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HLA type-independent generation of antigen-specific T cells for adoptive immunotherapy

Markus H. Hammer[1,2], Sonja Meyer[1], Gordon Brestrich[1], Andreas Moosmann[3], Florian Kern[2], Lydia Tesfa[2], Nina Babel[1], Alexa Mittenzweig[1], Cliona M. Rooney[4], Wolfgang Hammerschmidt[3], Hans-Dieter Volk[2] and Petra Reinke[1]

[1] Department of Nephrology and Internal Intensive Care, Universitätsmedizin Berlin, Berlin, Germany
[2] Institute of Medical Immunology, Charité, Universitätsmedizin Berlin, Berlin, Germany
[3] Clinical Cooperative Group Molecular Oncology, GSF Department of Gene Vectors and Department of Otorhinolaryngology, Ludwig-Maximilian Universität, Munich, Germany
[4] Center for Cell and Gene Therapy, Departments of Pediatrics and Medicine, Baylor College of Medicine, Houston, USA

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Adoptive immunotherapy with antigen-specific T cells has been successfully used to treat certain infectious diseases and cancers. Although more patients may profit from T cell therapy, its more frequent use is restricted by limitations in current T cell generation strategies. The most commonly applied peptide-based approaches rely on the knowledge of relevant epitopes. Therefore, T cells cannot be generated for diseases with unknown epitopes or for patients with unfavorable HLA types. We developed a peptide-based approach for HLA type-independent generation of specific T cells against various proteins. It is based on short-time stimulation with peptide libraries that cover most $CD4^+$ and $CD8^+$ T cell epitopes of given proteins. The procedure requires no prior knowledge of epitopes because libraries are synthesized solely on the basis of the protein's amino acid sequence. Stimulation is followed by immunomagnetic selection of activated IFN-γ-secreting cells and nonspecific expansion. To evaluate the protocol, we generated autologous T cells specific for a well-characterized antigen, the human cytomegalovirus phosphoprotein 65 (pp65). Generated T cell lines consisted of pp65-specific $CD4^+$ and $CD8^+$ lymphocytes that displayed antigen-specific killing and proliferation. The protocol combines the biosafety of peptide-based approaches with HLA type independence and may help to advance adoptive immunotherapy in the future.

Key words: Adoptive immunotherapy · Antigen-specific T cells · Peptide libraries · Generation procedure

Introduction

Adoptive immunotherapy using *ex vivo* expanded antigen-specific T cells is a promising therapy for certain diseases, but for broader application, obstacles still have to be overcome [1, 2]. In previous work, we and other colleagues described the successful treatment of patients suffering from therapy-resistant viral infections with Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV) [3–5]. Both diseases are predominantly found in immunosuppressed patients, underlining the role of diminished cellular immunity as main risk factor. (Re-)establishing a functional cellular immunity by adoptive transfer of EBV-specific T cells was also remarkably effective in treatment and prevention of EBV-associated lymphomas after transplantation, *i.e.* post-transplant lymphoproliferative disease (PTLD) [6]. Despite these encouraging results, only a small number of different diseases and patients were treated in clinical studies. A limiting factor is the time- and cost-intensive

The first two authors contributed equally to this work.

Correspondence: Markus H. Hammer, Interdisciplinary Laboratory Transplant Research, Nephrology/Medical Immunology, Department of Nephrology, Charité – Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany
Fax: +49–30–450–524907
e-mail: markus.hammer@charite.de

Abbreviations: **GMP:** Good manufacturing practice · **HCMV:** Human CMV · **IE-1:** Immediate early protein 1 · **LCL:** Lymphoblastoid cell lines · **PHA:** Phytohemagglutinin · **pp65:** Phosphoprotein 65 · **VP-1:** Viral protein 1 (polyoma virus)

production of antigen-specific T cells under good manufacturing practice (GMP) conditions.

The aim of this study was to establish an HLA type-independent protocol allowing T cell generation for an increased number of patients and diseases. Further demands were the generation of T cell lines consisting of cytotoxic $CD8^+$ and helper $CD4^+$ lymphocytes, both with the ability to recognize multiple epitopes of immunogenic proteins. To facilitate T cell production under GMP conditions, antigen-specific stimulation should be delivered by peptides instead of recombinant proteins or transgenic cells, because of superior biosafety and practicability.

Previous experiments of our group showed that overlapping peptide libraries that cover most existing T cell epitopes can be used to monitor frequencies of antigen-specific $CD8^+$ and $CD4^+$ lymphocytes in peripheral blood [7, 8]. Complete libraries are built of overlapping peptides with 15-aa length. Each peptide shares 11-aa overlap with the previous one, thereby spanning the entire protein's amino acid sequence (Fig. 1). Libraries designed this way contain, theoretically, all possible 9–12-mer epitopes for all HLA types. No knowledge of single epitopes is required for T cell stimulation. Because stimulation with peptide libraries could be used to monitor frequencies of antigen-specific T cells, we decided to integrate the stimulation step in an HLA type-independent T cell generation protocol. After short-time stimulation, frequencies of T cells specific for most viral and tumor antigens [*e.g.* phosphoprotein 65 (pp65), immediate early protein 1 (IE-1), viral protein 1 (VP-1), Wilms' tumor 1 (WT1)] were too low for immediate therapeutic use [2, 5, 7]. Therefore, specific enrichment and expansion of the infrequent antigen-specific T cells was required. Our strategy to solve this problem was to select antigen-specific cells after short-time stimulation and expand them nonspecifically afterwards (Fig. 1). For specific selection, we decided to enrich T cells that actively secrete IFN-γ by using an IFN-γ cytokine secretion assay [9].

The novel protocol was challenged by generating $CD4^+$ and $CD8^+$ lymphocytes specific for pp65, a known immunogenic matrix protein of HCMV. Generated T cell lines were checked for antigen-specific killing, proliferation and multiple epitope specificity. In addition, we compared the protocol with two alternative HLA type-independent T cell generation procedures, both based on repetitive antigen-specific stimulation with autologous, transgenic APC (Table 1) [10].

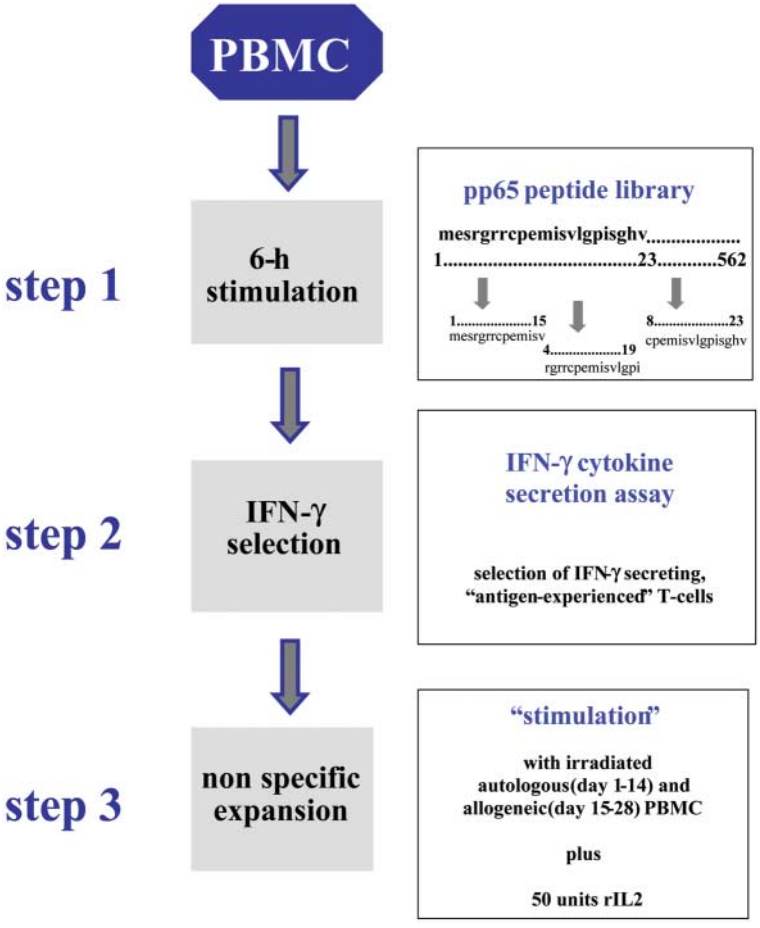

Fig. 1. T cell generation protocol. Schematic illustration of specific stimulation, magnetic cell selection and expansion of antigen-specific T cell lines. For short-time stimulation, overlapping peptide libraries were loaded on PBMC. Next, the specifically activated, IFN-γ-secreting cells were selected by an IFN-γ cytokine secretion assay. Finally, T cell expansion by weekly nonspecific stimulation was necessary to obtain sufficient cell numbers for adoptive immunotherapy.

Table 1. Experimental T cell generation protocols

Protocol	Stimulation	Selection	Expansion
I	pp65 peptide library	IFN-γ cytokine secretion assay	Nonspecific expansion (auto + allo PBMC)
II	pp65 peptide library	IFN-γ cytokine secretion assay	Specific expansion (pp65 mini-LCL)
III	pp65 mini-LCL	No selection step	Specific expansion (pp65 mini-LCL)

[a] PBMC were divided into three equal portions, one for each protocol

Results

Culture time and cell expansion rates

The expansion of autologous T cells with distinctive antigen specificity to numbers sufficient for clinical application is time and cost consuming. Therefore, cell numbers and culture time were closely monitored during the generation process. To allow pair-wise comparison, PBMC from each donor were divided into three equal fractions of 5×10^7 PBMC for T cell generation protocols I–III. Cell numbers were below 2×10^5 cells after elution of the enriched T cells from the magnetic column in all experiments. To allow the transfer of the protocol into the clinical setting, the expansion rate was calculated in relation to the donor's number of PBMC at culture start (Fig. 2a). Most efficient expansion was found after nonspecific expansion of IFN-

A

Volunteer	Protocol	Cell expansion: Cell number	Phenotype: CD4 (IFN-γ+)	CD8 (IFN-γ+)	CD16 (IFN-γ+)
1	I	3.11×10^9	62 (24.6)	18 (5.3)	7
	II	8.33×10^8	5 (0.6)	17 (2.1)	40
	III	2.25×10^8	1 (n.d.)	80 (8.8)	10
2	I	8.70×10^7	7 (3.3)	83 (49.2)	2
	II	8.00×10^7	1 (n.d.)	65 (34.3)	24
	III	1.40×10^9	8 (0.0)	60 (11.4)	24
3	I	2.08×10^8	12 (n.d.)	85 (n.d.)	0.1
	II	1.05×10^8	4 (n.d.)	87 (n.d.)	1.5
	III	2.92×10^7	7 (n.d.)	88 (n.d.)	0.6
4	I	2.20×10^9	92 (31.8)	12 (2.9)	0
	II	3.14×10^8	32 (9.1)	48 (2.4)	6
	III	9.30×10^7	4 (0.4)	94 (4.3)	1
5	I	3.29×10^8	5 (0.2)	93 (8.9)	1
	II	3.14×10^8	5 (0.0)	63 (3.8)	17
	III	4.00×10^7	2 (n.d.)	62 (5.1)	15
6	I	2.30×10^7	1 (n.d.)	93 (18.4)	0
	II	4.00×10^6	1 (n.d.)	63 (14.6)	10
	III	6.30×10^8	9 (3.2)	62 (8.3)	1
7	I	5.43×10^8	83 (n.d.)	10 (n.d.)	13
	II	1.72×10^8	37 (n.d.)	26 (n.d.)	42
	III	8.00×10^8	9 (n.d.)	80 (n.d.)	13
8	I	2.22×10^9	4 (0.2)	80 (8.5)	1
	II	5.00×10^8	4 (0.4)	95 (3.9)	1
	III	n.d.	n.d	n.d	n.d

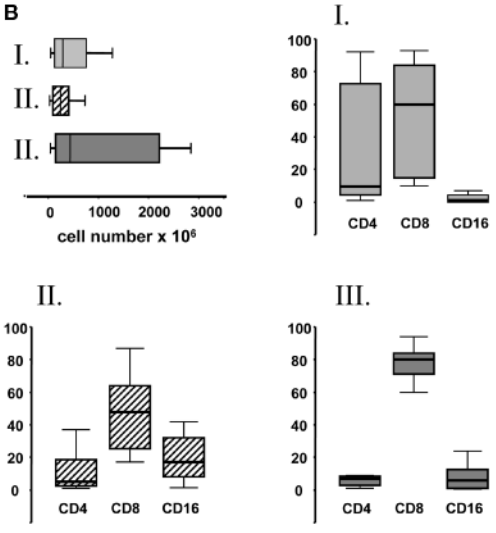

Fig. 2. (a) Cell expansion and phenotype. Pair-wise comparison of cell expansion and phenotype. Starting with an equal cell fraction of 5.0×10^7 PBMC per protocol (I–III), final cell numbers were counted after four stimulation steps. Phenotyping was done by fluorocytometric analysis. Cell lines were subdivided into $CD3^+/CD4^+$ (CD4), $CD3^+/CD8^+$ (CD8) and $CD3^-/CD16^+$ (CD16) populations. Intracellular staining measured the frequency of IFN-γ-positive cells; shown in brackets. Data are expressed in % of life gate cells. (b) Cell numbers and phenotypes are expressed as box plots. A comparison of cell lines generated by protocols I–III is shown.

γ-selected T cells (protocol I). Specific expansion of IFN-γ-selected cells with pp65 mini-lymphoblastoid cell lines (LCL) resulted in the lowest cell expansion (protocol II) whereas co-cultivation was in between (protocol III). Protocol III was not applied for donor 8, because stably growing autologous pp65 mini-LCL necessary for T cell expansion could not be established (Fig. 2a). Culture time took 25 days in protocol I, in opposite to an average of 66 days in protocols II and III. The delay of 41 days was based on the culture time needed to establish stably growing autologous pp65 mini-LCL required in protocols II and III.

Phenotype of expanded T cell lines

T cell phenotype was determined after four expansion steps. As expected, all generation protocols generated predominantly $CD3^+$ lymphocytes (Fig. 2). The ratio between $CD4^+$ and $CD8^+$ T lymphocytes was highly variable between different donors and protocols. The highest numbers of $CD4^+$ T cells were achieved in T cell lines generated with protocol I. Cell lines from three donors (nos. 1, 4, and 7) were predominantly $CD4^+$, with only 10–15% $CD8^+$ T lymphocytes. Although to a lesser extent, cell lines from the same three donors also showed increased $CD4^+$ T lymphocytes when generated with protocol II but not III. All T cell lines from the other donors consisted predominantly of $CD8^+$ T lymphocytes, independently of the generation protocol used. Surprisingly, high numbers of NK cells were found after specific expansion of IFN-γ-selected cells with transgenic LCL in protocol II (median 13.5%) and III (median 10.5%), whereas low numbers were present in protocol I (median 1%) (Fig. 2a, b). No significant differences in the $CD8^+/CD56^+$ population size were found (data not shown).

Antigen-specific T cell function

Cytotoxicity is the most important effector function of T cell lines generated for adoptive immunotherapy. T cells generated by nonspecific expansion of peptide library-stimulated and IFN-γ-selected cells (protocol I) showed pp65-specific responses in 7/8 cell lines. A mean killing rate of 57% (range 33–79%) was found at an effector-to-target cell ratio of 20:1 (Fig. 3a). Only one single cell line (no. 6) showed significant killing of autologous and allogeneic LCL, as defined by >20% cell lysis. However, autologous phytohemagglutinin (PHA) blasts were not killed, indicating that this cell line could attack transformed cell lines but not autologous primary cells. Since all T cell lines are checked for antigen specificity before adoptive immunotherapy, this cell line would be identified and excluded.

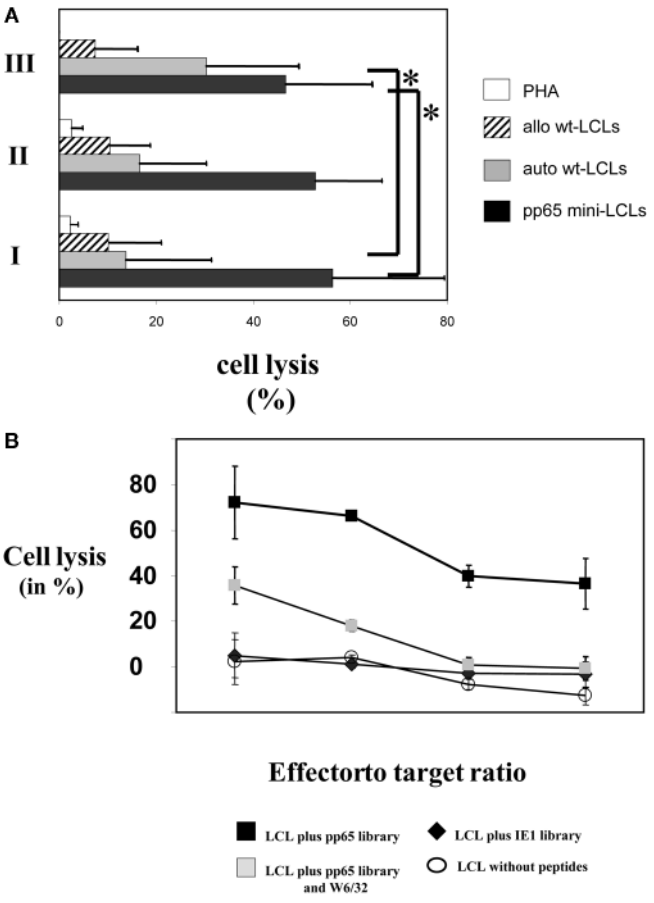

Fig. 3. pp65-specific cytotoxicity. (a) Cytotoxicity testing of T cell lines generated with protocols I–III. Autologous $pp65^+$ (pp65 mini-LCL), autologous $pp65^-$ (auto wt-LCL), allogeneic $pp65^-$ (allo wt-LCL) and autologous PHA blasts (PHA) were used as target cells. Cell lysis between different protocols (mean values) is compared at an effector-to-target cell ratio of 20:1. Error bars represent standard deviations for all generated cell lines classified into protocols I–III. In protocol I, the single nonspecific T cell line (1/8) is included, elevating the standard error in the wt- and allo wt-LCL group. Without this cell line, standard errors would be <10% in both groups. In protocol III, lysis of PHA blasts was ≤0%. *Pair-wise statistical analysis (Wilcox-Test) between I/II and I/III ($p<0.05$) is shown ($n=8$). (b) Cytotoxicity testing of T cell lines generated with protocol I against peptide-loaded autologous target cells. Autologous LCL were pulsed with pp65 and IE-1 peptide libraries ± MHC class I-neutralizing mAb W6/32. Cell lysis at different effector-to-target cell ratios is shown. Samples were run in triplicate. Error bars represent the standard deviation of the triplicates. One representative cell line (donor 5) is shown ($n=4$).

T cell lines generated according to protocols II and III also showed pp65-specific cytotoxicity with a mean killing of 52% (range: 29–71%) and 46% (range: 25–74%), respectively. Interestingly, the cell line from donor 6 also demonstrated significant killing of autologous and allogeneic LCL. Again, no lysis of autologous PHA blasts was detectable. T cell generation in protocol III was based on repetitive stimulation with autologous pp65 mini-LCL that also express latent EBV

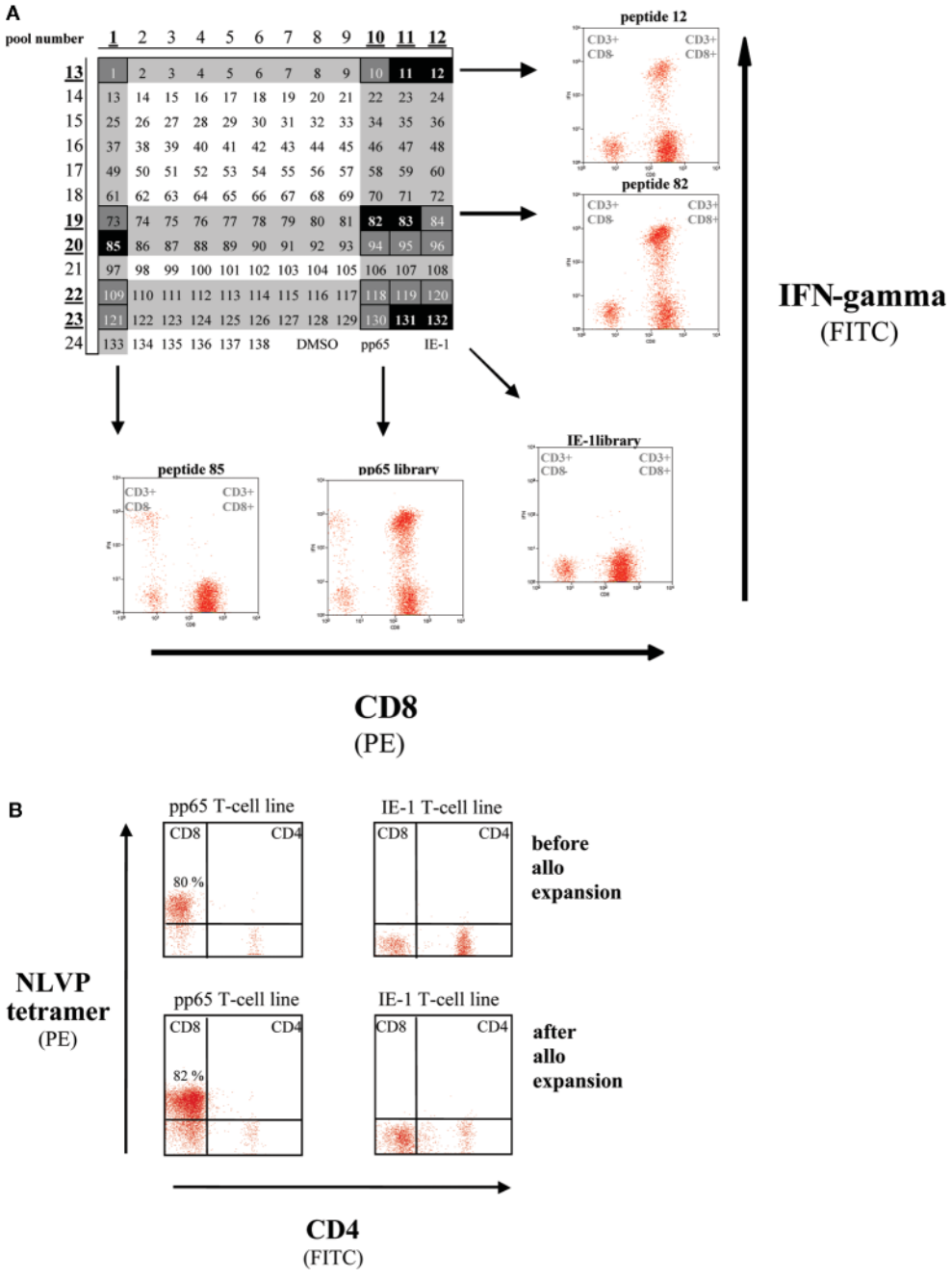

Fig. 4. Antigen specificity. (a) Epitope mapping was performed to confirm multiple target specificity. T cell lines generated by peptide library stimulation, IFN-γ selection and nonspecific expansion (protocol I) were stimulated with 24 peptide sub-pools and measured for intracellular IFN-γ responses. Positive pools are marked in light grey, epitope candidates in dark grey. Next, stimulation and IFN-γ staining was repeated with all single candidates. Single peptides that delivered IFN-γ^+ T cell responses are marked in black. The illustrated T cell line (donor 2) showed at least three $CD8^+$ responses against the following 15-aa peptides: GIHVRVSQPSLILVS/RVSQPSLILVSQYTP, TLGSDVEEDLTMTRN/DVEEDLTMTRNPQPF, KYGEFFWDANDIYRI/FFWDANDIYRIFAEL*; and one CD4 response against the 15-aa peptide TLGSDVEEDLTMTRN was found. Cell lines were also stimulated with the complete pp65 library, DMSO and a control peptide library (IE-1). One representative T cell line is shown (n=3). *Note that 11/15 aa were present twice in two consecutive overlapping peptides. (b) Epitope-specific T cells are monitored with A2 NLVP tetramers in two T cell lines from one HLA-A02 volunteer generated with pp65 and IE-1 peptide library stimulation. Dot blots illustrate the frequencies of NLVP tetramer$^+$ T cells before and after nonspecific expansion with allogeneic irradiated feeder cells. $CD8^+$ NLVP tetramer$^+$ cells are calculated in % of live gate cells.

antigens. The same EBV antigens were also present in wild-type (wt)-LCL, explaining the lysis of autologous LCL in protocol III (Fig. 3a).

In all cytotoxicity experiments described above, pp65 mini-LCL were used as pp65-expressing target cells. Mini-LCL resembled infected cells, because they endogenously process and present the viral pp65 protein. To exclude T cell responses against additional proteins of the vector construct, wt-LCL pulsed with pp65 peptide libraries were also used as targets. T cells generated with protocol I lysed pp65 peptide-pulsed targets, but not autologous cells pulsed with control libraries (IE-1). Blocking interactions between MHC and TCR revealed that the antigen-specific killing of $CD8^+$-dominated T cell lines was MHC class I restricted (Fig. 3b).

In addition to cytotoxicity testing, proliferation responses against different stimulator cells were determined. T cell lines generated with protocol I proliferated against autologous PBMC pulsed with pp65 but not with control peptide (IE-1) libraries. No alloantigen-driven proliferation was found, because T cell lines did not proliferate when stimulated with allogeneic PBMC. Even allogeneic PBMC from the same cell batch as had been used for nonspecific T cell expansion of the particular cell line during the generation process could not induce proliferation. In contrast to our selectively enriched T cell lines, unselected autologous PBMC from the same donors were able to proliferate against allogeneic stimulator cells (data not shown).

Multiple epitope specificity of generated T cell lines

Achieving specificity against multiple target epitopes and antigens could be a critical factor to avoid immune escape mechanisms by viruses and tumors. T cell lines generated with protocol I reacted against two, four and five different epitopes, confirmed by stimulation with the corresponding single peptides (Fig. 4a). Note that due to the design of the peptide library (15-aa peptides with 11 aa overlap) the 9-aa $CD8^+$ epitopes can be present in two consecutive 15-aa peptides. In parallel, epitope mapping was performed on the same donor's PBMC to evaluate the epitope specificities before T cell line generation. We found T cell responses against identical epitopes in two donors at the PBMC and T cell line level. In one donor, one initially weak epitope response ($<0.05\%$ of $CD3^+$ T cells) was lost during T cell production, whereas a second epitope answer was undetectable in PBMC before expansion, but present in the generated T cell line (data not shown).

To follow the fate of T cell responses against known epitopes through the entire generation process, we analyzed a defined answer against a common HLA-A2 epitope (**NLVP**MVATV). Of our donors, 3/8 were HLA-A02 positive, but specific binding of NLVP A02 tetramers was found in only one of them – a well-known phenomenon [7, 11]. Next, we generated a new T cell line from this donor by stimulating with our pp65 peptide library and NLVP peptides according to protocol I. In the multi-specific (pp65 peptide library) cell line, frequencies of T cells against the NLVP epitope accounted for 80% of the response, and the frequency did not change during the unspecific expansion procedure (Fig. 4b). In the monospecific cell line, generated by stimulation with NLVP peptides, frequencies of NLVP tetramer-positive T cells reached 95% throughout the whole process.

Discussion

An advantage of adoptive T cell therapy over vaccination strategies is the ability to influence and control T cell function and specificity during *in vitro* culturing. Repetitive stimulation with single 9-aa peptides pulsed on autologous APC is the most clear-cut procedure for generating antigen-specific, cytotoxic $CD8^+$ lymphocytes [7, 9]. Pure peptides pulsed on autologous APC do not raise critical biosafety and reliability questions and can readily be transferred into strict GMP production for clinical use. Major disadvantages of current peptide-based approaches arise from the restricted knowledge of immunodominant epitopes; a limitation also present in newer strategies using MHC tetramers or artificial APC [12, 13]. In most viral diseases, relevant epitopes are known for certain HLA types like HLA-A1, -A2 or -B7, but knowledge is limited for others. In our protocol, specific stimulation was provided by peptide libraries loaded on autologous PBMC. Peptide libraries are designed and synthesized solely on the basis of the protein's amino acid sequence and, theoretically, contain all T cell epitopes for all existing HLA types. Thus, in contrast to most peptide-based approaches, the protocol does not depend on previously identified epitopes. Furthermore, a library can be augmented by including sequences of identified viral or tumor mutations, allowing specific lymphocyte generation against mutated proteins. T cell generation under GMP conditions is a cost-intensive procedure. The novel strategy presented here relies on the stimulation with peptide libraries built of many peptides. The initial library synthesis is an expensive asset. But once established, many T cell lines for many patients can be produced, thereby cutting prices with every generated cell line. Li Pira and Rauser et al. generated pp65-specific T cells by repetitively stimulating them with a mixture of previously identified T cell epitopes. This strategy cuts the total number of peptides, but still depends on the knowledge of relevant epitopes [14, 15]. For single proteins like pp65, a variety of data

on relevant epitopes allow the design of a peptide mixture containing the most relevant candidates. For many other proteins, limited epitope information will not allow this strategy. The use of overlapping peptide libraries allowed us to generate T cell lines specific for proteins with restricted epitope knowledge, like the HCMV protein IE-1 or the polyoma virus protein VP-1 (data not shown).

After cell infusion, $CD4^+$ T cell help is thought to be critical for sustained cellular and possibly humoral immunity. Recent studies by Greenberg and colleagues showed that $CD4^+$ T cell help was necessary to achieve sustained survival and enhanced cytotoxic function of HCMV-specific $CD8^+$ T cells after adoptive transfer in man [16]. The generation of antigen-specific $CD4^+$ T lymphocytes depends on specific stimulation by MHC class II-presented antigens. For MHC class II presentation, current generation protocols work with viral/tumor lysates or recombinant proteins, which are taken up and processed by APC, preferentially DC [17, 18]. Beside serious biosafety questions raised by viral lysates, reliability is also critical, because APC function can differ between patients. In our previous work, we found that peptide libraries comprising 15-aa peptides successfully stimulated $CD4^+$ and $CD8^+$ T lymphocytes [7, 11]. In this report, we integrated peptide stimulation in a T cell generation process for antigen-specific $CD4^+$ and $CD8^+$ lymphocytes. Specific stimulation of $CD4^+$ lymphocytes is provided by peptides loaded on PBMC, circumventing the need for unsafe viral lysates or recombinant proteins used in most other approaches. The percentages of $CD4^+$ and $CD8^+$ lymphocytes in the generated T cell lines were highly variable. Because of short-time stimulation, the protocol is based on the expansion of the preexisting antigen-experienced T cells. Therefore, initial T cell response patterns determine the phenotype of the expanded cell lines. Similar variability was found in the peripheral blood of healthy volunteers and patients [7].

Under selective pressure exerted by adoptively transferred T cells, various immune escape mechanisms like viral mutation or antigen loss are described to limit therapeutic efficiency [19–22]. These mechanisms are less likely to occur after infusion of T cell lines that are able to recognize multiple epitopes or proteins. In most viral diseases, a patient's T cell responses are directed against different epitopes [7]. Remarkably, this naturally occurring multi-specificity was maintained by our protocol. Each tested T cell line generated with our novel protocol retained most of the previously identified epitope specificities. With such T cell lines, immune evasion by mutation of single epitopes could be avoided. However, clinical trials are required to determine the benefit of T cells with multiple target specificity compared to monospecific (single peptide) or tetramer approaches.

The danger of nonspecific adoptive T cell therapy became evident as severe graft-*vs.*-host diseases occurred in bone marrow-transplanted patients after receiving unselected donor lymphocyte infusions to treat infections or residual tumor mass [23]. Rooney et al. [24] were among the first to show that specifically expanded antigen-specific T cells restricted the immune response toward infected cells, and lacked harmful auto- and alloreactivity. Therefore, achievement of antigen specificity was a key demand in the development of our T cell generation protocol. To obtain antigen specificity, Trivedi et al. used overlapping peptide pools to generate pp65-specific T cells. Instead of positive selection, their protocol relied on repetitive stimulation of selected $CD3^+$ cells with peptide-pulsed, autologous DC [25]. Using autologous DC for repetitive stimulation of growing T cell cultures to numbers required in adoptive immunotherapy is problematic in the clinical situation. In addition, generation of DC will add additional time to the generation procedure. To optimize time and cost efficiency, the protocol described here is based on short-time stimulation of a patient's PBMC, followed by selection and nonspecific expansion to high numbers of antigen-specific T cells. Combining initial DC stimulation with nonspecific expansion is another possible option. However, recent work described the failure of inducing antigen-specific T cell lines by nonspecific expansion of T cells initially stimulated with pp65-transgenic DC [26]. In our protocol, nonspecific expansion of peptide library-stimulated and IFN-γ-selected T cells did not result in any loss of antigen specificity in terms of cytotoxicity and proliferation responses, most probably due to the enrichment of a highly specific T cell population. In addition, pp65-specific killing was similar to that demonstrated in T cell lines expanded by repetitive specific stimulation with pp65-transgenic APC. Comparable results were also described by Rauser et al. after expansion of selected pp65-specific T cells with autologous PBMC [15]. We also monitored antigen-specific T cells with MHC class I tetramers during the generation process. Interestingly, frequencies of tetramer-positive T cells were always higher than the numbers of IFN-γ producers (data not shown). These results question the usage of IFN-γ production after specific stimulation as single marker for T cell specificity. Despite the restricted IFN-γ production, T cell lines demonstrated high antigen-specific killing potential and proliferation. Further experiments analyzing the expression of the effector molecules perforin and the degranulation marker CD107a are in progress.

In summary, the presented T cell generation protocol combines the biosafety of peptide-based approaches with HLA type independence. The core of the protocol is the use of peptide libraries in combination with

immunomagnetic selection and nonspecific expansion. Peptide libraries are designed only on the basis of amino acid sequences of proteins. Neither knowledge of immunodominant epitopes, nor the presence of certain HLA types are needed to generate specific $CD8^+$ and $CD4^+$ lymphocytes for viral and possibly other pathogen/tumor proteins. The protocol may help to advance adoptive immunotherapy in the near future.

Material and methods

Generation of mini- and wt-LCL

The study was approved by local ethic authorities, and informed consent was obtained from all donors. Peripheral blood samples were donated by eight healthy CMV IgG-seropositive adult donors with random HLA types. wt-LCL were generated by infecting PBMC with concentrated supernatant of the marmoset cell line B95.8 [4]. pp65-transgenic mini-LCL were generated as recently described by our group [10]. Briefly, we constructed a pp65 mini-EBV plasmid that contained the necessary EBV genes to immortalize B cells *in vitro*. Infectious virions carrying pp65 mini-EBV DNA were produced by transfecting the plasmid into a packaging cell line for EBV vectors. Supernatants were harvested and used to infect PBMC, yielding pp65 mini-LCL. All cell lines were cultured in complete medium (RPMI 1640-stable glutamine, 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin).

Peptide library stimulation, IFN-γ selection and T cell expansion

The amino acid sequence from the AD169 CMV strain was used to synthesize the pp65 library (Jerini Biotools, Berlin, Germany). Each consecutive 15-aa peptide shared an overlap of 11 aa with the previous one, thereby spanning the entire pp65 protein sequence. PBMC (1×10^8) were stimulated in complete medium containing the pp65 peptide library (1 μg/ml of each peptide; 138 15-aa peptides) for 6 h in a humidified incubator. Cells were harvested and the IFN-γ Cytokine Secretion Assay (Miltenyi Biotech, Bergisch-Gladbach, Germany) was performed according to the manufacturer's instructions. Enriched cells were washed twice and seeded in 96-well flat-bottom plates (2×10^4–5×10^4 cells per well) together with irradiated autologous PBMC (5×10^5 cells/well, 20 Gy) in protocol I and with pp65 mini-LCL (1×10^5 cells/well, 30 Gy) in protocol II. Cell suspensions were cultured in complete medium supplemented with rIL-2 (50 U/ml Proleukin; Chiron, Emeryville, CA). For expansion, T cells were stimulated weekly in IL-2-supplemented complete medium with irradiated autologous PBMC (2^{nd} stimulation, 1:4 ratio) and allogeneic PBMC (3^{rd} and 4^{th} stimulation, 1:4 ratio) in protocol I or with autologous pp65 mini-LCL (4:1 ratio) in protocol II. In protocol III, PBMC were co-cultured with autologous pp65 mini-LCL without IL-2 for the first 10 days and then expanded according to protocol II [10].

Surface and intracellular cytofluorometry (FACS)

Surface staining was performed with fluorescent dye-labeled mAb against CD3, CD4, CD8, CD16, CD45, CD56, and CD69 (Becton Dickinson). For intracellular IFN-γ staining, cells were stimulated for 6 h with complete peptide libraries or single peptides pulsed on autologous cells (PBMC/LCL) as described for peptide library stimulation. Brefeldin A was added for the last 4 h. After fixation and permeabilization using FixPerm kit (Becton Dickinson), intracellular staining was performed with FITC-labeled IFN-γ and IgG1 isotype control mAb (Becton Dickinson).

For epitope mapping experiments, T cell lines from three donors with different HLA types were stimulated with autologous cells loaded with different sub-pools and single peptides of the pp65 peptide library, as described [27]. The IFN-γ production of the specifically activated T cells was then analyzed by intracellular cytokine staining. Staining with A2-NLVP tetramer was performed according to the manufacturer's protocol (Immunomics; Beckmann Coulter, Marseille, France). Becton Dickinson's FACScan was used for cytofluorometric analysis, and data were analyzed with CellQuest (Becton Dickinson) and Cytomation Summit software (Cytomation, Inc., Fort Collins, CO).

Cytotoxicity assay and imaging experiments

For cytotoxicity testing, a modified Calcein-AM release assay was used [11]. Target cells were labeled with Calcein-AM (15 mM) according to the manufacturer's instructions (Molecular Probes, Eugene, OR). T cell lysis of the following targets was analyzed: (i) autologous pp65 mini-LCL, (ii) autologous wt-LCL, (iii) allogeneic wt-LCL (full MHC class I and II mismatch), (iv) autologous PHA blasts, (v) allogeneic PHA blasts, and (vi) autologous wt-LCL pulsed with 1 μg/ml pp65 or IE-1 peptide libraries ± 50 ng/ml of the MHC class I-neutralizing antibody W6/32. Targets were co-cultured (1×10^4 cells/well) with the generated T cell lines for 4 h at effector-to-target cell ratios of 40:1, 20:1, 10:1 and 5:1 in 96-well flat-bottom plates. Triplicates were used for all tested samples, including controls with media for spontaneous and Triton X-100 for maximum release. Lytic activity was calculated as follows: % specific cytotoxicity = (sample release – spontaneous release)/(maximum release – spontaneous release) ×100. For the assessment of proliferation responses, T cells (4×10^4/well) were co-cultured with irradiated autologous and allogeneic LCL (1×10^4/well) in complete media with or without pp65 peptide libraries (1 μg/ml pp65 or IE-1 peptide libraries) and cultured for 6 days. Proliferation was quantified by measuring [^{3}H]thymidine incorporation for the last 16 h.

Statistic analysis

Statistic analysis was done with SPSS software (SPSS Inc., Chicago, IL). Because three T cell lines (protocol I–III) were generated for each donor, pair-wise analysis of these cell lines was performed by using the non-parametric Wilcoxon test. Differences with p values <0.05 were considered significant.

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