European Journal of Immunology

Selection of CMV-specific CD8$^+$ and CD4$^+$ T cells by mini-EBV-transformed B cell lines

Martina Wiesner[1], Caroline Zentz[1], Markus H. Hammer[2,3], Mark Cobbold[4], Florian Kern[3], Hans-Jochem Kolb[5], Wolfgang Hammerschmidt[1], Reinhard Zeidler[6,7] and Andreas Moosmann[7,8]

[1] Department of Gene Vectors, GSF - National Research Center for Environment and Health, Munich, Germany
[2] Department of Nephrology and Internal Intensive Care, Charité, University Medicine Berlin, Berlin, Germany
[3] Institute of Medical Immunology, Charité, University Medicine Berlin, Berlin, Germany
[4] Cancer Research UK Institute for Cancer Studies, University of Birmingham, Birmingham, UK
[5] Clinical Cooperative Group Hematopoietic Cell Transplantation, GSF – Institute of Molecular Immunology, and Department of Medicine III, Ludwig-Maximilians-Universität, Munich, Germany
[6] Vaecgene Biotech, Munich, Germany
[7] Department of Otorhinolaryngology, Ludwig-Maximilians-Universität, Munich, Germany
[8] Clinical Cooperative Group Molecular Oncology, GSF – Department of Gene Vectors, and Ludwig-Maximilians-Universität, Munich, Germany

Efficient protocols to generate cytomegalovirus (CMV)-specific T cells are required for adoptive immunotherapy. Recombinant Epstein-Barr virus (EBV) vectors called mini-EBV can be used to establish permanent B cell lines in a single step, which present the CMV antigen pp65 in a constitutive manner. These B cell lines, coined pp65 mini-LCL, were successfully used to reactivate and expand CMV-specific cytotoxic T cells. Here we evaluate this pp65 mini-EBV system in closer detail, focusing on (1) the quantification of T cells with specific effector function and (2) the identification of CMV-specific CD4$^+$ helper T cells. The co-expansion of various functional CMV epitope specificities was demonstrated by IFN-γ enzyme-linked immunospot assay (ELISPOT) assays and HLA-peptide tetramer staining. Single-cell cloning resulted in both CD4$^+$ and CD8$^+$ T cell clones, the majority of which was CMV specific. Thus, mini-LCL present the pp65 antigen on HLA class I and II, mobilizing both arms of the T cell response. Using a peptide library covering the pp65 sequence for further analysis of T cell clones, we identified new pp65 CD8$^+$ and CD4$^+$ T cell epitopes.

Received 20/12/04
Revised 11/5/05
Accepted 30/5/05

[DOI 10.1002/eji.200425936]

Key words:
Cytomegalovirus · T cells · Mini-EBV · Mini-LCL · Adoptive immunotherapy

Introduction

Cytomegalovirus (CMV) continues to be a major clinical problem in immunocompromised patients, like those undergoing allogeneic hematopoietic stem cell transplantation (SCT) [1]. CMV disease is considered to result from the lack of virus-specific T cells, since the presence of CMV-specific T cells in sufficient numbers was shown to be associated with protection [2]. Antiviral medication can protect from fatal CMV disease, but comes with side effects which limit overall benefit [3]. Antivirals also favor late-onset CMV disease [4], and the occurrence of resistant CMV strains [5]. In search of therapeutic alternatives, the reconstitution of protective T cell immunity by adoptive transfer of CMV-specific T cells has been successfully performed [5–8].

To select for and expand CMV-specific T cells from peripheral blood cells *in vitro*, various kinds of antigen-presenting cells (APC) and methods of antigen delivery to these APC have been used, often focusing on the dominant CMV antigen pp65. CMV-infected fibroblasts as APC [6] have generally been replaced either by

Molecular Oncology, Marchioninistr. 25, 81377 München, Germany
Fax: +49-89-7099-225
e-mail: andreas.moosmann@med.uni-muenchen.de
Abbreviation: **SCT:** stem cell transplantation

unseparated PBMC [5] or by defined professional APC populations such as dendritic cells (DC) [9–12] or activated B cells [13, 14]. The CMV antigenic stimulus has been provided by infection of APC with recombinant viral vectors introducing relevant CMV antigens [9, 10, 13, 14], loading with epitope peptides [12] or CMV protein lysates [5, 11].

Many of these studies focused on demonstrating the generation of $CD8^+$ cytotoxic T cell cultures *in vitro*. However, clinical studies on CMV indicated that the acquisition of both $CD8^+$ and $CD4^+$ antigen-specific T cells was necessary for long-term maintenance of specific T cell immunity and therapeutic efficacy [5, 7, 8]. $CD4^+$ T cells have an important role in the establishment of a functional $CD8^+$ T cell memory [15, 16]. In addition, $CD4^+$ T cells have a direct antiviral effect due to their secretion of effector cytokines, and can also exert direct virus-specific cytolytic activity [17]. Therefore, the use of both $CD4^+$ and $CD8^+$ T cells, preferably recognizing various different CMV epitopes, would be highly desirable for immunotherapy. However, whether a proliferating cell line with a constant phenotype, serving as a permanent source of APC, can be used for the generation of both $CD8^+$ and $CD4^+$ CMV-specific T cells recognizing various epitopes remains to be shown.

We have previously presented a method for expanding CMV-specific cytotoxic $CD8^+$ T cells using a novel kind of renewable APC, called mini-LCL [18]. Mini-LCL are generated with the aid of a multifunctional "mini"-Epstein-Barr virus vector (mini-EBV) that mediates growth transformation of B cells and expression of the pp65 antigen. Here, we reinvestigated the *in vitro* stimulation of CMV-specific T cells with the pp65 mini-EBV system, with a focus on (1) the monitoring of the co-expansion of CMV- and EBV-specific $CD8^+$ T cells by a combination of functional and phenotypic assays, (2) the quest for CMV-specific $CD4^+$ helper T cells, and (3) the use of the mini-LCL system as a tool to identify new CMV-specific $CD8^+$ and $CD4^+$ T cell epitopes.

Results

Co-expansion of CMV- and EBV-specific $CD8^+$ T cells with pp65 mini-LCL

We have shown previously that stimulation of PBMC from CMV/EBV-seropositive donors with autologous pp65 mini-LCL yielded CMV/EBV-specific T cell cultures that displayed a predominantly pp65-specific cytotoxic reactivity. The presence of T cells specific for $CD8^+$ T cell epitopes of CMV and EBV was shown by HLA-peptide tetramer staining [18]. Now we wanted to determine the proportions of functional CMV and EBV antigen-specific T cells in such cultures, and relate these numbers to the initial frequencies in PBMC *ex vivo*. In a pilot experiment, we used cells from donor 1, with an HLA type for which $CD8^+$ T cell epitopes of pp65 and an EBV latent cycle protein were known (the A1-restricted pp65 epitope YSE, and the B8-restricted EBNA3A epitope QAK; see also Table 1). To quantify functional epitope-

Table 1. $CD8^+$ T cell epitopes investigated in this study[a)]

Abbreviation	HLA	Antigen	Position, aa	Sequence
YSE	A1	pp65	363-373	YSEHPTFTSQY
NLV	A2	pp65	495-503	NLVPMVATV
ATV	A11	pp65	501-509	ATVQGQNLK
TPR	B7	pp65	417-426	TPRVTGGGAM
RPH	B7	pp65	265-274	RPHERNGFTV
IPS	B35	pp65	123-131	IPSINVHHY
FPT	B3502	pp65	188-195	FPTKDVAL
VAF	Cw12	pp65	294-302	VAFTSHEHF
RRR [b)]	B14	pp65	539-547	RRRHRQDAL
NQW [b)]	B3501	pp65	173-181	NQWKEPDVY
CLG	A2	EBV LMP2	426-434	CLGGLLTMV
RPP	B7	EBV EBNA3A	379-387	RPPIFIRRL
QAK	B8	EBV EBNA3A	158-166	QAKWRLYTL
YPL	B35	EBV EBNA3A	458-466	YPLHEYHGM

a) For an overview of known CMV pp65 $CD8^+$ epitopes, see [13, 29]; for known EBV epitopes, see [30].
b) Epitopes identified in this study.

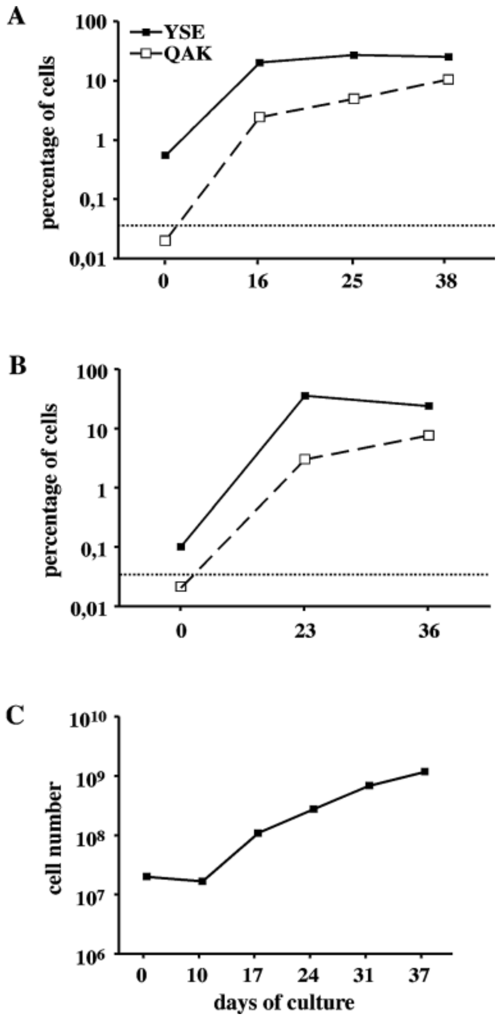

Fig. 1. Expansion of CMV- and EBV-specific T cells by pp65 mini-LCL stimulation. T cells specific for the CMV pp65 epitope YSE and the EBV EBNA3A epitope QAK (see Table 1) were quantified by HLA/peptide tetramer staining (A) or IFN-γ ELISPOT assay (B), *ex vivo* (day 0) and after the indicated periods of expansion. The detection limit is indicated by a dotted line. Total cell numbers (C) were determined by counting trypan blue-excluding cells. T cells and stimulators were from donor 1.

specific T cells *ex vivo* and after different periods of cultivation, we used an IFN-γ ELISPOT assay. Fig. 1 shows the combined functional (ELISPOT) and phenotypic (tetramers) analysis of epitope-specific T cells from donor 1 *ex vivo* and at various times of cultivation. Both methods of analysis consistently showed that T cells specific for the CMV pp65 epitope YSE were more frequent than T cells specific for the EBV latent protein epitope QAK. A considerable absolute expansion of the T cell culture as well as a relative increase in T cells specific for either of both epitopes was obvious. On day 0, numbers of phenotypically detectable T cells were higher than numbers of functional T cells for the pp65 epitope (no T cells were detectable for the EBV epitope). At later times, however, numbers of tetramer-staining T cells and of IFN-γ–secreting T cells approximately correlated.

Analysis of polyclonal T cell cultures from donors with diverse HLA backgrounds

Next, we generated T cell cultures from four additional donors seropositive for both CMV and EBV (donors F45, F46, F60 and F62). The donors covered a range of different HLA types (Table 2). We ensured peptides and tetramers for at least one CMV and one EBV epitope for each donor's HLA type were available, but had no prior knowledge about epitope specificities actually recognized by the donors' T cells. For this series of experiments, we used cell culture medium supplemented with 2% human AB serum only. We found that T cell cultures could be maintained and expanded by pp65 mini-LCL stimulation under these conditions. However, expansion was not as effective as under conditions using 10% FCS (Fig. 2A). Flow cytometric analysis showed that the cultures contained $CD8^+$ T cells, $CD4^+$ T cells and $CD4^-CD8^-$ cells, usually in this order of decreasing frequency (Fig. 2B).

In all of these T cell cultures, CMV and EBV epitope-specific T cells were quantified *ex vivo* and after several rounds of cultivation, using a functional assay (IFN-γ ELISPOT) and a phenotypic assay (HLA-peptide tetramer staining; for an example, see Fig. 2C). In all four donors, an expansion of T cells specific for at least one pp65 epitope was demonstrated by tetramer staining and the ELISPOT assay. In most cases, the order of

Table 2. HLA types of donors

Donor	HLA-A*	HLA-B*	HLA-C*	HLA-DQ*	HLA-DRB1*	DRB3/4/5
TW	1	8, 62	3, 7	n. d.[a)]	n. d.	n. d.
F45	1, 68	8, 14	7, 8	3, 6	13, 15	3, 5
F46	3, 11	7, 3501	4, 7	6	13, 15	3, 5
F60	2, 26	7, 38	7, 1203	1, 6	8, 13	3
F62	3	3501, 52	4, 1202	1, 6	1, 15	3

a) n. d., not determined.

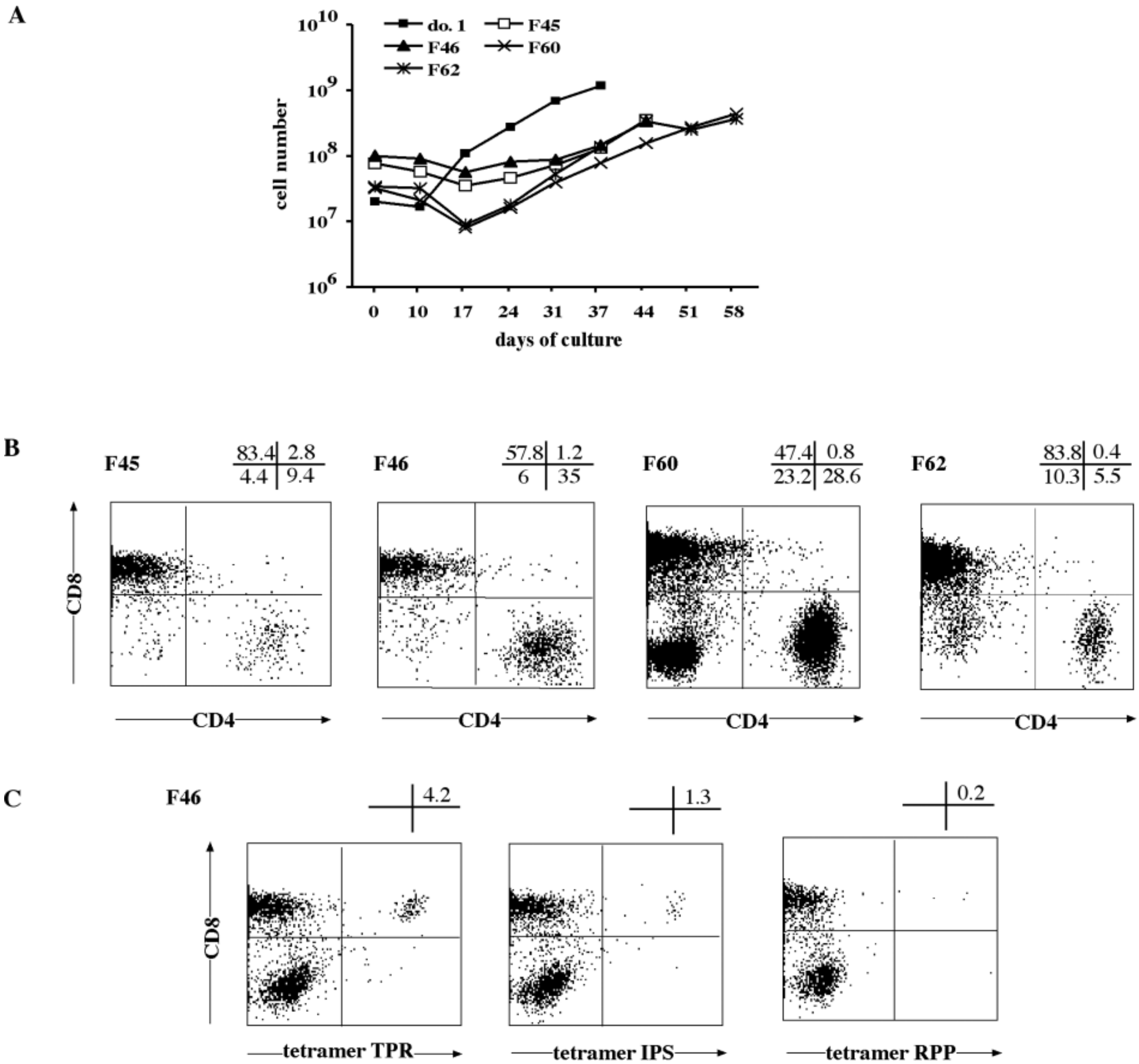

Fig. 2. Establishment of T cell cultures of four donors under xenogeneic serum–free conditions. In (A) the expansion of T cell cultures from 4 donors (F45, F46, F60, F62) is shown. For comparison, cell numbers for the T cell culture of donor 1 (cultivated with FCS) are also given. The phenotype of T cell cultures was analyzed by flow cytometry near the end of the cultivation period (B), on day 53 (donors F45 and F46) or day 44 (donors F60 and F62) of cultivation. Epitope-specific T cells were detected by HLA/peptide tetramer staining. Such an analysis is shown for donor F46 (C). Tetramers representing two pp65 epitopes (TPR and IPS) and an epitope from an EBV latent cycle antigen (RPP) were used. For T cell epitopes, see Table 1.

frequencies of CMV epitope specificities was preserved during cultivation. For a given epitope and donor, numbers of active effector (IFN-secreting) T cells tended to be lower than numbers of tetramer-staining T cells, but usually represented a significant proportion of the latter. Unlike CMV-specific T cells, EBV epitope-specific T cells tended to expand less strongly or in some cases even to decrease over time. Additionally, we performed intracellular IFN-γ staining of T cells after brief stimulation with pp65 mini-LCL or control mini-LCL (Fig. 3C). While a quantitative interpretation was hindered by the lack of a clear separation of IFN-γ-positive and -negative T cells, the results gave a first indication that both CMV-specific CD8$^+$ and CD4$^+$ T cells were present.

Consistent with these results, there was a strong cytotoxic reactivity of the T cell cultures against targets endogenously expressing pp65 or exogenously loaded with pp65 peptides, as shown in Fig. 4 for donor F45 (top) and donor F60 (bottom). Lysis of targets expressing only EBV antigens was weaker. The cytotoxic reactivity was HLA restricted, mismatched target cells were not lysed.

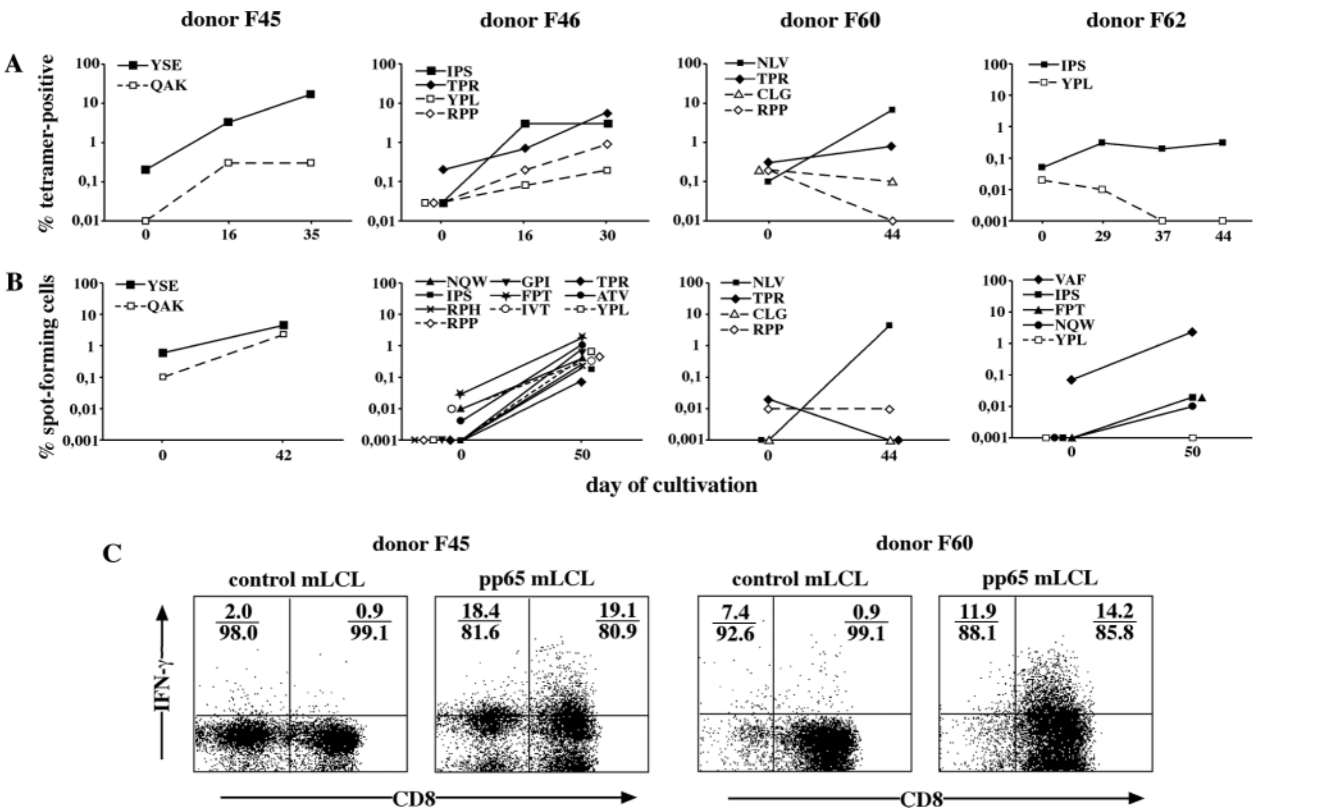

Fig. 3. Virus-specific T cells in cultures from four donors. Numbers of T cells specific for various epitopes from pp65 and immunodominant EBV latent proteins were determined by HLA/peptide tetramer staining (A) or IFN-γ ELISPOT assay (B) at the start of cultivation (day 0) and at later times. Epitopes are designated with their three-letter abbreviation (see Table 1). CMV pp65 epitopes are represented by closed symbols and continuous lines, EBV latent cycle epitopes by open symbols and dashed lines. For two of these T cell lines, CMV-specific T cells were also visualized by increased intracellular staining for IFN-γ after short-term stimulation with pp65 mini-LCL as compared to stimulation with control mini-LCL (C).

Clonal analysis of pp65 mini-LCL-stimulated T cell cultures

While there was a clear pp65-specific reaction pattern of the pp65 mini-LCL-stimulated T cell cultures, epitope-specific assays never accounted for more than 20% of total cell numbers (Fig. 3). Therefore, we were interested in characterizing the specificity of the remaining T cells. Formal single-cell clones were established from the T cell cultures of three donors. Cloning was performed at day 45 (donors F45 and F46) or day 31 (F62) of polyclonal cultivation, and cloned T cells were nonspecifically expanded. Between 44 and 85 clones of each donor were screened for pp65 specificity by an IFN-γ ELISA with pp65 mini-LCL and control mini-LCL as stimulators, and tested for their expression of CD4 and CD8. In each of the three donors, the majority of T cell clones displayed CMV-specific IFN-γ release (Table 3), showing that among those T cells with sufficient proliferative potential to grow out as clones, CMV-specific T cells indeed predominated. Clones displaying the $CD8^+$ phenotype were predominant among CMV-specific T cell clones, but, in two of the three donors, $CD4^+$ T cell clones were also found in significant proportions, demonstrating that pp65 mini-LCL could reactivate and select for CMV-specific $CD4^+$ as well as $CD8^+$ T cells.

To determine the HLA restriction of the $CD4^+$ and $CD8^+$ T cell clones, we tested their reactivity against allogeneic target cells with a panel of HLA class II and class I allele matches. In donor F46, individual CMV-specific $CD8^+$ T cell clones exhibited one of three different reactivity patterns, consistent with HLA restrictions through HLA-B*07, A*11 and B*3501, respectively (Fig. 5A). For $CD4^+$ clones from the same donor three patterns were found, indicating restriction through HLA-DRB1*13, DRB1*15 and DRB5 (Fig. 5B). HLA restrictions of all T cell clones analyzed are summarized in Table 4. HLA restriction was especially diverse in donor F46 (six different HLA class I and II restrictions), but the other two donors' clones also displayed at least three different HLA restriction patterns. Remarkably, several clones from donor 45 showed reactivity restricted through HLA-B*14, a

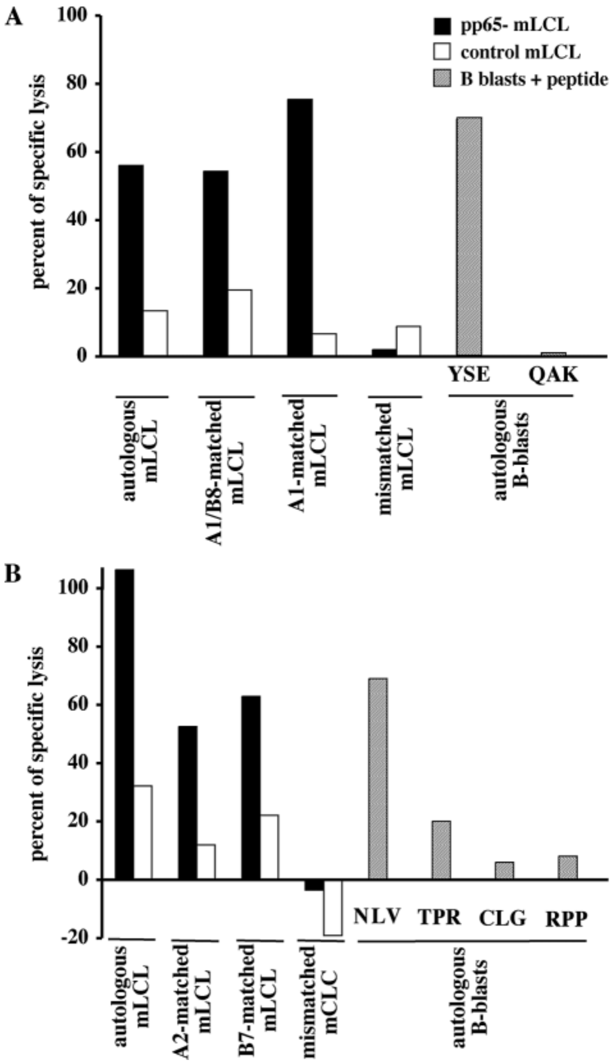

Fig. 4. Cytotoxic reactivity of T cell cultures from donor F45 (A) and donor F60 (B). Reactivity was tested against autologous target cells (pp65 mini-LCL, black bars, and control mini-LCL, white bars) and allogeneic targets matched for HLA class I alleles as indicated. To test for epitope-specific cytotoxic reactivity, autologous EBV-free B lymphoblasts (hatched bars), loaded with epitope peptides as indicated, were used as targets. Experiments were performed at day 36 (donor f45) or day 37 (donor F60) of cultivation. An effector-to-target ratio of 8 was used.

restriction element for which no pp65 epitope has been described so far. The same is true for the DRB5 restriction displayed by clones from donor F46.

Selected CMV-specific $CD4^+$ T cell clones from donors F46 and F62 were also tested for their cytokine secretion profile by ELISA assays. Besides IFN-γ, both tested T cell clones secreted the effector cytokines TNF-α, GM-CSF and IL-2 in an antigen-specific and HLA-restricted manner, but differed in the amounts of the individual cytokines (Fig. 6).

Identification of pp65 $CD8^+$ and $CD4^+$ epitopes

Since we were unable to detect any reactivity of some of the $CD8^+$ T cell clones from donor F46 against well-established CMV epitopes for the relevant restriction elements (see Table 1), we sought to determine the epitope specificity of these clones using an overlapping peptide library covering the complete pp65 sequence. As previously described [19], the 138 peptides of this library were used to compose two sets of 12 peptide sub-pools each in a cross-matrix orientation, which enabled the pp65 subsequence recognized by the T cell clone to be define using only 24 individual reactions. Fig. 7 A shows some typical results. For example, clone 14 from donor F46 only reacted with "vertical" pools 7 and 8 and "horizontal" pool 16, making it likely that the epitope recognized by this clone was to be found in the overlap region of two neighboring peptides, one present in pools 7 and 16, the other in pools 8 and 16; this overlap region has the sequence NQWKEPDVYYT, previously suspected of containing an HLA-B*3501-restricted epitope [19]. This region contains the nonameric sequence NQWKEPDVY carrying the typical C-terminal tyrosine "anchor residue". We tested the reactivity of our T cell clones against this nonamer and confirmed NQWKEPDVY as an HLA-B*3501 epitope (Fig. 7B). Two out of two B*3501-restricted T cell clones analyzed from donor F46 recognized this epitope, consistent with ELISPOT data indicating dominance of this epitope over the B*3501-restricted epitope IPS in donor 46 (Fig. 3). Analogously,

Table 3. Clonal analysis of pp65 mini-LCL-generated T cell cultures

	Antigen specificity					
	CMV [a)]		EBV or other [b)]		Nonreactive	
Donor	CD8+	CD4+	CD8+	CD4+	CD8+	CD4+
F45	61	0	9	1	7	7
F46	16	11	8	2	4	3
F62	59	4	2	0	12	3

a) T cell clones were considered CMV-specific when they secreted at least three times the amount of IFN-γ after challenge with pp65 mini-LCL than after challenge with mini-LCL.
b) T cell clones recognizing both pp65 mini-LCL and control mini-LCL.

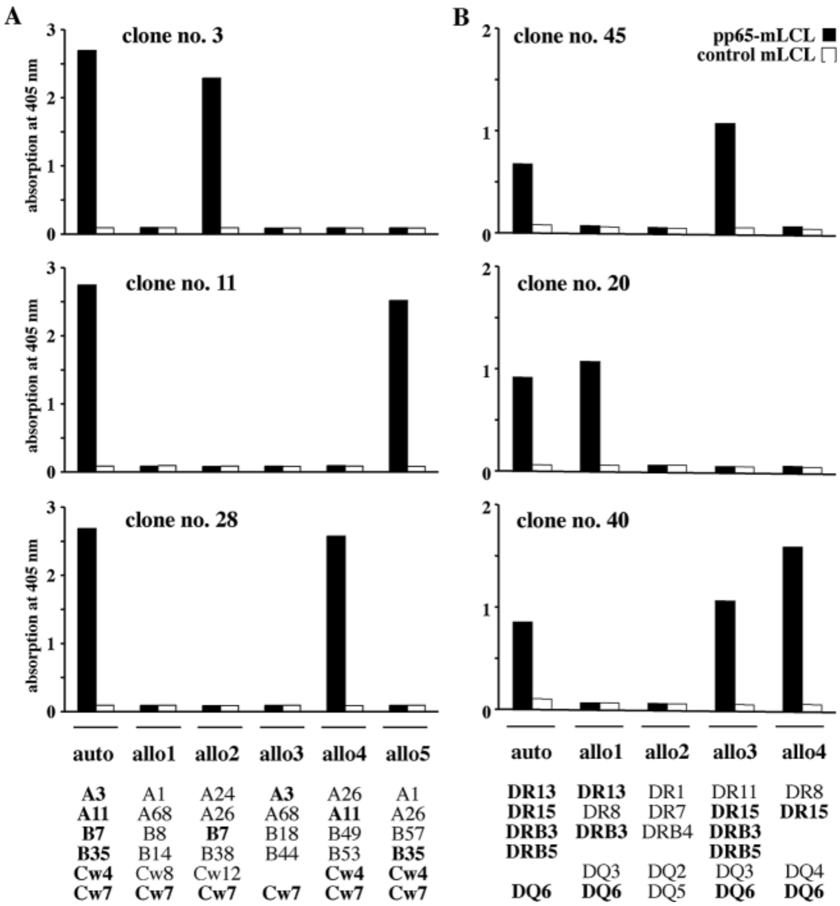

Fig. 5. Antigen specificity and HLA restriction of pp65-specific $CD8^+$ and $CD4^+$ T cell clones, generated from pp65 mini-LCL–stimulated T cell cultures from donor F46. Three $CD8^+$ T cell clones (A) and three $CD4^+$ T cell clones (B) were tested in an interferon-γ ELISA for their activation by cocultivation with various stimulator cells. Stimulators were pp65 mini-LCL (black bars) or control mini-LCL (white bars) from the autologous donor ("auto") or from diverse allogeneic donors ("allo1" etc.), whose HLA class I or class II alleles are stated for the experiments involving $CD8^+$ or $CD4^+$ T cells, respectively. HLA alleles that match with those of the T cell donor are written in boldface.

Table 4. HLA restriction and epitope specificity of pp65-specific clones

Donor	HLA restriction	No. of HLA-restricted clones	Epitope recognized	Amino acid position in pp65	No. of epitope-specific clones	Reference
F45	A*68	8	n.d.	-	-	-
	B*14	11	RRRHRQDAL	539-547	5/5	This study
	n.d.[a)]	20	n.d.	-	-	-
F46	A*11	4	ATVQGQNLK	501-509	3/3	[13]
	B*07	4	TPRVTGGGAM	417-426	1/4	[31]
	B*35	8	NQWKEPDVY	173-181	2/2	This study
	DRB1*13	5	(QPFM)RPHERNGFTVL(CPKN) [b)]	261-279	1/1	[22]
	DRB1*15	1	MSIYVYALPLKMLNI	109-123	1/1	This study
	DRB5	2	(AGIL)ARNLVPMVATV(QGQN) [b)]	489-507	1/1	[21, 22]
F62	A*03	3	n.d.	-	-	-
	Cw*12	15	VAFTSHEHF	294-302	1/3	[13]
	DRB1*01	4	(GQNL)KYQEFFWDAND(IYRI) [b)]	505-523	1/1	[21, 22]

a) $CD8^+$ T cell clones that did not recognize any available partially HLA class I-matched target cells.
b) Two 15-mer peptides, overlapping by 11 amino acids, were both recognized.

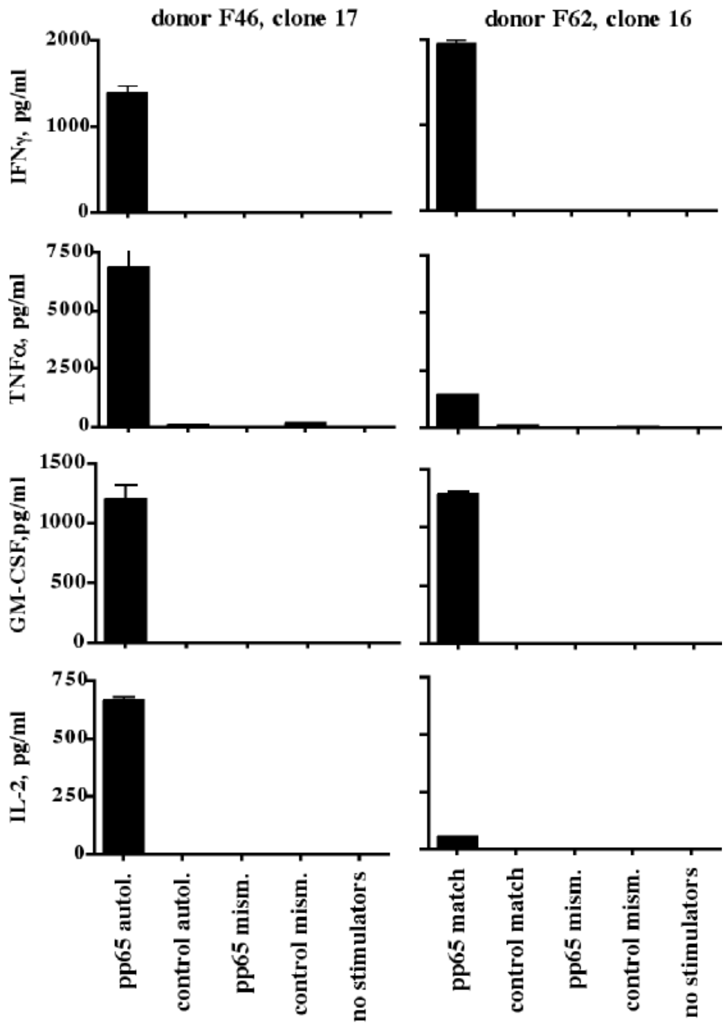

Fig. 6. Cytokine secretion profile of CMV-specific T cell clones. T cells were stimulated with various mini-LCL targets for 32 h or left unstimulated, as indicated. Cytokine concentrations were determined by ELISA: 1,000 pg/ml correspond to 0.005 pg cytokine per T cell.

we identified the HLA-A*11-restricted epitope ATVQGQNLK and the HLA-Cw*12-restricted epitope VAFTSHEHF, both recently described by Kondo et al. [13], to be target epitopes of our T cell clones.

For donor 45, the T cell clones displaying the novel HLA-B*14 restriction were analyzed with the peptide library (Fig. 6A), and the 15-mer PAAQPKRRRHRQDAL was found to be recognized. The nonameric sequence RRRHRQDAL, being in very good accordance with the established HLA-B*14 motif [20] (xRxxRxxxL), was found to be recognized by 5 out of 5 evaluable HLA-B*14-restricted T cell clones, confirming RRRHRQDAL as a new CD8$^+$ T cell epitope of pp65.

For each HLA class II restriction displayed by our CMV-specific CD4$^+$ T cell clones, a representative clone was tested for epitope specificity using the pp65 peptide library (Table 4). Two previously described CMV epitope specificities [21, 22], restricted through HLA-DRB1*01 and DRB1*13, were confirmed. Our DRB5-restricted T cell clone recognized amino acids 489–507, already described as a presumably DR11-restricted epitope [21, 22]. This sequence, which also contains the HLA class I (HLA-A2) epitope NLVPMVATV, can therefore be presented by at least two different class II molecules. Another pp65 subsequence (amino acids 109–123), to our knowledge previously undescribed, represents a new CD4$^+$ T cell epitope restricted through HLA-DRB1*15.

Discussion

In this study, we present a refined analysis of CMV-specific T cell cultures generated by stimulation of PBMC with permanent APC lines, the autologous pp65 mini-LCL. These mini-LCL are activated B cell lines stably carrying a recombinant mini-EBV episome that simultaneously mediates expression of the foreign antigen pp65 and growth transformation of the cell line [18]. The efficient routine generation of autologous mini-LCL that are free from wild-type EBV or helper virus is based on a packaging system for EBV vectors [23], by which EBV vector plasmids are enveloped with an intact EBV coat. To our knowledge, this is to date the

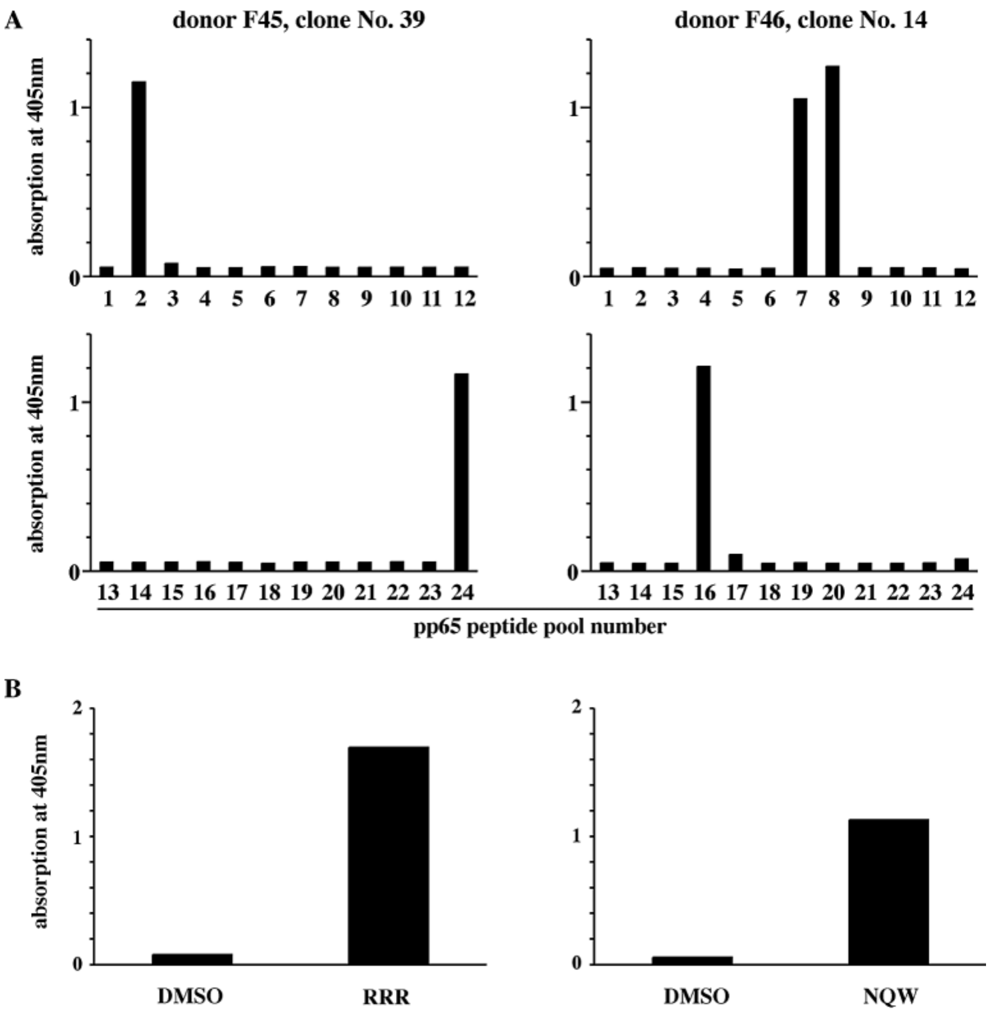

Fig. 7. Identification of the epitopes recognized by pp65-specific CD8+ T cell clones. Results are shown for an HLA-B*14-restricted T cell clone from donor F45 (A and B, left) and an HLA-B*3501-restricted clone from donor F46 (A and B, right). The epitope-containing region was determined using a 15-mer peptide library covering the pp65 sequence, as described in the main text. T cell clones were tested for reactivity against autologous mini-LCL pulsed with peptide sub-pools (A) in an IFN-γ ELISA. Candidate nonameric epitope peptides were then prepared and similarly tested in ELISA assays (B).

only gene vector system that can confer these three constitutive properties by means of a single infection step: foreign antigen expression, induction of a professional APC phenotype, and growth transformation. Therefore, we anticipate that pp65 mini-LCL are likely to facilitate CMV-specific immunotherapy in the near future, much like standard LCL facilitated EBV-specific cellular therapy [24].

To investigate the potential of pp65 mini-LCL to expand both $CD4^+$ and $CD8^+$ T cells of various HLA restrictions, we randomly selected four CMV-seropositive donors, excluding only such donors whose HLA type did not permit any epitope-specific T cell analysis. We found that, except for one epitope in one donor (donor F60, TPR epitope), all CMV epitope specificities in these four donors that could be monitored with the available HLA/peptide tetramers and peptides were indeed expanded by pp65 mini-LCL stimulation. In contrast, the expansion of EBV epitope-specific T cells could only be demonstrated in some of the donors, a result consistent with the generally less pronounced lysis of target cells expressing only EBV antigens, in contrast to the efficient lysis of targets expressing pp65 (Fig. 3). Thus, pp65 mini-LCL are a reliable tool to expand a wide spectrum of CMV pp65-specific, but not necessarily EBV epitope-specific T cells.

We used single-cell cloning at limiting dilution under non-antigen-specific conditions to analyze pp65 mini-LCL-stimulated polyclonal T cells. In all three donors tested, a clear majority of T cell clones was found to be CMV-specific (Fig. 5 and Table 3). Therefore, pp65 mini-LCL stimulation generates T cell cultures that contain T cells capable of extended proliferation *in vitro*, and these T cells are mainly CMV specific. Most CMV-specific T cell clones were CD8 positive, but in two out of three donors, CMV-specific $CD4^+$ T cells clones were also found. It follows that pp65 mini-LCL can stimulate both CMV-specific CD4$^+$ and CD8$^+$ T cells, presenting pp65 epitopes through HLA class I and II. According to the classical picture, MHC class II processing and presentation operates on exogenous antigens that have been endocytosed by the APC. However, examples of class II presentation of cytosolic antigens by endogenous pathways are accumulating. For example, a model cytosolic antigen [25] and the EBV antigen EBNA1 [26] have been recently shown to access vesicular compartments by autophagy, resulting in their presentation on HLA class II by EBV-immortalized B cells. Similar mechanisms might be operative in mini-LCL.

A set of pp65 mini-LCL and control mini-LCL displaying single HLA allelic matches with the T cell donors enabled convenient testing of the HLA restriction of the T cell clones. Among the CMV-specific CD8$^+$ T cell clones, at least two different HLA class I restrictions were identified for each donor. In one donor (F46), three different HLA class I restrictions and three class II restrictions were found, demonstrating that a diverse HLA restriction pattern of pp65-specific T cells can be maintained in pp65 mini-LCL-stimulated T cell cultures.

The presentation of the pp65 antigen by mini-LCL through both HLA class I and II enabled us to identify two new HLA class I-restricted epitopes, one new class II epitope, and to confirm several recently described pp65 epitopes at the level of T cell clones (Table 4). Remarkably, some epitope specificities prominent in the cloned T cell populations had been undetectable in PBMC *ex vivo*, indicating the capability of the mini-EBV system to expand T cell populations with low precursor frequencies in peripheral blood.

To conclude, here we have shown that the pp65 mini-LCL system permits the *in vitro* reactivation and expansion of CMV-specific CD8$^+$ and CD4$^+$ T cells recognizing a variety of CMV epitopes restricted through different HLA alleles. Therefore, pp65 mini-LCL are likely to prove useful in clinical application for adoptive T cell transfer. In addition, they provide useful tools for the identification of new target structures of the CMV-specific cellular immune response, the knowledge of which will facilitate future applications in immunotherapy, vaccination and immunomonitoring.

Materials and methods

Cell culture

Standard cell culture medium was RPMI 1640 with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). For mini-LCL generation, medium was supplemented with cyclosporin A (1 μg/ml), for the first 4 weeks of cultivation. Unless otherwise noted, T cell medium contained 2% human serum type AB (Cambrex Bioproducts) instead of FCS and was supplemented with recombinant interleukin-2 ("Proleukin," Chiron) as specified. Medium for CD40-stimulated B blasts contained IL-4 (2 ng/ml; R&D) and cyclosporin A (1 μg/ml).

Mini-EBV vectors, mini-LCL, and CD40 B lymphoblasts

The construction of the pp65 mini-EBV and the control mini-EBV plasmid, the preparation of packaged mini-EBV vectors, and the generation of wild-type EBV-free mini-LCL have been described previously [18]. Mini-EBV vectors for B cell infection were generated by packaging of mini-EBV plasmids, which are replication incompetent, in the complementing cell line TR-2/293 [23]. Mini-LCL-expressing pp65, and control mini-LCL, were generated by infection of unseparated PBMC with packaged mini-EBV vector. CD40-stimulated B blast cultures were established by weekly replating PBMC on irradiated feeder cells, which were murine L fibroblasts stably expressing the human CD40 ligand [27], in the presence of IL-4 (2 ng/ml) and cyclosporin A (1 μg/ml).

T cell lines and clones

T cells were derived from peripheral blood of five human adult CMV-seropositive donors. Except for donor 1 (from whom freshly drawn blood was available), material was obtained in the form of a buffy coat preparation containing enriched PBMC from a routine 500-ml blood donation. PBMC were further purified by Ficoll centrifugation and immediately used to set up mini-LCL and CD40-stimulated B blast cultures. The remaining PBMC were cryoconserved for later use in T cell stimulation. Low-resolution HLA class I and II typing was performed by PCR-based methods (Labor Dr. Klein, Martinsried, Germany). CMV IgG antibody status was determined on plasma-containing buffy coat supernatants with a CMV IgG latex agglutination test kit (CMVScan, Becton-Dickinson).

T cells were reactivated from cryoconserved (for donor 1, freshly isolated) autologous PBMC by restimulation with the irradiated autologous pp65 mLCL [18]. Per well of a 24-well plate, 2×10^6 PBMC and 5×10^4 irradiated mini-LCL (50 Gy) were cocultivated in 2 ml medium. On day 10 and then after intervals of 7 days, cells were pooled, counted, and replated at 1×10^6 cells/2 ml medium per well, adding freshly irradiated mLCL cells as stimulators at an effector-stimulator ratio of 4:1. Cells were re-fed or expanded at least every 4 days. From day 15 onward, culture medium was supplemented with rIL-2 (90 U/ml).

T cell clones were generated in 96-well round-bottom plates by seeding 0.5 T cells/well in medium containing 10% FCS and supplemented with 1,000 U recombinant IL-2, 0.5 μg/ml PHA, 1×10^4 irradiated mLCL, and 1×10^5 irradiated feeder cells consisting of a mix of allogeneic PBMC from four donors. Restimulations of T cell clones were performed every 2–3 weeks, and outgrowing clones were expanded in 96-well plates under same conditions.

IFN-γ ELISPOT and ELISA

An ELISPOT was used to determine the frequency of pp65-specific IFN-γ-secreting cells in freshly prepared PBMC or in

T cell cultures after various cultivation periods. IFN-γ ELISPOT assays were performed according to the manufacturer's instructions (Mabtech, Nacka, Sweden) in 96-well cellulose ester plates (MAHAS4510, Millipore). For analysis of PBMC, 200,000-10,000 cells were distributed to each well. Antigenic peptide was added at 5 μg/ml. For analysis of cultivated T cells, stimulator cells (autologous CD40 B lymphoblasts) were loaded with antigenic peptide (at 5 μg/ml for 2 h at 37°C), washed and used in ELISPOT assays at 1×10^4 cells/well, together with various numbers of T cells ranging from 1,000 to 100,000 per well. In both cases, stimulation was performed in 200 μl medium overnight. T cell clones were also analyzed with ELISA assays for IFN-γ, TNF-α, IL-2 (Mabtech) and GM-CSF (Becton Dickinson).

Cytotoxicity assay

The cytotoxic activity of T cells was analyzed as described [18], by an assay based on the time-resolved fluorometric quantification of a complex of Europium (Eu^{3+}) and the chelate ligand TDA (terpyridine dicarboxylic acid), which is released from labeled target cells upon cell lysis.

Flow cytometry

The HLA/peptide tetrameric complexes representing CMV and EBV epitopes were prepared as described [28] or purchased from Proimmune (Oxford, UK). Staining, data acquisition and analysis were performed as described [18]. For intracellular cytokine staining, resting T cell cultures were added to mini-LCL as stimulators at a ratio of 4:1. Cytokine export was blocked by addition of brefeldin A after 1 h. After additional 5 h, cells were harvested and stained using "Fix&Perm" reagent (Caltag) according to the manufacturer. Antibodies IFNg-Alexa488 and CD4-PE were from Caltag, antibodies CD8-FITC, CD3-CyChrome and CD56-APC were from Becton Dickinson.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft (SFB455), Wilhelm Sander-Stiftung, H.W. & J. Hector-Stiftung, and Fonds der Chemischen Industrie.

References

1 **Boeckh, M., Nichols, W. G., Papanicolaou, G., Rubin, R., Wingard, J. R. and Zaia, J.,** Cytomegalovirus in hematopoietic stem cell transplant recipients: current status, known challenges, and future strategies. *Biol. Blood Marrow Transplant.* 2003. **9:** 543–558.

2 **Cwynarski, K., Ainsworth, J., Cobbold, M., Wagner, S., Mahendra, P., Apperley, J., Goldman, J. et al.,** Direct visualization of cytomegalovirus-specific T cell reconstitution after allogeneic stem cell transplantation. *Blood* 2001. **97:** 1232–1240.

3 **Broers, A. E., van Der Holt, R., van Esser, J. W., Gratama, J. W., Henzen-Logmans, S., Kuenen-Boumeester, V., Lowenberg, B. and Cornelissen, J. J.,** Increased transplant-related morbidity and mortality in CMV- seropositive patients despite highly effective prevention of CMV disease after allogeneic T cell-depleted stem cell transplantation. *Blood* 2000. **95:** 2240–2245.

4 **Einsele, H., Hebart, H., Kauffmann-Schneider, C., Sinzger, C., Jahn, G., Bader, P., Klingebiel, T. et al.,** Risk factors for treatment failures in patients receiving PCR-based preemptive therapy for CMV infection. *Bone Marrow Transplant.* 2000. **25:** 757–763.

5 **Einsele, H., Roosnek, E., Rufer, N., Sinzger, C., Riegler, S., Loffler, J., Grigoleit, U., Moris, A. et al.,** Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. *Blood* 2002. **99:** 3916–3922.

6 **Riddell, S. R., Watanabe, K. S., Goodrich, J. M., Li, C. R., Agha, M. E. and Greenberg, P. D.,** Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science* 1992. **257:** 238–241.

7 **Walter, E. A., Greenberg, P. D., Gilbert, M. J., Finch, R. J., Watanabe, K. S., Thomas, E. D. and Riddell, S. R.,** Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T cell clones from the donor. *N. Engl. J. Med.* 1995. **333:** 1038–1044.

8 **Peggs, K. S., Verfuerth, S., Pizzey, A., Khan, N., Guiver, M., Moss, P. A. and Mackinnon, S.,** Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T cell lines. *Lancet* 2003. **362:** 1375–1377.

9 **Hamel, Y., Blake, N., Gabrielsson, S., Haigh, T., Jooss, K., Martinache, C., Caillat-Zucman, S. et al.,** Adenovirally transduced dendritic cells induce bispecific cytotoxic T lymphocyte responses against adenovirus and cytomegalovirus pp65 or against adenovirus and Epstein-Barr virus EBNA3C protein: a novel approach for immunotherapy. *Hum. Gene Ther.* 2002. **13:** 855–866.

10 **Keever-Taylor, C. A., Margolis, D., Konings, S., Sandford, G. R., Nicolette, C. A., Lawendowski, C. and Burns, W. H.,** Cytomegalovirus-specific cytolytic T cell lines and clones generated against adenovirus-pp65-infected dendritic cells. *Biol. Blood Marrow Transplant.* 2001. **7:** 247–256.

11 **Peggs, K., Verfuerth, S. and Mackinnon, S.,** Induction of cytomegalovirus (CMV)-specific T cell responses using dendritic cells pulsed with CMV antigen: a novel culture system free of live CMV virions. *Blood* 2001. **97:** 994–1000.

12 **Szmania, S., Galloway, A., Bruorton, M., Musk, P., Aubert, G., Arthur, A., Pyle, H. et al.,** Isolation and expansion of cytomegalovirus-specific cytotoxic T lymphocytes to clinical scale from a single blood draw using dendritic cells and HLA-tetramers. *Blood* 2001. **98:** 505–512.

13 **Kondo, E., Akatsuka, Y., Kuzushima, K., Tsujimura, K., Asakura, S., Tajima, K., Kagami, Y., et al.,** Identification of novel CTL epitopes of CMV-pp65 presented by a variety of HLA alleles. *Blood* 2004. **103:** 630–638.

14 **Sun, Q., Pollok, K. E., Burton, R. L., Dai, L. J., Britt, W., Emanuel, D. J. and Lucas, K. G.,** Simultaneous ex vivo expansion of cytomegalovirus and Epstein-Barr virus-specific cytotoxic T lymphocytes using B-lymphoblastoid cell lines expressing cytomegalovirus pp65. *Blood* 1999. **94:** 3242–3250.

15 **Shedlock, D. J. and Shen, H.,** Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* 2003. **300:** 337–339.

16 **Janssen, E. M., Lemmens, E. E., Wolfe, T., Christen, U., von Herrath, M. G. and Schoenberger, S. P.,** $CD4^+$ T cells are required for secondary expansion and memory in $CD8^+$ T lymphocytes. *Nature* 2003. **421:** 852–856.

17 **Appay, V., Zaunders, J. J., Papagno, L., Sutton, J., Jaramillo, A., Waters, A., Easterbrook, P. et al.,** Characterization of CD4(+) CTLs *ex vivo*. *J. Immunol* 2002. **168:** 5954–5958.

18 **Moosmann, A., Khan, N., Cobbold, M., Zentz, C., Delecluse, H. J., Hollweck, G., Hislop, A. D. et al.,** B cells immortalized by a mini-Epstein-Barr virus encoding a foreign antigen efficiently reactivate specific cytotoxic T cells. *Blood* 2002. **100:** 1755–1764.

19 **Kern, F., Bunde, T., Faulhaber, N., Kiecker, F., Khatamzas, E., Rudawski, I. M., Pruss, A. et al.,** Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T cell repertoire in CMV-exposed individuals. *J. Infect. Dis.* 2002. **185:** 1709–1716.

20 **DiBrino, M., Parker, K. C., Margulies, D. H., Shiloach, J., Turner, R. V., Biddison, W. E. and Coligan, J. E.,** The HLA-B14 peptide binding site can accommodate peptides with different combinations of anchor residues. *J. Biol. Chem.* 1994. **269:** 32426–32434.

21 **Khattab, B. A., Lindenmaier, W., Frank, R. and Link, H.,** Three T cell epitopes within the C-terminal 265 amino acids of the matrix protein pp65 of human cytomegalovirus recognized by human lymphocytes. *J. Med. Virol.* 1997. **52:** 68–76.

22 **Li Pira, G., Bottone, L., Ivaldi, F., Pelizzoli, R., Del Galdo, F., Lozzi, L., Bracci, L. et al.,** Identification of new Th peptides from the cytomegalovirus protein pp65 to design a peptide library for generation of CD4 T cell lines for cellular immunoreconstitution. *Int. Immunol* 2004. **16:** 635–642.

23 **Delecluse, H. J., Pich, D., Hilsendegen, T., Baum, C. and Hammerschmidt, W.,** A first-generation packaging cell line for Epstein-Barr virus-derived vectors. *Proc. Natl. Acad. Sci. USA* 1999. **96:** 5188–5193.

24 **Rooney, C. M., Smith, C. A., Ng, C. Y., Loftin, S. K., Sixbey, J. W., Gan, Y., Srivastava, D. K. et al.,** Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood* 1998. **92:** 1549–1555.

25 **Nimmerjahn, F., Milosevic, S., Behrends, U., Jaffee, E. M., Pardoll, D. M., Bornkamm, G. W. and Mautner, J.,** Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. *Eur. J. Immunol* 2003. **33:** 1250–1259.

26 **Paludan, C., Schmid, D., Landthaler, M., Vockerodt, M., Kube, D., Tuschl, T. and Munz, C.,** Endogenous MHC class II processing of a viral nuclear antigen after autophagy. *Science* 2005. **307:** 593–596.

27 **Garrone, P., Neidhardt, E. M., Garcia, E., Galibert, L., van Kooten, C. and Banchereau, J.,** Fas ligation induces apoptosis of CD40-activated human B lymphocytes. *J. Exp. Med.* 1995. **182:** 1265–1273.

28 **Khan, N., Cobbold, M., Keenan, R. and Moss, P. A.,** Comparative analysis of $CD8^+$ T cell responses against human cytomegalovirus proteins pp65 and immediate early 1 shows similarities in precursor frequency, oligoclonality, and phenotype. *J. Infect. Dis.* 2002. **185:** 1025–1034.

29 **Paston, S. J., Dodi, I. A. and Madrigal, J. A.,** Progress made towards the development of a CMV peptide vaccine. *Hum. Immunol* 2004. **65:** 544–549.

30 **Khanna, R. and Burrows, S. R.,** Role of cytotoxic T lymphocytes in Epstein-Barr virus-associated diseases. *Annu. Rev. Microbiol.* 2000. **54:** 19–48.

31 **Weekes, M. P., Wills, M. R., Mynard, K., Carmichael, A. J. and Sissons, J. G.,** The memory cytotoxic T-lymphocyte (CTL) response to human cytomegalovirus infection contains individual peptide-specific CTL clones that have undergone extensive expansion *in vivo*. *J. Virol.* 1999. **73:** 2099–2108.

