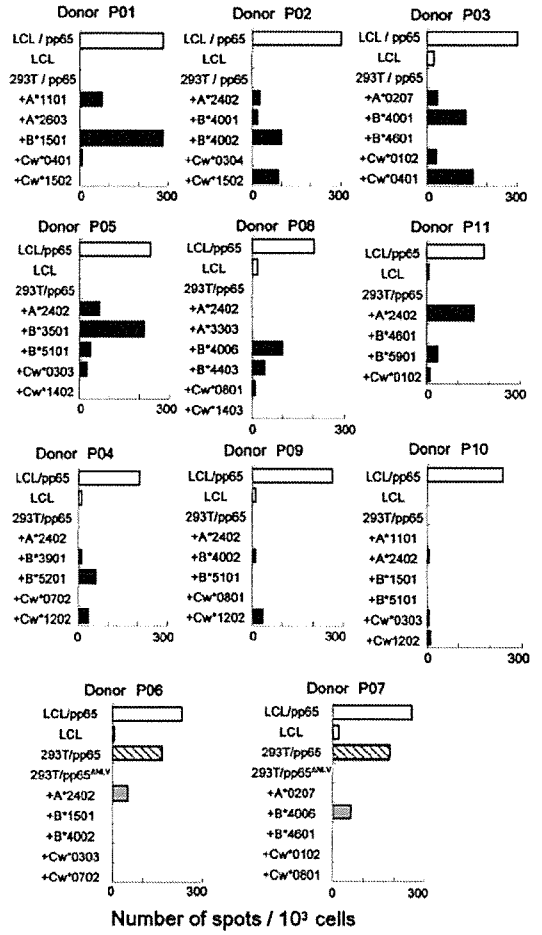

Figure 1. ELISPOT assay for the determination of HLA restriction of the CTL lines generated from CMV seropositive donors. CTL lines generated after the third stimulation with pp65-transduced CD40-B cells were tested for HLA restriction. ELISPOT assays were performed by incubating the CTL line with one of the following: autologous LCL, pp65-transduced autologous LCL (□), 293T cells transfected with both the pp65 gene and the individual HLA cDNA (■), or the *pp65* gene alone (▨). In the case of P06 and P07, CTL lines were found to recognize 293T cells transfected with the *pp65* gene alone, due to their endogenous expression of HLA-A*0201. Thus, 293T cells transfected with both mutant *pp65* gene (pcDNA3-pp65$^{\Delta NLV}$), lacking a dominant A*0201-restricted epitope (NLVPMVATV), and individual HLA cDNA were used in these cases (▨) (see "Materials and methods"). Each bar represents the number of spots per 10^3 cells.

pp65 gene plus one of the HLA class I alleles belonging to donors as stimulator cells (Figure 1). For instance, a major population of the CTL line generated from donor P01 was stimulated by 293T cells transfected with HLA-B*1501 and the *pp65* gene, while minor populations were restricted by HLA-A*1101 or -Cw*0401. Responses associated with the other HLA class I alleles were not detected when 10^3 cells of the CTL line were used. The results demonstrated that the P01 CTL lines recognized multiple pp65-derived epitopes presented by at least 3 different HLA class I alleles. Similarly in the cases of P02, P03, P05, P08, and P11, the CTL lines were restricted by multiple HLA class I alleles and the sum of the spots restricted to each allele was comparable to those produced with autologous LCL/pp65. In contrast, in the cases of P04, P09, and P10, the sum of the spots restricted to each HLA class I allele was much smaller than those with pp65-transduced LCLs (Figure 1).

The CTL lines from HLA-A*0201–positive donors (P06, P07) well recognized the transfectant with pp65 alone (Figure 1 ▨)

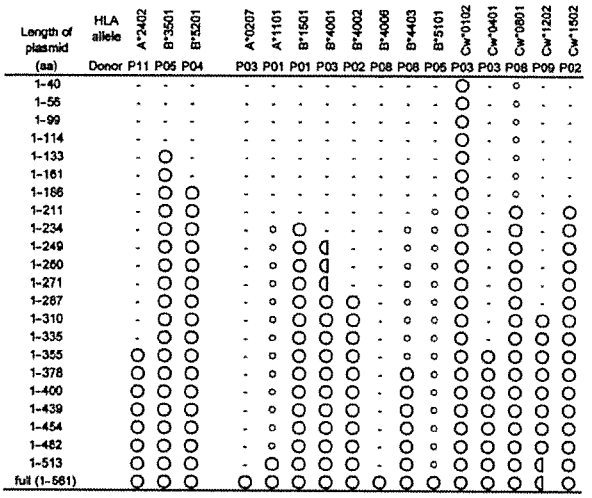

Figure 2. Localization of pp65-derived CTL epitopes estimated by ELISPOT assay using various pp65-deletion mutants. Amino acids (aa's) are numbered from the initial methionine. Each deletion mutant was transfected into 293T cells together with restricting HLA cDNA and was tested for the recognition by corresponding pp65-specific CTL line by ELISPOT assay. The number of spots over that of full-length pp65 or Δpp65(1-482) (only in Cw*1202) is indicated as follows: greater than 70% (large-sized circles), 20% to 70% (semicircles), less than 20% (small-sized circles). "—" indicates the absence of spots.

because 293T cells express HLA-A*0201 endogenously (data not shown). The mutant pp65 cDNA expression plasmid (pcDNA3-pp65$^{\Delta NLV}$), lacking the HLA-A*0201–restricted immunodominant epitope, enabled us to detect CTL responses associated with HLA alleles other than HLA-A*0201. The results revealed that HLA-A*2402 and -B*4006 were subdominant restricting HLA alleles for P06 and P07 CTL lines, respectively (Figure 1 ▨).

Identification of the regions encoding the pp65 epitopes restricted by individual HLA class I alleles

As an initial step toward defining the epitopes recognized by these pp65-specific CTL lines, C-terminus–truncated *pp65* genes were generated using the conventional exonuclease III deletion method to locate the regions encoding the epitopes (Figure 2). Thereafter, 293T cells were transfected with each of the deletion mutants plus restricting HLA class I cDNA and used for stimulation of each CTL line.

The P11 CTL line could recognize 293T cells transfected with HLA-A*2402 cDNA plus Δpp65(1-355) or longer deletion mutants, but not Δpp65(1-335) or shorter deletion mutants (Figure 2), indicating that the pp65 epitope presented by HLA-A*2402 should fully or partially be contained inside the region spanning between amino acid residues aa336 and aa355. Indeed, an HLA-A*2402–restricted epitope QYDPVAALF (aa's 341-349)[15] is found within the region. The results for HLA-B*3501 and -B*5201 were also consistent with the reported epitopes (IPSINVHHY [aa's 123-131][26] and QMWQARLTV [aa's 155-163],[27] respectively). These observations indicate that our strategy using deletion mutants worked effectively.

Next, we attempted to locate the regions containing pp65 CTL epitopes presented by other HLA class I alleles. As shown in Figure 2, such regions existed between the following amino acid residues: 514-561 (A*0207), 483-513 (A*1101), 212-234 (B*1501), 272-287(B*4002), 514-561 (B*4006), 356-378 (B*4403), 514-561 (B*5101), 1-40 (Cw*0102), 336-355 (Cw*0401), 187-211 (Cw*0801), 288-310 (Cw*1202), 187-211(Cw*1502). In the case of HLA-B*4001, the P03 CTL line strongly recognized not only Δpp65(1-287) or longer deletion mutants but also Δpp65(1-249), Δpp65(1-260), and Δpp65(1-271) to a lesser extent, suggesting the presence of 2 different HLA-B*4001–restricted epitopes. The existence of an additional subdominant epitope was also suggested in the cases of HLA-A*1101, -B*4403, -B*5101, and -Cw*0801 (Figure 2). As for HLA-Cw*1202, it is of note that the transfectants with the full-length plasmid produced a smaller number of spots than those with the shorter deletion mutants, such as Δpp65(1-482).

Identification of the pp65 epitopes presented by HLA alleles whose binding motif is predictable by computer algorithm

To predict the epitopes within the regions narrowed down by the deletion mutant experiments (Figure 2), amino acid sequences of the determined regions with a 10-aa extension to the N-terminus were analyzed by online computer algorithm software. The prediction results are listed in Table 2. For the HLA-A*0207–restricted epitope, A*0201 was alternatively selected because A*0207 was not available on the computer algorithm we used.[23,24] As for HLA-B*1501, the computer algorithm on the BIMAS website[22,23] depicted 3 candidate epitopes with similar scores (6, 4.4, and 4), one of which was also depicted by another algorithm[24] and subsequently adopted.

Table 2. Candidate epitopes predicted by computer algorithms

HLA restricted	Parameter submitted		Result predicted‡				Rank in whole pp65
	Range, aa†	HLA type	Position, aa	Sequence	Length	Score	
A*0207	504-561	A*0201	522-530	RIFAELEGV	9	39.8	12
A*1101	473-513	A*1101	501-509	ATVQGQNLK	9	1.5	3
B*1501	202-234	B*1501§	215-223	KMQVIGDQY§‖	9	117§	4
B*4001	225-249	B60	232-240	CEDVPSGKL	9	176	1
B*4001	262-287	B60	267-275	HERNGFTVL	9	160	2
B*4002	262-287	B61	267-275	HERNGFTVL	9	8	6
B*4006	504-561	B40	525-534	AELEGVWQPA	10	80	1
B*4403	346-380	B*4403	364-373	SEHPTFTSQY	10	720	1
B*5101	504-561	B*5101	547-555	LPGPCIAST	9	14.3	25
Cw*0102	1-40	Cw*0102§	7-15	RCPEMISVL§	9	200§	3
Cw*0401	336-378	Cw*0401	341-349	QYDPVAALF	9	216	4

†The different region between the shortest positive deletion mutant and the longest negative one (Figure 2) with a 10 aa extension to the N-terminus was submitted.

‡All results except for the marked ones§ were predicted by "HLA Peptide Binding Predictions" on BIMAS website.[23]

§Predicted by "HLA Epitope binding prediction" on the website of University of Oklahoma Health Sciences Center.[24]

‖By computer algorithm on BIMAS website, the peptide was assigned 3rd rank (score = 4) within the submitted range and 21st rank in the full-length pp65 gene, respectively.

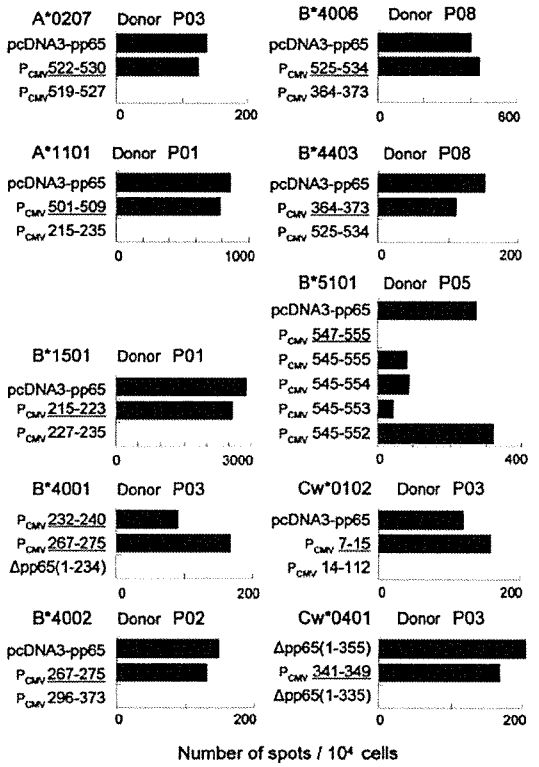

Figure 3. Recognition of the epitopes predicted by computer algorithm by the pp65-specific CTL lines. Linear expression fragments encoding various peptides including the predicted epitopes were generated and transfected into 293T cells together with restricting HLA cDNA. Recognition by the pp65-specific CTL lines was evaluated 48 hours later by ELISPOT assay. Numbers indicate the amino acid position of pp65 encoded by each construct. Linear expression fragments encoding the predicted epitopes are underlined. Each bar represents the number of spots per 10^4 cells.

To test the recognition of the predicted epitopes by each CTL line, linear expression fragments encoding various peptides including the predicted epitopes were generated by overlapping PCR (see "Materials and methods"), transfected into 293T cells together with restricting HLA class I cDNA, and evaluated by ELISPOT assay. As shown in Figure 3, all predicted epitopes except for the HLA-B*5101–restricted one were well recognized by the corresponding pp65-specific CTL, and the specificity was confirmed using irrelevant fragments. For instance, the P01 CTL line could be stimulated by 293T cells cotransfected with a fragment encoding aa's 215 to 223 and HLA-B*1501 comparably to the case with the full-length pp65 gene, but not at all with aa's 227 to 235, indicating that aa's 215 to 223 (KMQVIGDQY; nonamer) is at least one of the HLA-B*1501–restricted epitopes. With HLA-B*4001, both of the 2 fragments encoding the predicted epitopes (aa's 232-240 and aa's 267-275) were well recognized by P03 CTL line (Figure 3), confirming the results of ELISPOT assay using deletion mutants (Figure 2).

In the case of HLA-B*5101, the fragments encoding the predicted epitope (aa's 547-555) or the one with the second highest score (aa's 545-553) were not or only poorly recognized by the P05 CTL line (Figure 3). Thus various other fragments were tested and the one encoding octamer peptide (DALPGPCI; aa's 545-552) was found to be well recognized. This octamer has a binding motif consistent with that for HLA-B*5101.[28]

In summary, we successfully identified 11 new pp65-derived epitopes presented by 10 distinct HLA alleles (Table 3). Of interest is the fact that QYDPVAALF (aa's 341-349) restricted by HLA-Cw*0401 is identical to that restricted by HLA-A*2402.[15]

Identification of the pp65 epitopes presented by HLA alleles whose binding motif is not predictable by computer algorithm

Since algorithms that predict peptides binding to HLA-Cw*0801, -Cw*1202, and -Cw*1502 are currently not available, we performed step-by-step epitope mapping using the linear expression fragments. Based on the results with deletion mutants and HLA-Cw*1202 (Figure 2), fragments encoding the region from aa 267 to aa 292 or to aa 302 were tested. The P04 CTL line recognized the transfectant with the fragment encoding aa's 267 to 302, but not that with aa's 267 to 292 (Figure 4A left), suggesting that the epitope should be fully or partially contained between aa 293 and aa 302. Next, various fragments encoding the septamer to dodecamer peptides within this region were generated and tested. The fragments whose C-terminus was Phe302 were well recognized, but those with His301 or Gly303 at the C-terminus were not or only

Table 3. Summary of CTL epitopes derived from CMV-pp65

Newly identified epitopes				Previously reported epitopes				
HLA	Position, aa	Sequence	Length	HLA	Position, aa	Sequence	Length	Reference
A*0207	522-530	RIFAELEGV	9	A*0101	363-373	YSEHPTFTSQY	12	29,30
A*1101	501-509	ATVQGQNLK	9	A2	14-22	VLGPISGHV	10	31
B*1501	215-223	KMQVIGDQY	9	A2	120-138	MLNIPSINV	9	31
B*4001	232-240	CEDVPSGKL	9	A2	495-503	NLVPMVATV	9	14,32
B*4001	267-275	HERNGFTVL†	9	A*1101	16-24	GPISGHVLK	9	9,30
B*4002	267-275	HERNGFTVL†	9	A*2402	341-349	QYDPVAALF‡	9	15
B*4006	525-534	AELEGVWQPA	10	A*2402	369-379	FTSQYRIQGKL	11	9,30
B*4403	364-373	SEHPTFTSQY	10	A*2402	113-121	VYALPLKML	9	33
B*5101	545-552	DALPGPCI	8	A*6801/2	186-196	FVFPTKDVALR	11	30
Cw*0102	7-15	RCPEMISVL	9	B7	265-274	RPHERNGFTV	10	14,34
Cw*0401	341-349	QYDPVAALF‡	9	B7	417-426	TPRVTGGGAM	10	14,34
Cw*0801	198-206	VVCAHELVC§	9	B35(B*3501)	123-131	IPSINVHHY	9	26
Cw*1202	294-302	VAFTSHEHF	9	B35(B*3502)	188-195	FPTKDVAL	8	9,14,30
Cw*1502	198-206	VVCAHELVC§	9	B35(B*3503)	187-195	VFPTKDVAL	9	14,35
				B*3801/2	367-379	PTFTSQYRIQGKL	13	14,34
				B44(B*4402)	512-521	EFFWDANDIY	10	14,34,35
				B*5201	155-163	QMWQARLTV	9	27

†,‡,§ Same peptide found to be presented by different alleles or subtypes of HLA.

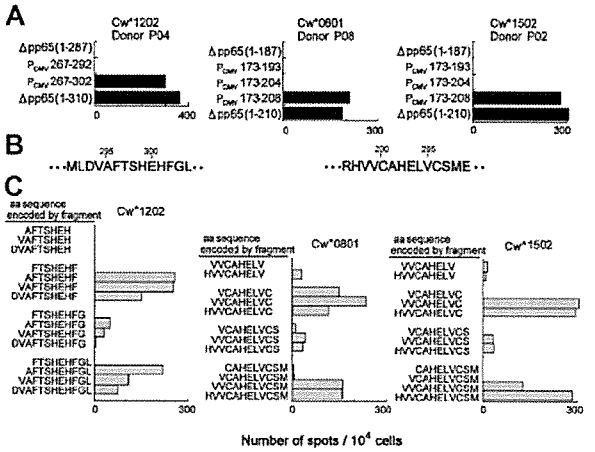

Figure 4. Identification of pp65-derived CTL epitopes for which a computer algorithm was not applicable. To narrow down the region containing the epitope, the linear expression fragments encoding further truncated forms of the deletion mutant, Δpp65(1-310) for HLA-Cw*1202 or Δpp65(1-210) for HLA-Cw*0801 and -Cw*1502, were generated and transfected into 293T cells together with restricting HLA cDNA. (A) Recognition by the pp65-specific CTL lines was evaluated 48 hours later by ELISPOT assay. (B) Amino acid sequence of pp65 around the defined region. (C) Based on the results shown in the upper panels, various linear expression fragments within the region were generated, transfected into 293T cells together with restricting HLA cDNA, and then tested for the recognition by corresponding pp65-specific CTL line using ELISPOT assay. Each bar represents the number of spots per 10^4 cells.

poorly recognized (Figure 4C left). The CTL lines were partially activated by the fragments ending at Leu304. Progressive N-terminal deletion revealed that Ala295 was crucial for recognition. The same results were obtained with the P09 CTL line (data not shown). Additional experiments with synthetic peptides revealed that the CTL clone could recognize VAFTSHEHF (aa's 294-302) at 2-log lower concentrations than AFTSHEHF (aa's 295-302) (data not shown), indicating that the nonamer VAFTSHEHF is the minimal epitope presented by HLA-Cw*1202.

In the case of HLA-Cw*0801, fragments encoding the region up to aa 193, aa 204, or aa 208 were also constructed for further narrowing the regions identified (Figures 2 and 4). Because the fragment encoding aa's 173 to 208, but not aa's 173 to 204, was recognized by the P08 CTL line, the C-terminus of the epitope should be located between aa's 205 and 208 (Figure 4A middle). ELISPOT assay using transfectants with various linear expression fragments revealed that those encoding the peptides ending at Cys206 or Met208 were well recognized (Figure 4C middle). The results also indicated that Val198 was required for recognition. Thus we determined that VVCAHELVC (aa's 198-206) is the minimal epitope presented by HLA-Cw*0801. Interestingly, the same nonamer (VVCAHELVC) was independently identified as a minimal epitope presented by HLA-Cw*1502 (Figure 4C right).

Generation of CTL lines from CMV-seropositive donors with CD40-B cells pulsed with newly identified pp65 epitopes

To confirm the immunogenicity of the newly identified epitopes, we tried to generate pp65-specific CTL using synthetic peptide-pulsed CD40-B cells as APCs. After the third stimulation cytolytic activity of the CTL lines was assessed. A CTL line from donor P03, induced by CEDVPSGKL presented by HLA-B*4001, could lyse HLA-B*4001–positive allogeneic LCLs (from donor N06) expressing pp65 antigen and also the peptide-pulsed LCL/EGFPs (Figure 5A). Similar results were also obtained with the other 5 epitopes.

Generation of pp65-specific CTL lines from seronegative donors with CD40-B cells pulsed with a mixture of newly or previously identified pp65 epitopes

In the current and previous studies we failed to generate pp65-specific CTL lines from all 8 seronegative donors using pp65-transduced CD40-B cells as APCs. To improve stimulation efficiency, we applied CD40-B cells pulsed with a mixture of newly or previously identified epitopes. One million $CD8^+$ T cells were stimulated 3 times with autologous CD40-B cells pulsed with a mixture of 2 to 4 synthetic peptides, which are presentable by HLA alleles belonging to each donor. The T-cell line from N02 produced IFN-γ spots after stimulation with 2 of the 4 peptides (Figure 6A). Peptide titration assay revealed that half-maximal number of spots for N02 line was obtained with approximately 3.0 nM of peptide IPSINVHHY, while that for P05 CTL line from a seropositive donor sharing HLA-B*3501 was 80 pM (Figure 6B top), indicating that a 38-fold higher peptide concentration was required in the N02 line. Nevertheless, the N02 could lyse autologous LCL/pp65s and also dermal fibroblasts infected with CMV although a lesser extent (9% at the effector-target [E/T] ratio of 25:1) (Figure 6C top). Two of the 4 peptides were recognized by the N03 T-cell line whose half-maximal spots for both peptides were obtained with 2.9 nM (peptide QYDPVAALF) and 14 nM (peptide HERNGFTVL), which were 35- and 77-fold higher than those for P02 CTL line from a seropositive donor sharing HLA-A*2402 and -B*4002, respectively. This N03 line lysed autologous LCL/pp65s and peptide-pulsed fibroblasts, but failed to lyse CMV-infected fibroblasts, suggesting the avidity of the line was not high enough for exerting cytotoxic activity against CMV-infected fibroblasts. On the other hand, in the case of N06 and N08, exogenously pulsed

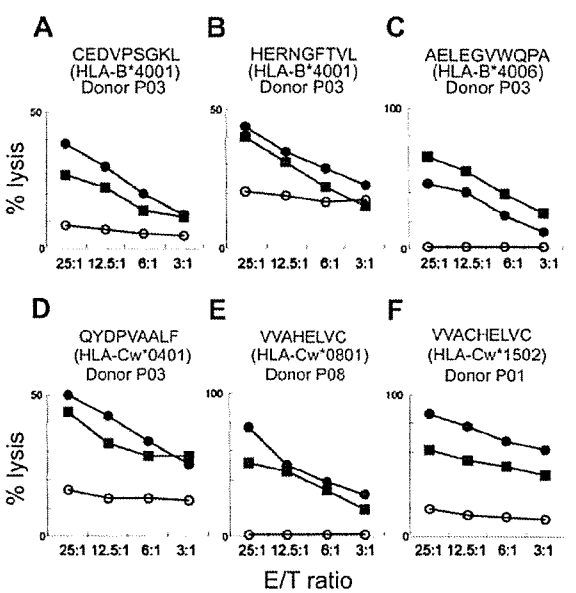

Figure 5. Cytolytic activity of the CTL lines generated by stimulation with CD40-B cells pulsed with newly identified epitopes. $CD8^+$ T cells from CMV-seropositive donors were stimulated with peptide-pulsed autologous CD40-B cells. CMVpp65-derived synthetic peptides used were CEDVPSGKL ($pp65^{232-240}$) to be presented by HLA-B*4001 (A), HERNGFTVL ($pp65^{267-275}$) by HLA-B*4001 (B), AELEGVWQPA ($pp65^{525-534}$) by HLA-B*4006 (C), QYDPVAALF ($pp65^{341-345}$) by HLA-Cw*0401 (D), VVCAHELVC ($pp65^{198-206}$) by HLA-Cw*0801 (E) or Cw*1502 (F). One week after the third stimulation, cytolysis of CTL lines was assessed by ^{51}Cr release assay against LCL/pp65 (■), LCL/EGFP (○), or 1 μM peptide-pulsed LCL/EGFP (●) over a range of E/T ratios as indicated. The origin of target cells was autologous (panel F) or allogeneic [panels A-B, N06 (B*4001+); panel D, P01(Cw*0401+); panels C and E, N03(B*4006, Cw*0801+)].

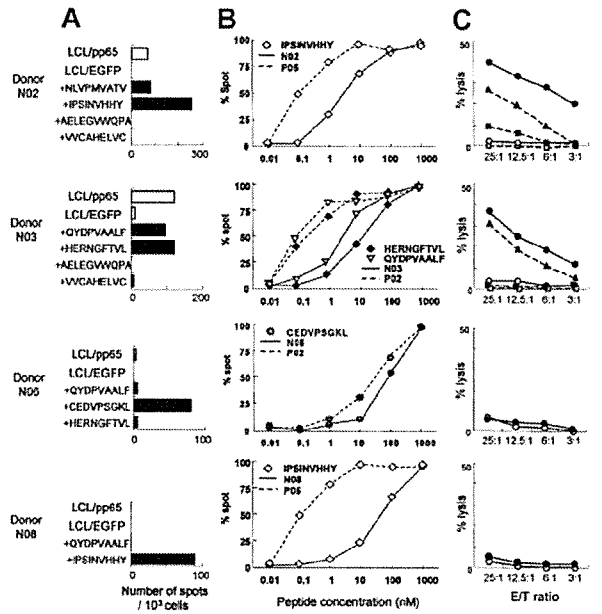

Figure 6. Effector cell activity of the T-cell lines generated from seronegative donors after stimulation with CD40-B cells pulsed with a mixture of antigenic peptides. $CD8^+$ T cells from CMV-seronegative donors were stimulated 3 times with autologous CD40-B cells pulsed with a mixture of 2 to 4 peptides and tested for effector activity. CMVpp65-derived synthetic peptides used are listed in Table 3. The antigenicity of these peptides was proven in the experiments whose results are shown in Figure 5. (A) ELISPOT assay was conducted using LCL/pp65, LCL/EGFP (□), or LCL/EGFP pulsed with 1 μM of each peptide indicated (■). Each bar represents the number of spots per 10^3 cells. (B) Peptide titration was conducted using LCL/EGFP pulsed with various concentrations of the peptides indicated by ELISPOT assay. Percent spot was calculated for individual T-cell lines by dividing the number of spots at indicated peptide concentrations by the maximal number of spots × 100%. (C) Cytolysis of CTL lines was assessed against autologous LCL/pp65 (●), LCL/EGFP (○), HLA-matched dermal fibroblasts infected with CMV supernatant (■), with mock supernatant (□), or with 1 μM peptide mixture showing spots in the ELISPOT assay (▲) over a range of E/T ratios by ^{51}Cr release assay.

peptides (N06, CEDVPSGKL; N08, IPSINVHHY) on LCL/EGFPs were recognized (Figure 6A), whereas endogenously expressed pp65 in LCLs was not recognized (Figure 6A,C), which might partly be explained by the 10- to 20-fold higher peptide concentrations necessary for the half-maximal spots (65 nM for CEDVPSGKL in N06 and 36 nM for IPSNVHHY in N08; Figure 6B).

Discussion

The present study focused on the systematic identification of novel CTL epitopes derived from CMV-pp65. We combined biotechnology with 22 C-terminal truncations of the *pp65* gene at an average 22–amino acid interval (range, 11-48 aa's) and computer technology with algorithm-based prediction of peptide binding to certain HLA alleles. We were able to identify CTL epitopes using linear expression fragments constructed by overlapping PCR, even if a computer algorithm was not available for HLA alleles of interest. In general, to identify CTL epitopes, establishment of CTL clones and verification using ^{51}Cr release assay have frequently been used. In this study, for efficient detection of CTL responses, we adopted the ELISPOT assay[15] so that even responses to subdominant epitopes could be detected, such as the HLA-Cw*0102–restricted one in donor P03.

In the cases of P04, P09, and P10, the sum of the spots against 293T cells transfected with *pp65* gene plus each HLA class I allele was unexpectedly smaller than that against pp65-transduced LCLs (Figure 1). This could be due to differential processing and/or presentation of pp65 proteins in LCLs and 293T cells. In this sense, HLA-A*2402 and -Cw*1202 are the focus as presenting molecules because both are present in the cases. Since the CTL lines were poorly stained with HLA-A*2402 tetramers incorporating peptide, QYDPVAALF (data not shown), it is unlikely that HLA-A*2402–restricted epitopes are concerned in this issue. Indeed, defective processing and/or presentation of the HLA-Cw*1202–restricted epitope from full-length pp65 in 293T cells was demonstrated (Figure 2). Probably, in LCLs, the pp65 is more efficiently processed and presented to yield the HLA-Cw*1202–restricted epitope. The better processing might attribute to so-called immunoproteasomes equipped by LCLs. So far, simultaneous transfection with immunoproteasome components, such as large multifunctional protease 2 (LMP2), LMP7, and LMP10, or IFN-γ treatment did not improve the recognition of 293T transfectants (data not shown), suggesting that additional factors are involved. The reason why truncated pp65 were processed more efficiently is unclear but this could be due to relatively unstable pp65 protein prone to degradation and entry into the processing pathway. Additional studies are now in progress to address the question.

One of the interesting findings of this study is that linear expression fragments encoding the right epitopes with only a single amino acid extension at the C-terminus were not recognized by each CTL line efficiently, whereas N-terminal extension rarely affected the recognition (Figure 4). Aminopeptidases, such as endoplasmic reticulum aminopeptidase 1 (ERAP1)[36-38] or leucine aminopeptidase,[39] may be able to trim N-terminal extensions and create peptides with optimal length for binding to major histocompatibility complex (MHC). However, mammalian cells lack carboxypeptidases,[39-41] thus proteasome is solely responsible for creating the correct C-terminus of the epitopes. Although a very limited number of amino acids adjacent to both sides of a proteasomal cleavage site contribute to cleavage site selection,[42] only a single amino acid extension at the C-terminus may be too short to be removed efficiently by proteasomes. In addition, constitutive proteasomes might be dominant in 293T cells[43] rather than immunoproteasomes, which have greater efficacy and are expressed in LCLs or mature dendritic cells. This may partially explain why C-terminal–extended epitopes were poorly recognized in our experiments.

We identified 2 new epitopes presented by HLA-A molecules. One is RIFAELEGV, dominantly presented by HLA-A*0207 but not by HLA-A*0201. This result underscores differential peptide repertories that bind to HLA-A*0207 and -A*0201, probably influenced by a single amino acid substitution at the floor of the binding groove.[44] The other is presented by HLA-A*1101 (ATVQGQNLK; aa's 501-509): this seems to be the dominant epitope presented by the allele, although a subdominant one may be located between aa 211 and aa 234. Both the dominant and undefined subdominant epitopes are, however, different from those reported previously, GPISGHVLK; aa 16-24.[9,30] For HLA-B alleles we identified 7 new epitopes, 4 of which are restricted by the HLA-B40 group. Among them, HERNGFTVI (aa's 267-275) is presented by both HLA-B*4001 and -B*4002. HLA-B*4001 presents an additional epitope, CEDVPSGKL (aa's 232-240). An HLA-B*4403–restricted epitope found in this study (SEHPTFTSQY; aa364-373) differs from the HLA-B44–restricted one reported earlier (EFFWDANDIY; aa's 512-521).[14,34] HLA-B*4402 and -B*4403 are 2 major HLA-B44 subtypes in white individuals,[45,46] and HLA-B*4403 is most frequent HLA-B44 subtype in

Japanese individuals.[47-49] Because the reported epitope, EFFWDANDIY, was listed as the HLA-B*4402–restricted one,[35] these 2 epitopes might be restricted by a different subtype of HLA-B44. All the data imply that subtle differences in amino acid residues facing the groove of HLAs have an impact on the peptide binding and subsequent CMV-pp65–specific T-cell responses.

This paper describes, for the first time to our knowledge, pp65-specific epitopes presented by HLA-C alleles. A unique epitope, VVCAHELVC (aa's 198-206), was presented by both HLA-Cw*0801 and -Cw*1502. Since there seems to be no information on the peptide binding motif for these HLA alleles, the epitope was determined by gene engineering of pp65, followed by probing with CTL restricted to each HLA-C allele. The results should shed light on the structural basis of understanding the HLA-C molecules. Interestingly, an HLA-Cw*0401–restricted epitope, QYDPVAALF (aa's 341-349),[15] is also dominantly presented by HLA-A*2402 allele. The binding motifs of those 2 alleles are similar to each other (ie, Tyr, Pro, or Phe at the second position and Leu or Phe at the C-terminus in HLA-Cw*0401; and Tyr at the second position, and Ile, Leu, or Phe at the C-terminus in HLA-A*2402). From the point of immunotherapy, this single peptide has great advantages among populations where HLA-A*2402 and -Cw*0401 are common.

There are only a few reports of successful generation of pp65-specific CTL from CMV-seronegative donors. Kleihauer et al[50] showed that cytotoxic T-cell lines were generated from 2 of 11 seronegative donors starting with 3×10^6 PBMCs on stimulation with pp65 peptide–pulsed monocyte-derived dendritic cells. In our previous study, we failed to generate pp65-specific CTL lines from seronegative donors using pp65-transduced CD40-B cells as APCs, but in this study CTL lines that could lyse LCLs expressing endogenously processed peptides from the transduced pp65 gene were generated in 2 of 4 cases (N02 and N03) by using CD40-B cells pulsed with a mixture of the peptides as APCs. However CMV-infected fibroblasts were recognized weakly only by the CTL line from N02. Again, the better antigen processing by LCLs than fibroblasts might contribute to the better recognition by the CTL lines. Such CTLs may be able to only lyse CMV-infected hematopoietic cells in vivo. It is noted in this regard that CMV is reported to infect not only stroma and epithelial cells but also hematopoietic cells including $CD34^+$ stem cells, monocytes, and dendritic cells.[51-55] In the other 2 cases, the CTL lines could recognize only peptide-pulsed LCLs. This observation seems to reflect the results of peptide titration experiments by ELISPOT assay; the peptide concentration to yield half-maximal spots was found to be 10- to 20-fold lower in N02 and N03 lines compared with N06 and N08 lines, suggesting the lower avidity of the lines generated from N06 and N08. To overcome this problem, initial stimulation with peptides at a lower concentration may induce higher avidity CTLs.[56] Alternatively, more T-cell input at the time of initial stimulation may be needed to induce CTL lines from rare precursor T cells with higher affinity T-cell receptor since estimated precursor frequency of naive T cells against a single epitope is very low (for example, one in 5×10^6 of $CD8^+$ cells for lymphocytic choriomeningitis virus in mice[57]). Further efforts are now underway to establish better CTL induction conditions from seronegative donors.

In summary, we here identified 14 novel CTL epitopes derived from CMV-pp65 antigen restricted by HLA-A, -B, and -C alleles. These should be useful for immunologic monitoring of individuals expressing these HLA class I alleles and also for generation of pp65-specific CTLs from not only seropositive but also seronegative donors. In addition, our present approach of epitope identification applying deletion mutants and linear expression fragments, together with the efficient generation of CTL lines, may be applicable for other tumor-specific or viral antigens.

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