

# Peptide microarrays for the profiling of cytotoxic T-lymphocyte activity using minimum numbers of cells

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**Abstract** The identification of epitopes that elicit cytotoxic T-lymphocyte activity is a prerequisite for the development of cancer-specific immunotherapies. However, especially the parallel characterization of several epitopes is limited by the availability of T cells. Microarrays have enabled an unprecedented miniaturization and

parallelization in biological assays. Here, we developed peptide microarrays for the detection of CTL activity. MHC class I-binding peptide epitopes were pipetted onto polymer-coated glass slides. Target cells, loaded with the cell-impermeant dye calcein, were incubated on these arrays, followed by incubation with antigen-expanded CTLs. Cytotoxic activity was detected by release of calcein and detachment of target cells. With only 200,000 cells per microarray, CTLs could be detected at a frequency of 0.5% corresponding to 1,000 antigen-specific T cells. Target cells and CTLs only settled on peptide spots enabling a clear separation of individual epitopes. Even though no physical boundaries were present between the individual spots, peptide loading only occurred locally and cytolytic activity was confined to the spots carrying the specific epitope. The peptide microarrays provide a robust platform that implements the whole process from antigen presentation to the detection of CTL activity in a miniaturized format. The method surpasses all established methods in the minimum numbers of cells required. With antigen uptake occurring on the microarray, further applications are foreseen in the testing of antigen precursors that require uptake and processing prior to presentation.

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## Introduction

The initiation and enhancement of cancer-specific CTL responses hold great promise in the development of effective novel cancer therapies [1–3]. In combination with agents intended to promote the induction of immunity such as adjuvants or dendritic cells, antigens are administered

that, in the ideal case, are specific to the tumor cells. The administration of HLA class I-restricted antigenic peptides corresponding to cancer-related epitopes for recognition by CTL is one potential strategy for tumor vaccination [4]. In the immunostimulatory context, these antigens then activate T cells that finally enter the periphery of the body and target the tumor. The complexity of the molecular and cellular mechanisms involved in antigen processing, presentation and recognition renders this approach highly vulnerable to failure. Concomitant with the development of immunotherapeutic protocols, there is therefore an urgent need for efficient approaches to identify the functionally most relevant peptide epitopes and monitor the presence of antigen-specific T cells in a patient [5].

At present, various functional assays exist to quantitate and characterize antigen-specific T-cell reactivities. Enzyme-linked immunospot (ELISpot) [6], intracellular cytokine staining (ICS) [7] as well as quantitative reverse transcription polymerase chain reaction (qRT-PCR) [8] represent functional T-cell assays that analyze antigen-induced cytokine profiles. ELISpot provides the best characterized and most robust assay with a sensitivity of down to 1:100,000 to detect a tumor antigen-specific T-cell response [9]. In contrast to the functional assays, HLA multimer (tetramer)-based assays yield quantitative, but not functional data by staining epitope-specific T-cell receptors with an intermediate detection limit. However, for each T-cell epitope, an individual tetramer has to be produced and characterized, making labor-intensive controls necessary [5].

For the characterization of T cells, current data support the use of a functional assay such as the ELISpot or ICS in conjunction with a phenotyping assay like HLA multimers [9]. However, in the clinical situation the sampling of blood is restricted and most T-cell assays are highly cell consuming, thus limiting extensive immune monitoring. Similarly, for epitope identification, it is highly advantageous to employ T cells that have undergone a minimum number of expansion cycles.

In the analysis of gene expression, microarrays have revolutionized the way experiments are conducted with respect to miniaturization and parallelization. Given the minimum of biological material required, also attempts have been made to exploit the benefits of microarrays for cellular assays including the detection of MHC-specific T-cell responses. In one case, tetramers of MHC-peptide complexes were spotted and the antigen-specific T cells detected by cell capture on the spots and activation of calcium signaling [10]. In a modification of this approach, capture antibodies for cytokines were co-immobilized with the MHC-peptide complexes. In this way, physiologically relevant T-cell responses could be detected [11, 12]. Nevertheless, the ultimate goal for the detection and characterization of CTL should be a miniaturized assay that

directly probes for cell killing. Moreover, MHC-peptide complexes and antibody molecules, once spotted on the array are prone to denaturation. In contrast, MHC-binding peptides are highly stable. Therefore, the spotting of peptides instead of protein complexes would be very advantageous.

Here, we show that the spotting of mixtures of MHC-binding peptide epitopes and polylysine on hydrogel-coated glass slides provides a powerful answer to these challenges. Target cells adhere to peptide spots only and presentation of the spotted peptides is confined to each spot. As a readout of cytolytic activity, cellular release of the fluorescent dye calcein [13] and detachment of cells are employed. Cytolytic activity could be detected down to 0.5% antigen-specific cytotoxic T cells corresponding to only 1,000 antigen-specific T cells on three peptide spots. Moreover, we could also detect the presence of different cytolytic activities in parallel, thereby enabling a profiling of T-cell activities.

## Materials and methods

### Cells and peptides

The MUC-1- and CMV-negative and HLA-A\*0201-positive [14] human melanoma cell line SK-Mel-24 [15] was a kind gift from P. Walden, Charité, Berlin. CMV- and MUC-1-specific CTLs were isolated and expanded as described below. All peptides were kindly provided by S. Stevanović (Department of Immunology, Interfaculty Institute for Cell Biology, University of Tübingen, Germany). The following HLA-A\*0201-binding peptides were used: the human cytomegalovirus-derived epitope HCMV pp65<sub>495-503</sub> (NLVPMVATV) (protein accession number (swissprot) P06725) [16] and the RNA-dependent helicase-derived peptide DDX17<sub>146-154</sub> (p68) (Q92841) (YLLPAIVHI) [17]. The mucin (MUC)-1 derived epitope M1.2 (P15941) [18] was modified in anchor position 12 (L to K) to enhance HLA-A\*0201 binding (KLLLTVLTV).

### Antigen-presenting cells (APCs):

DCs and CD40L-activated B-cell lines

Monocyte-derived DCs were generated as previously described [19] and activated with 10 ng/ml tumor necrosis factor- $\alpha$ , 10 ng/ml interleukin (IL)-1 $\beta$ , 1,000 U/ml IL-6 (all R&D Systems) and 1  $\mu$ g/ml prostaglandin E2 (Calbiochem, La Jolla, CA, USA) for 24 h. DCs were characterized by CD83 and CD80 expression and analyzed by flow cytometry. The culture of B cells was performed as described by Schultze et al. [20], who provided the cell line t-CD154 (NIH-3T3 cells expressing human CD154). After 3 weeks, the population contained predominantly B cells (>90%

CD19<sup>+</sup> cells). APCs were washed twice with serum-free X-Vivo 15 medium (Biowhittaker) and loaded for 1–2 h with 5 µg/ml of the HLA-A\*0201-binding epitopes of interest. In 24-well plates (Nunc),  $1 \times 10^5$  effector cells were cocultured with antigen-pulsed  $\gamma$ -irradiated (32 Gray) APCs.

#### In vitro priming and expansion of peptide-specific T cells

Healthy donors were selected on the basis of HLA-A\*02 antigen expression as well as CMV seropositivity. Blood samples were obtained after written informed consent from the Department of Transfusion Medicine, University of Tübingen, Germany. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation. PBMCs were cocultured with autologous peptide-pulsed dendritic cells (DCs) at a ratio of 10:1 for 7 days followed by repeated stimulation cycles using peptide-pulsed CD40L-activated B cells (ratio: 4:1) in T-cell medium [20, 21]. Initially, cultures contained IL-12 p70 (5 ng/ml, PromoKine, Heidelberg, Germany) substituted by IL-7 (10 ng/ml, R&D Systems) during restimulation. Expansion of T cells was supported by addition of IL-2 (Chiron, Emeryville, CA, USA). After three to four rounds of stimulations, the cultures consisted predominantly of CD8<sup>+</sup> T cells. CD8<sup>+</sup> T cells were magnetically enriched by negative selection using a CD8 T-cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Enrichment was always >80%. Preparation of antigen-presenting cells (APCs) was performed as described in the supplements.

#### Cytotoxic T-cell assay

The cytolytic activities of T-cell lines were tested in a standardized chromium release assay. Specific lysis was calculated according to the following formula:

$$\% \text{ Lysis} = \left( \frac{[\text{experimental cpm} - \text{spontaneous cpm}]}{[\text{maximal cpm} - \text{spontaneous cpm}]} \right) \times 100.$$

Here, peptide-pulsed T2 cells, a transporter associated with antigen presentation-deficient HLA-A\*0201<sup>+</sup> cell line (American Type Cell Collection (ATCC), CRL-No.: 1992, Rockville, MD, USA), were used as target cells. T2 cells were pulsed for 1–2 h with 5 µg/ml peptides in serum-free media.

#### HLA-peptide monomers and flow cytometry

Biotinylated recombinant HLA-A\*0201-molecules and fluorescent HLA-tetramers were produced as described

previously [22]. Briefly, fluorescent tetramers were generated by incubating biotinylated HLA-monomers with streptavidin-PE or streptavidin-APC (Molecular Probes, Leiden, Netherlands) at a 4:1 M ratio. FACS analyses were performed with tetramer-PE/APC plus antibody CD8-PerCP (BD Bioscience, Heidelberg, Germany). Cells were incubated with antibodies at 4°C for 20 min in the dark, followed by 30 min incubation with fluorescent HLA-tetramers at the same conditions. Cells were analyzed on a four-color FACSCalibur cytometer (BD).

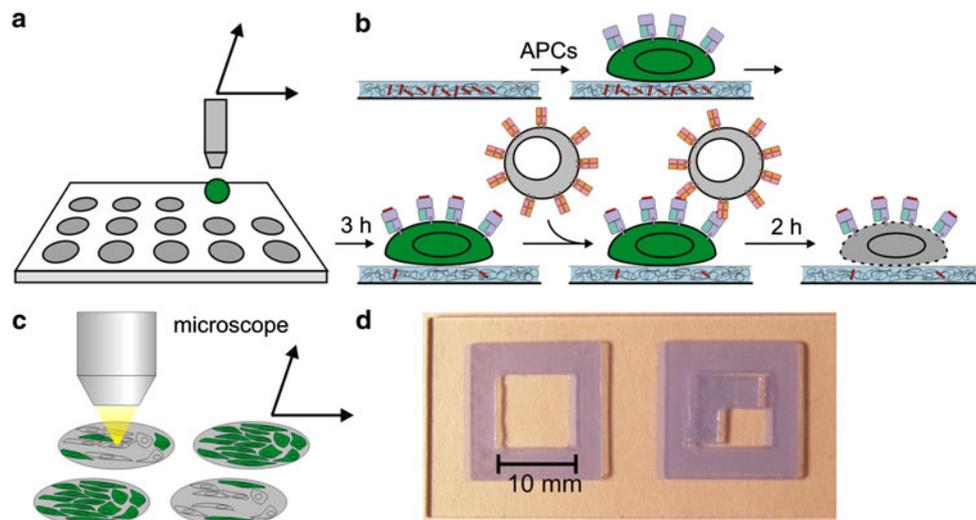
#### Preparation of microarrays

Hydrogel-coated glass slides were from Perkin Elmer (Jügesheim, Germany). Peptide microarrays were generated using a piezo-driven contact-free nanopipettor (Nanoplotter 2.0, GeSiM, Grosserkmannsdorf, Germany). Working dilutions of 1 mg/ml peptide stock solutions were prepared immediately before use, according to the following procedure: peptide stock solutions were placed in an ultrasonic bath at room temperature for 3 min, diluted to 60 µM in PBS containing 0.01% poly-L-lysine 70,000–150,000 g/mol (Sigma–Aldrich), mixed on a vortexer, placed in an ultrasound bath for 1 min and spun down. Into a 384-well plate, 30 µl of this peptide solution was transferred and used immediately for the production of peptide microarrays with the nanopipettor. Hydrogel slides were placed on a plate cooled to dew point (15°C) using a Polystat CC3 (Peter Huber Kältemaschinenbau GmbH, Offenburg, Germany). For each spot, a total volume of 1.2 nl was pipetted. The microarrays were dried at room temperature and used within 2 days after preparation. The peptide spots had a diameter of 200–300 µm and a center-to-center distance of 450 µm.

#### Microarray experiments

For the generation of incubation chambers, 10 × 10 mm GeneFrames (Peqlab, Erlangen, Germany) were taped on hydrogel-coated slides (Fig. 1). By taping a quarter corner of a GeneFrame into the first GeneFrame, 4 × 4 mm incubation chambers for 6 × 6 peptide arrays were obtained. One hydrogel-coated slide contained up to three such incubation chambers in parallel. The slide was then placed into a covered 24 cm sterile Petri dish containing wet filter papers placed to avoid dry fall of the arrays during incubation. Prior to the incubation with cells, the arrays were washed 2 × 1 min and 1 × 10 min with 50 µl PBS at room temperature to remove excessive peptide from the spots. Care was taken to avoid drying of the array.

SK-Mel-24 cells were harvested by trypsination using 0.05%/0.02% Trypsin/EDTA solution for 3 min at 37°C,



**Fig. 1** Detection of CTL activity using peptide microarrays. **a** Peptide microarrays are generated by spotting of mixtures of the peptide and poly-L-lysine on hydrogel-coated glass slides. **b** Target cells, loaded with the cell tracer calcein, only settle on peptide spots and locally take up and present the peptide. CTL activity is detected by

detachment of targets and calcein release using a confocal microscope (**c**). **d** Incubation chambers on hydrogel-coated slides. Smaller incubation chambers of a quarter of the original GeneFrame were obtained by taping an edge of another GeneFrame into the original incubation chamber

washed with medium, adjusted to  $1 \times 10^6$  cells/ml and stained with  $0.1 \mu\text{M}$  calcein-AM (Merck Biosciences, Darmstadt, Germany) in medium for 20 min at room temperature, washed again and re-adjusted to  $1 \times 10^6$  cells/ml. In  $25 \mu\text{l}$  medium,  $2.5 \times 10^4$  SK-Mel-24 was applied as targets to a  $4 \times 4$  mm array and incubated for 3 h at  $37^\circ\text{C}$ . Cells that had not attached at the end of this period were removed by careful washing of the array.

Before addition of T cells, the array was imaged by scanning in a mosaic-like fashion using an LSM510 laser scanning microscope equipped with a  $10\times 0.3$  NA objective. Detection was non-confocal. Fluorescence was excited using the 488 nm line of an argon-ion laser and detected using an LP505 long pass filter. T cells were washed once with fresh, preheated ( $37^\circ\text{C}$ ) T-cell medium (RPMI-1640, 10% HS, Pen/Strep,  $200 \mu\text{M}$  L-glutamine) and adjusted to a density of  $8 \times 10^6$  cells/ml. To each  $4 \times 4$  mm array,  $2 \times 10^5$  peptide-expanded,  $\text{CD8}^+$ -sorted T cells were added and incubated for 2 h at  $37^\circ\text{C}/5\%\text{CO}_2$ . Arrays were imaged every 30 min to determine time-dependent loss of target cells. After 2 h, the array was washed with warm X-vivo-15 medium to remove lost cells and imaged again to determine total cell loss. Image processing was carried out with Image Pro4.5.1 (MediaCybernetics, Silver Spring, USA) as described in the supplements.

#### Image processing

Imaged processing was performed with the confocal images of calcein fluorescence. Fluorescence background between cells was removed by multiplication with a binary

mask that was created from a copy of the original image using an intensity threshold so that only signals of cells were included as valid pixels. Holes in this mask were closed using morphological filters. Spot size, spot fluorescence intensity and cell count were measured on a spot-by-spot basis. Data were exported into Excel 2003 (Microsoft) for further data analysis. The fractions of the spot area, spot intensity and cell count after the end of the experiment were related on a spot-by-spot basis to the values obtained prior to incubation with T cells.

#### Results

##### Detection of cytotoxic activity in the microarray format

We had observed previously that peptides, immobilized non-covalently on the surface of a microarray substrate, may be transferred locally onto cells contacting the surface [23]. The use of hydrogel-coated slides in combination with low concentrations of polylysine in the spotting buffer of the peptide solution confined the cell attachment to the peptide spots. The co-administration of poly-L-lysine rendered the cell attachment independent from the physicochemical characteristics of the peptide. A hydrogel is a polymer network with the ability to take up large amounts of water. In this case, a polyacrylamide-based hydrogel was employed [24]. In the dry state, this gel had a thickness of  $4 \mu\text{m}$ . According to the manufacturer's specifications, in the hydrated state the gel swelled to up to  $20 \mu\text{m}$ .

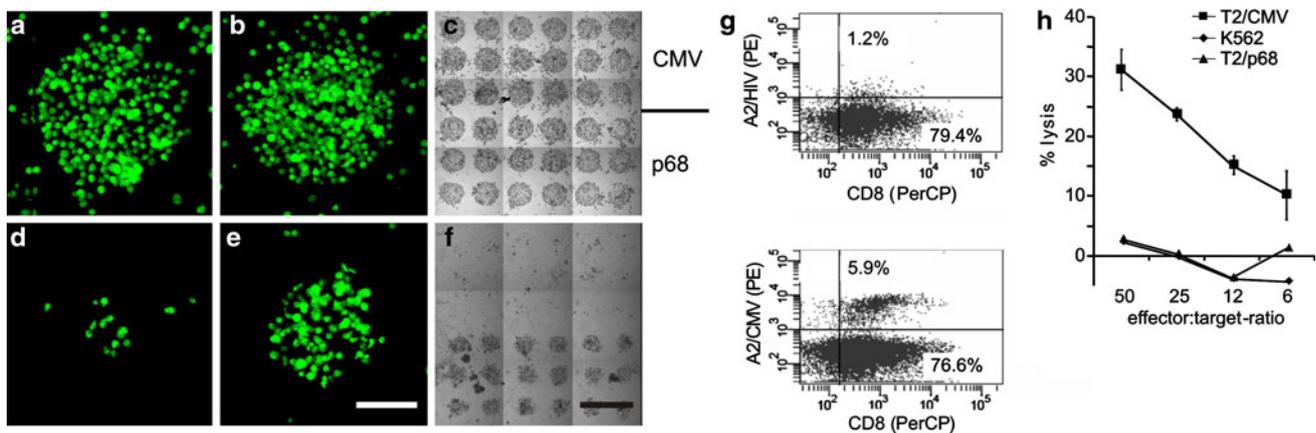
For the parallel testing of several peptides, peptide spots were pipetted in the form of a regular microarray. As much as 1.2 nl of peptide solution was pipetted for each spot. Peptides were spotted from a 60  $\mu\text{M}$  solution. In related experiments, this concentration had provided the best results for local T-cell activation without detectable cross-contamination between individual spots (results not shown). The peptide spots had a diameter of about 200–300  $\mu\text{m}$ . With a center-to-center spacing of 450  $\mu\text{m}$ , 36 spots could be tested on a surface of only  $4 \times 4$  mm. Incubation chambers corresponding to the size of the peptide microarray were created by using commercially available self-adhesive frames that are typically employed for the generation of incubation chambers for the hybridization of oligonucleotides (Fig. 1).

As a representative for an epitope overexpressed on tumor cells, a human mucin-1 (MUC-1)-derived peptide was employed [18]. MUC-1 is a membrane-associated glycoprotein that is overexpressed on epithelial tumors such as breast and ovarian cancer and might prove as a target for humoral and cellular immune responses [25]. One of the predominant viral antigens is the lower matrix 65-kDa phosphoprotein (pp65) of cytomegalovirus (CMV). CTLs may readily be generated from the blood of seropositive individuals by the use of antigen-presenting cells pulsed with defined virus epitopes such as HLA-A\*0201-restricted pp65<sub>495–503</sub> [16]. Finally, the RNA-dependent helicase-derived peptide DDX17<sub>146–154</sub> (p68) represents a

well-defined self ligand with high affinity to HLA-A\*0201 [17]. Because of the lack of corresponding T cells, p68 is commonly used as a negative control in several immunodiagnostic assays.

As target cells, adherently growing SK-Mel-24 cells were chosen [26]. This melanoma-derived cell line is positive for HLA-A\*0201 and negative for the antigens that we had selected as test antigens. Adherently growing cells were preferred over suspension cells. Even though the time for peptide loading was not sufficiently long to allow for spreading of the cells on the surface, also after careful washing, the SK-Mel-24 cells completely covered the spots (100–150 cells/spots). In comparison, suspension cells such as B cells were largely washed off the surface. SK-Mel-24 cells were loaded with the membrane-permeable acetoxymethyl ester (AM) of the fluorescent cell tracer calcein. Inside the cell, this ester is rapidly hydrolyzed rendering the dye membrane impermeable. Calcein release upon CTL-induced membrane damage had been used as a fluorescence-based alternative to the chromium-release assay previously [13].

In a first experiment, we were interested to learn whether using the microarray format and calcein-based detection of membrane damage, CTL activity could be specifically detected. For this purpose, on a  $6 \times 6$  spot array, three rows each of the CMV-derived epitope and the p68 peptide were spotted (Fig. 2). After a 3 h incubation period with SK-Mel-24 cells, CMV antigen-expanded,



**Fig. 2** Detection of CTL activity on cellular peptide microarrays. SK-Mel-24 cells, serving as targets, were stained with 0.1  $\mu\text{M}$  calcein (AM) and incubated at a density of  $2.5 \times 10^4$  cells for 3 h on a  $6 \times 6$  spot array containing 18 spots each of the CMV and of a p68 control. After careful washing to remove loose cells, incubation for 2 h with  $2 \times 10^5$  CMV-expanded CD8<sup>+</sup> T cells and a further washing step images were recorded. **a, b, d, e** Representative spots for CMV (**a, d**) and p68 (**b, e**) presenting targets before (**a, b**) and after (**d, e**) incubation with CMV-expanded T cells. **c, f** Overview of the cellular peptide microarray before (**c**) and after (**f**) incubation with CMV-expanded CTL. The scale bar in **e** corresponds to 100  $\mu\text{m}$ , the one in **f** to 400  $\mu\text{m}$ . **g, h** Characterization of CMV-reactive CTLs by standard

<sup>51</sup>Cr release assays. PBMCs of the same HLA-A\*0201-positive human donor were stimulated with CMV peptide loaded (pp65<sub>495–503</sub>) autologous APCs. After four rounds of stimulations, T cells were tested by **g** tetramer staining as well as by **h** <sup>51</sup>Cr release assay. **g** Double staining using anti-CD8-PerCP together with PE-labeled A2/CMV tetramers show 5.9% T cells. Staining with an irrelevant tetramer (A2/HIV) revealed 1.2% background staining. **h** Cytolytic activity was analyzed in a standard 4 h <sup>51</sup>Cr release assay against HLA-A\*0201+T2 cells loaded with CMV peptides (filled square), T2 cells loaded with p68 as negative control (filled triangle) or K562 cells (filled diamond) to show NK cell dependent lysis

CD8<sup>+</sup>-sorted T cells with a fraction of antigen-specific cells of 6%, corresponding to 25% antigen-specific killing in a <sup>51</sup>Cr release assay, were added. The efficiency of antigen presentation and T-cell activation was time dependent. A maximum was reached after 2–3 h (data not shown).

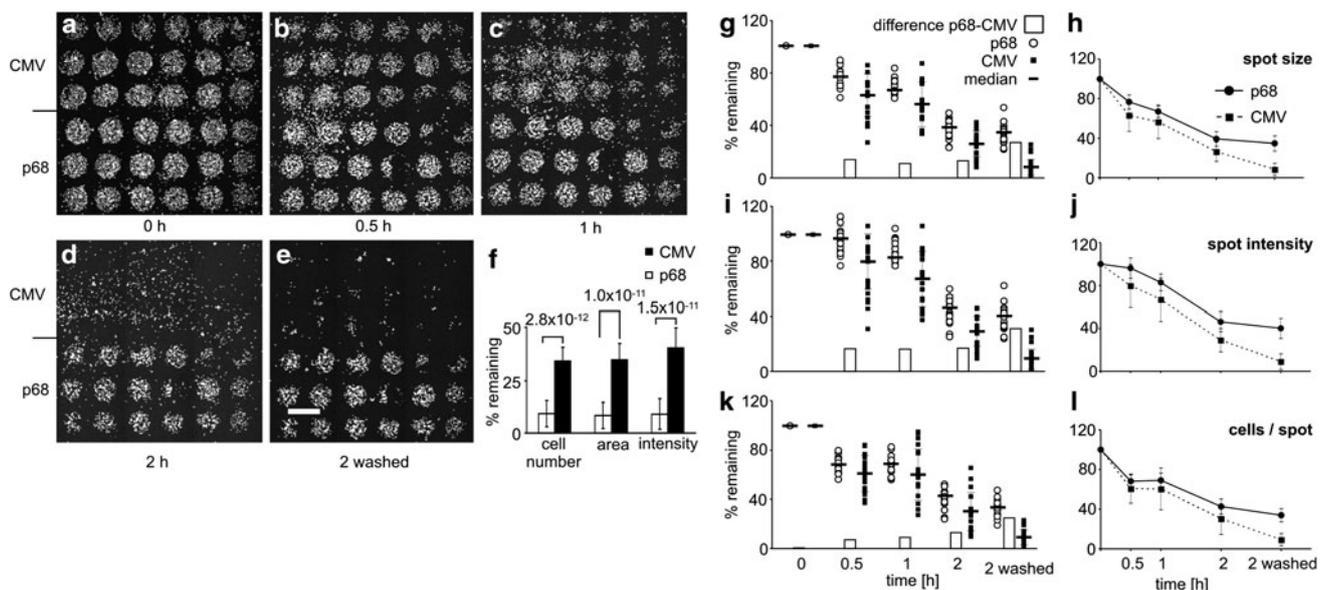
T cells were added at a density of  $2 \times 10^5$  cells on a  $6 \times 6$  cellular peptide microarray. This relatively high density enhanced the chances of an encounter of antigen-specific T cells with target cells on a cellular peptide spot displaying its specific antigen. After 2 h and a brief washing of the array, inspection by fluorescence and transmission light microscopy revealed a complete loss of targets on all CMV spots. In contrast, on the p68 spots, a major part of the cells and of the fluorescence was still left. This result demonstrates that on the hydrogel-coated slide, the peptides were present in a form competent for binding to MHC molecules. Though one may assume that a significant part of the peptides had been released into the medium, on the spot cells were exposed to a depot of the peptide at a higher active concentration. If peptides had been released into the medium before binding to the MHC molecules, cytolytic activity would not be confined to the spots bearing the CMV epitope. A parallel negative control of targets on a peptide array without T cells was used as control for cell loss over time. As much as 20% of cells were lost in a 2-h incubation period. Moreover, we were concerned by a possible influence of the calcein stain on

cytolytic activity. A reduction in cytolytic activity was observed when target cells were incubated with the dye at a concentration of 1  $\mu$ M. At a concentration of 0.1  $\mu$ M, no such effect could be detected (not shown).

#### Time dependence of cytolytic activity

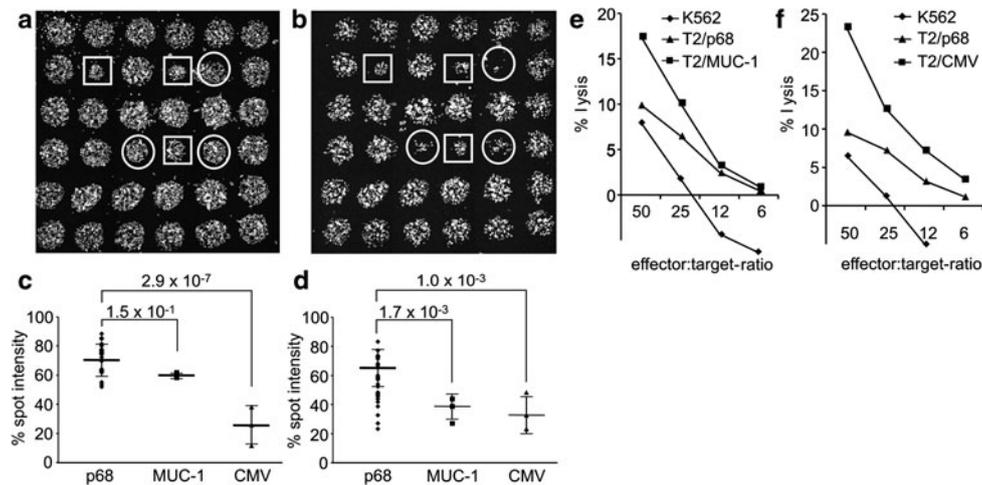
Next, we were interested to follow the cytolytic activity over time. Furthermore, we were interested in comparing quantitatively the lysis of cells on epitopes recognized by CTLs and controls. Thus, three rows of the CMV-derived and the p68-derived epitopes were spotted followed by incubation with calcein-loaded targets for peptide presentation. Then CTLs were added and the microarrays imaged over time (Fig. 3). For the CMV spots, there was a dispersion of cells already after 30 min. At 2 h after addition of CTLs, cells were completely dispersed. For the p68 controls, only 3 out of 18 spots had lost a major part of their cells. A brief washing of the array removed nearly all cells from the CMV spots. In contrast, for the p68 spots, except for three, the cell layers were still present.

The quantitative analysis of cell death proceeded on the basis of calcein fluorescence. Three parameters were considered: The spot size (cells on the spot), the total intensity of all cells of a spot and the cell count per spot relative to the respective original value. For both, the CMV spot as well as the p68 controls, the average of all three values decreased over time (Fig. 3h, j, l). However, for the CMV



**Fig. 3** Time course of cytolytic activity of CMV-expanded CD8<sup>+</sup> T cells on a cellular peptide microarray. **a–e** Time series of the cytolytic activity of CMV-expanded T cells against calcein-loaded SK-Mel-24 cells incubated on CMV and p68 peptide spots for 3 h. The array was scanned at the indicated time points and after a final washing step. The scale bar corresponds to 500  $\mu$ m. **f–l** Quantification of cytotoxic

activity; **f** averages of all spots after 2 h and washing. Error bars correspond to the standard deviations of all spots. Significance was determined by a paired Student's *t* test. **g–l** Time course of (**g, h**) spot size, (**i, j**) spot intensity and (**k, l**) cell numbers per spot relative to the initial value for or each individual spot (**g, i, k**) and the average of all spots (**h, j, l**)



**Fig. 4** Parallel detection of different CTL activities on a cellular peptide microarray. CMV and MUC-1-expanded CD8<sup>+</sup> T cells were added to SK-Mel-24 cells that had been incubated on a microarray containing three CMV- (squares) and MUC-1 (circles) peptide spots surrounded by spots of p68 control peptide for 3 h. Images of the array before (a) and after (b) 2 h incubation including washing with  $2 \times 10^5$  CMV-expanded T cells. **c, d** Fluorescence intensities of spots on arrays incubated (c) with  $2 \times 10^5$  CMV-expanded cells (5% tetramer positive cells, 10,000 cells), and (d) a mixture of  $1 \times 10^5$  CMV- and  $1 \times 10^5$  MUC-1-expanded CTL (2.5 and 0.5% tetramer positive T cells (corresponding to 5,000 and 1,000 antigen-

specific T cells, respectively). *P* values were determined by a paired Student's *t* test. **e, f** Characterization of CMV- and MUC-1-reactive CTLs by standard <sup>51</sup>Cr release assays. PBMCs of an HLA-A\*02-positive human donor were stimulated with either (e) MUC-1-peptide (M1.2mod) or (f) CMV peptide (pp65<sub>495-503</sub>) loaded to autologous APCs. After three rounds of stimulations, T cells were tested in a standard 4 h <sup>51</sup>Cr-release assay against HLA-A\*0201-positive T2 cells loaded with the respective antigenic peptides (filled square), T2 cells loaded with p68 as negative control (filled triangle) or K562 cells (filled diamond) to show NK cell-dependent lysis

spot, this decrease was stronger than for p68. In this case, the loss of signal from p68 spots was very likely due to some higher background killing by the CTL, as indicated by a standard <sup>51</sup>Cr release assay (Fig. 4f). For all three parameters, there was a constant difference over the time course of the experiment. When instead of the averages the values for individual spots were considered (Fig. 3g, i, k), it was apparent that for CMV the individual values varied much stronger than for the control peptide. For the control, there was a homogenous decrease for all three parameters over the time course of the experiment. The increased variation of the CMV spots was due to the fact that for some of the spots, detached cells were still present above the spot area. After washing, the values for the CMV and p68 spots clearly differed from one another. For only 7 out of 36 spots did the tested parameters overlap. All three parameters performed comparably well, with the straightforward assessment of total spot intensity yielding a slightly better separation of control versus epitope than the other two parameters.

#### Simultaneous detection of T-cell epitopes

After having established a quantitative protocol for the detection of cytolytic activity using the peptide microarrays, finally, we were interested to simultaneously detect different CTL activities and evaluate the detection limit.

Next to the CMV epitope, a breast cancer-associated MUC-1 epitope was included on the array (Fig. 4). In this case, instead of splitting the whole array into two different areas, the epitope spots were distributed over the whole array. In this way, we further addressed whether specific killing remained restricted to the epitope spots. In each case, the arrays were incubated with  $2 \times 10^5$  CD8<sup>+</sup> T cells. If only CMV-expanded CTL were present at a frequency of 5%, corresponding to 10,000 cells, killing was restricted to targets seeded on CMV spots. In contrast, as soon as MUC-1-specific CTL were added, killing was also observed for cells on MUC-1 spots. Specific killing was observed for as little as 0.5% epitope-specific CTL, as quantified by tetramer staining. It is noticeable that the presence of MUC-1-expanded cells led to more variation of remaining cells on the p68 spots. This observation may be explained by the fact that also in the standard <sup>51</sup>Cr release assay, the MUC-1-expanded CTL showed more unspecific killing of p68-loaded cells than the CMV-expanded cells.

#### Discussion

Our results demonstrate a full reconstitution of antigen presentation, recognition and cytolytic activity in a microarray-based format that only requires about 6 h for the total protocol and operates with an unprecedented low

number of cells. The simultaneous detection of two different T-cell activities illustrates that induction of antigen presentation and killing was not a serendipitous finding for the CMV peptide, but instead a principle that readily applies to other MHC class I-restricted epitopes.

On first sight, the use of peptides instead of peptide–MHC complexes as demonstrated in prior approaches [10–12] may be considered an enormous increase in the complexity of the assay. Using peptide microarrays, peptide uptake and presentation also have to occur on the array. This initial step requires the incubation of the array with target cells prior to the application of T cells. Due to the absence of physical boundaries between individual peptide spots, cross-contamination during antigen uptake therefore has to be considered. For RNA and small molecules, a localized transfer of molecules onto cells had been shown [27, 28]. However, for peptides such an application has not been published. The chemical stability and ease of synthesis of large collections of synthetic peptides [29] in comparison to the generation of MHC–peptide complexes constitutes a major advancement over published microarray-based strategies for the monitoring of T-cell activities.

Moreover, with respect to the monitoring of CTL responses, the detection of the actual cytolytic activity should be preferred over the detection of cytokines as surrogate markers. In pathologic situations such as chronic viral infection or cancer, many CTL, in spite of expressing cytokines, lack cytolytic activity [30–32]. Using a viral and a tumor-derived HLA-A\*0201-restricted epitope, the killing by antigen-expanded CTL was directly visualized on the array.

By performing standard  $^{51}\text{Cr}$  release assays on the same T-cell populations that were used for the microarray experiments, we confirmed that killing was MHC class I and peptide restricted. No killing was observed for K562 cells confirming the absence of NK cell-mediated lysis. In addition, we would like to point out that due to the presence of cells presenting specific and control epitopes side by side, the microarray-based format inherently controls for the restriction of killing for specific MHC–peptide complexes.

Currently, we foresee the major application of the peptide microarrays in epitope identification. In comparison to the identification of T-cell frequencies in patient samples, in epitope identification constraints on detection sensitivity are less severe. Still, these assays will benefit enormously from approaches that require rather little expansion of T cells from patient isolates and enable the acquisition of T-cell response profiles by parallel testing of several epitopes.

Nowadays, candidate tumor antigen-derived peptides for cancer vaccination may be predicted by computational algorithms [33] or by sequencing of naturally presented peptides eluted from HLAs of tumor tissues [34]. In

addition, epitope mapping of cancer and pathogen-associated proteins is a commonly employed method for the identification of relevant CTL epitopes [35, 36]. All methods critically depend on the methods for a functional validation of CTL activity. In the development of cancer vaccines, a direct testing of cytolytic activity, as provided by our arrays, represents the most relevant readout.

In summary, our peptide microarray-based approach implements the whole process of antigen presentation and recognition in a peptide microarray format. Currently, with respect to sensitivity, ELISpot remains the gold standard with a sensitivity of 1:50,000 to 1:100,000. However, this assay does not enable a detection of cytolytic activity. To our knowledge, there is currently no method that can functionally detect cytolytic activity with fewer cells than our microarrays. For the characterization of candidate epitopes for peptide-based cancer vaccines and ultimately also for monitoring of vaccination success in CTL from patients, our microarray-based method therefore enables functional studies that were not possible before.

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