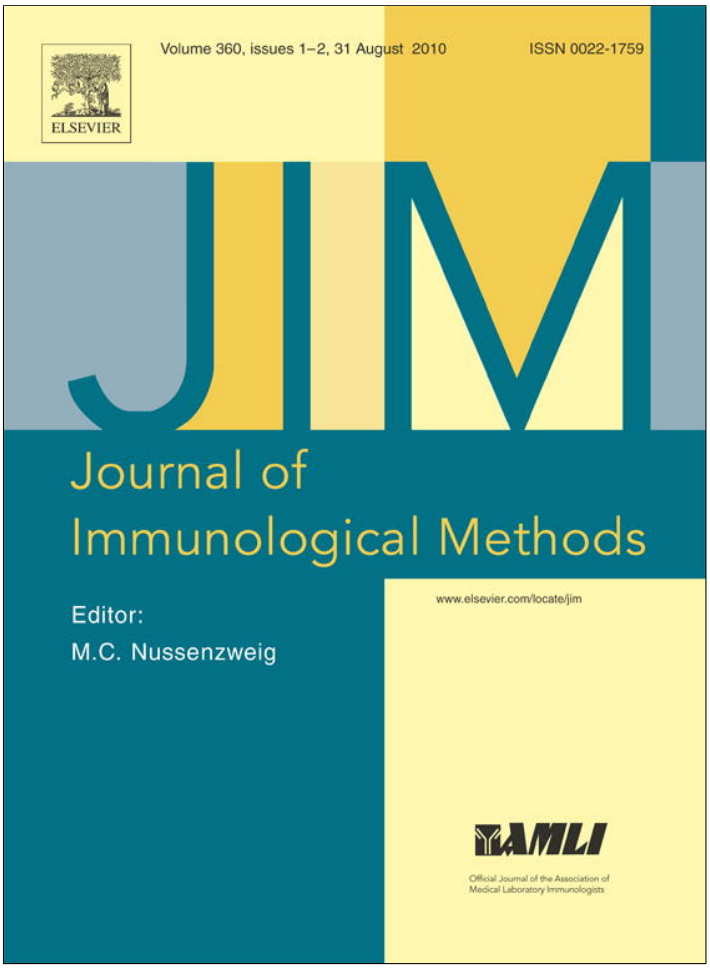

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Research paper

A flow cytometry-based assay to assess minute frequencies of CD8$^+$ T cells by their cytolytic function

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ABSTRACT

Limited sample size and low sensitivity of currently used functional assays challenge direct analysis of cytotoxic CD8$^+$ T lymphocyte activity to quantify antigen-specific immunity after infection or vaccination. Our flow cytometry-based assay reproducibly detects at least three epitope-specific CD8$^+$ T lymphocytes by their cytolytic function. As exemplified for viral epitopes restricted to the human leukocyte antigen (HLA)-A2, the HLA-A2$^+$ human somatic cell hybrid T2 provided an about 10-fold more sensitive readout as compared to autologous B-lymphoblastoid cells or the human erythroleukemia cell line K562 transfected to express HLA-A2 when used as target cells. We named our assay VITAL-FR assay, referring to Hermans et al. (2004) and indicating the modification of using Far Red (FR) dye instead of CMTMR. Under optimal conditions the VITAL-FR assay proved 30 times more sensitive than the ^{51}chromium-release assay to assess epitope-specific target cell lysis. The high overall sensitivity of the VITAL-FR assay basically depended on the negligible spectral overlap of the emission of a stable Far Red fluorescent reporter with the green tracer for target cell labelling. It also profited from long co-incubation of effector and target cells of up to 72 h, from prior *in-vitro* culture increasing the frequency of epitope-specific CD8$^+$ T cells and from generic, easily accessible standardized target cells that were used with only 10^3 specific and 10^3 control target cells per individual experimental reaction. Our functional approach with the VITAL-FR assay therefore ideally suits for monitoring CD8$^+$ T cell-mediated cytotoxicity in e.g. vaccination studies with known MHC-restricted immunogenic peptides in scientific and diagnostic applications.

1. Introduction

Identification and characterisation of disease-associated specific CD8$^+$ cytotoxic T lymphocytes (CTL) and antigenic peptide epitopes involved are central aims in T cell immunology. Once induced, these lymphocytes represent the most important quickly recallable defence against viral infections. Quantifying these cells and assessing their functionality is of paramount importance for specific immune therapies or for monitoring the immune status. Frequencies of epitope-specific CTL vary from percentages to parts per million therefore assays to detect CTL function have to be highly sensitive (Kather et al., 2003). The classical ^{51}chromium-release assay directly measures the extent of effector cell-mediated lysis that correlates to the release of target cell-bound radioactivity (Brunner et al., 1968). Necessity to work with radioactive material and previous time-consuming expansion of epitope-specific CTL populations to large numbers limits the CRA for routine clinical

Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; CMV, cytomegalovirus; CRA, chromium release assay; CTL, cytotoxic CD8$^+$ T cell; ELISpot, enzyme-linked immunosorbent spot; EBV, Epstein–Barr virus; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; HPV, human papilloma virus; IMP, influenza matrix protein; IL, interleukin; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; pp65, phosphoprotein 65.

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use especially for epitopes with low immunogenicity (Michel et al., 2002). A non-radioactive variant assessing released lactate dehydrogenase from lysed cells by conversion of a chromogenic substrate requires even more effector CTL for proper control (Korzeniewski et al., 1983). Interferon-γ expression is regarded a surrogate marker for CTL function (Ghanekar et al., 2001). Enzyme-linked immunosorbent spot (ELISpot) assay determines granzyme or cytokine secretion of individual cells but does not discriminate for the producing cell type in mixed cell populations (Shafer-Weaver et al., 2003; Schoenborn and Wilson, 2007; Zaritskaya et al., 2009). Intracellular cytokine staining for flow cytometry also monitors surrogate parameters instead of direct CTL function, but allows evaluation of additional specific markers e.g. the degranulation marker CD107a to further define the CTL properties and frequencies (Betts et al., 2003).

Flow cytometry-based assay systems were developed in an effort to overcome the limitations of the other experimental methods to assess CTL frequency and function (Ritchie et al., 2000; Hermans et al., 2004; Jedema et al., 2004; Abate et al., 2005; Stambas et al., 2007). As for the VITAL assay specific target cells labelled with fluorescent chloromethyl derivatives are exogenously loaded with defined immunogenic synthetic peptides to be presented in the context of major histocompatibility complex (MHC) molecules. Control target cells are labelled with an orange-red fluorescent dye and a peptide known to be non-immunogenic against the same MHC background. In co-cultures with CTL and peptide-loaded target cells, lysis can be determined by the ratio of the remaining viable control and target cells, which are quantified by flow cytometry after a certain incubation time of 4–24 h (Kienzle et al., 2002; Hermans et al., 2004). The VITAL assay uses the chloromethyl-benzoylamino tetramethylrhodamine derivative CMTMR for one of the target cell populations (Hermans et al., 2004). The mildly thiol-reactive dye reacts with intracellular components and establishes fluorescent cells.

We here aimed to provide an even more sensitive and flexible flow cytometry-based *in-vitro* assay for clinically relevant specific CTL function. In a model with strong viral antigenic peptides and well described associations with the human leukocyte antigen (HLA)-A2 we established an assay we called VITAL-FR assay as an extension to the assay according to Hermans et al. (2004). We studied the effect of prior short term HLA/peptide-specific CTL activation, alternative fluorescent cell dyes with optimized emission wavelength for discrimination by flow cytometry and with low toxicity, as well as generic target cell types with defined HLA-class I variants on the specificity and sensitivity for detection of epitope-specific CTL activity. Under optimal conditions, by the VITAL-FR assay we were able to significantly detect cytotoxicity caused by approximately three specific CTL.

2. Materials and methods

2.1. Cell lines and primary cells

The human erythroleukemia cell line K562 (DSMZ ACC 10) and the monkey lymphocyte cell line B95-8 (DSMZ ACC 100) releasing high titers of Epstein–Barr virus (EBV) were obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany). The HLA-A2$^+$ T2 human–human somatic cell hybrid (ATCC CRL-1992) was from the American Type Culture Collection (Bethesda, MD, USA). Peripheral blood mononuclear cells (PBMC) were prepared from buffy coats of HLA-A2$^+$ healthy donors (Deutsches Rotes Kreuz, Berlin, Germany) by Ficoll density gradient centrifugation ($\rho = 1.078$ g/ml; GE Healthcare, Freiburg, Germany). Autologous B-lymphoblastoid cell lines (B-LCL) were established as described earlier (Pelloquin et al., 1986). In brief, 2×10^6 freshly prepared PBMC were cultured in 1 ml standard culture medium consisting of RPMI1640 and 10% fetal bovine serum supplemented with 1 μg/ml of cyclosporin A, and 1 ml culture supernatant of B95-8 cells. Weekly, 1 ml culture supernatant was replaced by 1 ml fresh standard medium with cyclosporin A. Proliferating cells were stained with a CD19-specific monoclonal antibody (clone HIB19; BD Biosciences, Heidelberg, Germany) and assessed by flow cytometry (FACS-Calibur with the Cell Quest Pro software; BD Biosciences). When 98% of the cells were CD19$^+$, B-LCL were used as autologous target cell populations. Cell morphology was monitored by phase contrast microscopy (Zeiss, Oberkochen, Germany). If not noted otherwise, cell culture reagents were from Invitrogen (Karlsruhe, Germany). Chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany) or Merck (Darmstadt, Germany) and were of the highest purity available.

2.2. Generation of K562 cells stably expressing HLA-A2

Recombinant constitutive expression of HLA-A2 by K562 cells allows for presentation of HLA-A2-restricted peptides (Britten et al., 2002). In brief, complete cDNA of the heavy chain of the human HLA-A*0201 allele (NCBI accession AY365426) was a kind gift of Dr. Wolfgang Herr (University Medical Center, Mainz, Germany). The sequence was cloned into the pVITRO2-mcs plasmid (Invivogen, San Diego, CA, USA) under the control of the ferritin promoter. K562 cells transfected by electroporation with the plasmid to ectopically express HLA-A*0201 were incubated with 200 μg/ml Hygromycin to select for stable transfectants. A HLA-A2-specific monoclonal antibody (clone BB7.2; Parham and Brodsky, 1981) coupled to pan mouse anti-human immunoglobulin G paramagnetic beads (BD Biosciences) was used to enrich by magnet-activated cell sorting for HLA-A*0201 expression. About 95% of the cells of this population were HLA-A2$^+$ and the cell line now called K562-A2 was routinely maintained in standard culture medium, and was used as target cells for peptide presentation to CTL.

2.3. Generation of epitope-specific T cell lines

CD8$^+$ T cells specific for immunodominant epitopes of the cytomegalovirus (CMV)-derived phosphoprotein 65 (NCBI accession ACN52454.1) CMV pp65$_{495–503}$ (NLVPMVATV), the polyprotein from the human immunodeficiency virus (HIV; NCBI accession NC001802) HIV pol$_{510–518}$ (ILKEPVHGV), or from the matrix protein-M1 of the influenza virus A (IMP M1; NCBI accession M63527) IMP$_{58–66}$ (GILGFVFTL) were expanded from PBMC. Corresponding synthetic peptides were all from Biosyntan (Berlin, Germany). Autologous dendritic cells were obtained from the adherent PBMC and differentiated for 4 days in the presence of 50 ng/ml granulocyte-macrophage colony-

stimulating factor and 1000 U/ml interleukin (IL)-4. Dendritic cells were pulsed in serum free CellGrow DC medium (CellGenix, Freiburg, Germany) with 10 μg/ml specific peptide for 3 h at 37 °C and washed thoroughly. Co-cultures of 1×10^7 PBMC and 5×10^5 peptide-loaded autologous dendritic cells were set up in 6-well tissue culture plates. In some experiments cultures with dendritic cells without exogenous peptide served as controls. Weekly, cultures were restimulated with fresh dendritic cell preparations loaded with the respective specific peptide as described above. After 14 days, cells were stained with a CD8-specific monoclonal antibody (clone RPA-T8; BD Biosciences) and HLA-A2/CMV $pp65_{495-503}$ or HLA-A2/IMP_{58-66} tetramers. In these cultures, frequencies of HLA-A2 tetramer$^+$/CD8$^+$ T cells were in the range of 1.2–9.8% and these cell lines were used as effector cells.

2.4. ^{51}Chromium-release assay

The CRA measures the lysis of radioactively labelled target cells by natural killer cells or CTL by quantifying released radioactivity in culture supernatants (Brunner et al., 1968). In brief, $1–2\times10^6$ target cells in fetal bovine serum were loaded with 300 mCi $Na_2[^{51}Cr]O_4$ (Amersham, Freiburg, Germany) for 2 h at 37 °C. Target cells (5×10^3) mixed with titrated numbers of effector T cells in 96-well V-bottom plates were incubated for 4 h at 37 °C. Supernatants were harvested by means of a Skatron system (Skatron, Lier, Norway) and counted in a gamma counter (LKB Wallac, Bromma, Sweden). Percentages of specific ^{51}chromium-release were calculated using the formula: percentage of specific release = (ER − SR) × 100/(MR − SR), where ER was experimental ^{51}chromium release, SR the spontaneous ^{51}chromium release as measured in the supernatant of 5×10^3 target cells cultured in medium alone, and MR the maximum release after the addition of 100 μl 1% Triton X-100 (Sigma). SR had to be lower than 20% MR for results to be included into the final analysis.

2.5. VITAL-FR assay

Target cells (1×10^6) were incubated with 10 μM carboxyfluorescein succinimidyl ester (CFSE) or 5 μM Far Red dimethyldodecylamine oxide-succinimidyl ester (Far Red; both from Invitrogen) for 5 min in RPMI 1640 at 37 °C. The reaction was terminated by addition of 20% fetal bovine serum and cells were thoroughly washed with standard culture medium. Cells stained with CFSE were incubated with 10 μg/ml of either IMP_{58-66}, CMV $pp65_{495-503}$, or with HIV $pol_{510-518}$ in RPMI supplemented with 3% FBS and were thoroughly washed before being used as specific target cells. Far Red-stained cells were loaded with 10 μg/ml of either the human papillomavirus-derived HLA-A2-restricted peptide HPV16 $E7_{11-20}$ (NCBI accession PPH16; YMLDLQPETT; Biosyntan) or HIV $pol_{510-518}$ as control peptides. Effector T cells were titrated in 96-well V-bottom plates and 1×10^3 CFSE-labelled specific peptide-loaded and 1×10^3 Far Red-labelled control peptide-loaded target cells were added. Wells containing the target cells only served as a control. Final volumes were 200 μl of standard culture medium supplemented with 10 IU/ml IL-2. Cultures were incubated at 37 °C and resuspended by pipetting once every 24 h. After up to 72 h, all cells were collected and immediately assessed by flow cytometry (Fig. 1). The entire target cell population was defined by a live gate in a forward scatter/side scatter dot plot. Specific target cells were denoted by regions in Fl-1 (CFSE)/Fl-4 (Far Red) dot plots and detected and enumerated as specific target cells as CFSE$^+$ (R3) and control target cells as Far Red$^+$ (R2) as shown in Fig. 1. Non-fluorochrome labelled cells comprised the effector cell populations. Peptide-specific lysis was calculated from the ratio R3/R2 in cultures containing defined numbers (n) of effector T cells $(R3/R2)_n$ in comparison to control (co) wells without T cells $(R3/R2)_{co}$ using the formula: $100\% - [(R3/R2)_n/(R3/R2)_{co}]\times100\%$. Numbers of epitope-specific effector T cells were calculated from the HLA-A2 tetramer$^+$/CD8$^+$ T cells determined one day prior to the setup of a VITAL-FR assay.

2.6. Statistical analysis

The extent of epitope-specific lysis was determined from the variation coefficients and lysis was considered significant when it was higher than the threefold standard deviation of the control wells with the target cells only. For statistical comparison, we used the SPSS software for Windows (version 15; SPSS, Chicago, IL, USA).

3. Results

3.1. Long-term co-incubation of effector and Far Red-labelled target cells determines the sensitivity of the VITAL-FR assay

We first assessed the kinetics of peptide-specific target cell lysis. To activate and enrich CTL specific for the HLA-A2 restricted peptide CMV $pp65_{495-503}$ PBMC (from CMV-tetramer screened donors) were restimulated once by peptide-pulsed autologous dendritic cells. Effector cell populations containing at least 2% HLA-A2/CMV $pp65_{495-503}$ tetramer$^+$ CTL were co-incubated with autologous B-LCL or with HLA-A2$^+$ T2 cells as target cells in VITAL-FR assays (Fig. 2). As with CFSE or CMTMR, staining with Far Red resulted in about 95% target cell viability prior to assay setup. While mean fluorescence intensities of CMTMR-labelled cells declined over time eventually overlapping with the negative control population, both CFSE and Far Red remained clearly separated (Supplementary Fig. 1).

Using autologous CMV $pp65_{495-503}$-loaded B-LCL providing a completely matched HLA haplotype of a given donor, 100 HLA-A2/CMV $pp65_{495-503}$ tetramer$^+$ effector cells relating to an effector:target ratio of 1:10 mediated about 20% peptide-specific lysis within 4 h (Fig. 2, left panel). Half-maximum lysis was reached after 12–14 h. A plateau level of about 90% maximal lysis of target cells that sustained for at least up to 72 h was reached after 24 h. While 30 peptide-specific effector T cells conferred comparable maximal lysis after 48 h, 10 CMV $pp65_{495-503}$-specific CTL were sufficient to reproducibly assess cytolytic function with autologous B-LCL target cells. In parallel experiments, T2 cells were tested as target cells again with CMV $pp65_{495-503}$ as specific peptide or HIV $pol_{510-518}$ as control peptide (Fig. 2, right panel). In contrast to B-LCL, within 4 h of incubation with 100 specific effector cells a half maximal specific lysis of T2 cells was reached. Kinetics using 30 HLA-A2/CMV $pp65_{495-503}$ tetramer$^+$ CTL and T2 as target cells compared to lysis of autologous B-LCL by 100 specific effector cells. Thus,

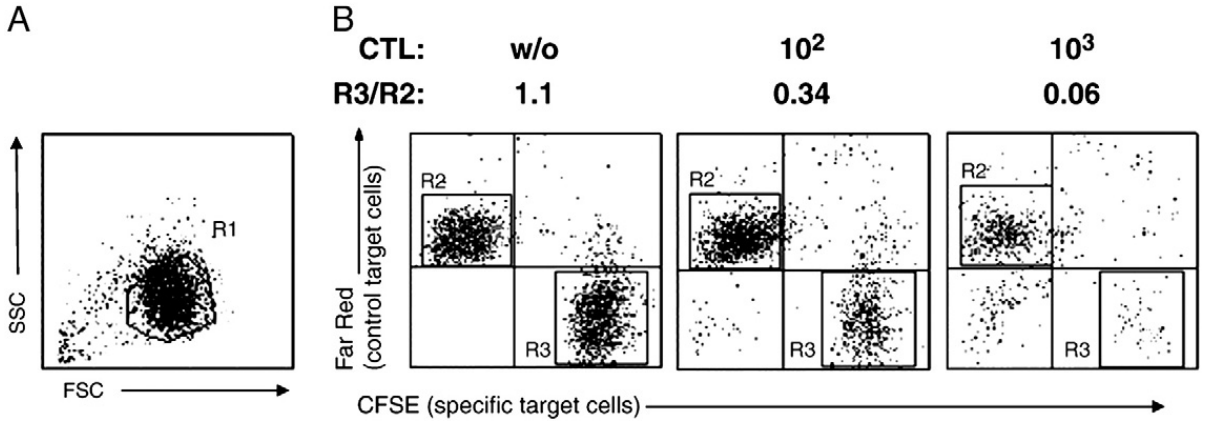

Fig. 1. Flow cytometric readout of target cell lysis by VITAL-FR assay. Specific target cells were stained with CFSE and loaded with an antigenic peptide. For control, cells of the same line were labelled with Far Red and loaded with the respective control peptide. Mixtures of 10^3 specific and 10^3 control target cells were incubated either alone or in the presence of titrated CTL numbers. After 72 h target cells were acquired in a live gate by flow cytometry (A). Ratios of Far Red$^+$ (R2) and CFSE$^+$ (R3) labelled target cell numbers were directly determined and their relative amount defined the lysis within individual cultures (B). Epitope-specific CTL-mediated target cell lysis was calculated in comparison to control cultures without CTL.

T2 cells were advantageous as target cells in this setting. Emphasizing the impact of the duration of the co-culture on the sensitivity of the VITAL-FR assay, as few as 1–3 CMV pp65$_{495–503}$-specific CTL reproducibly caused around 20% specific lysis of T2 target cells after 72 h.

3.2. The VITAL-FR assay is highly specific for activated CTL and for the cognate peptide epitope

During the incubation period of several days there is a potential risk for unspecific lysis or intra-assay activation of T cells. To exclude this we tested the peptide specificity of the VITAL-FR assay. We first compared freshly isolated PBMC and PBMC of the same donor that were stimulated twice with CMV pp65$_{495–503}$ peptide-loaded autologous dendritic cells. Frequencies of CMV pp65$_{495–503}$ specific CTL were 0.03% or 2.2%, respectively, according to HLA-A2 tetramer analysis. After 72 h of incubation in the VITAL-FR experiment, target cell ratios were measured and specific lysis was calculated (Fig. 3A). For wells containing about 300 restimulated effector cells, average specific target cell lysis was approximately 50%. About 1000 effector cells, corresponding to 20–30 HLA-A2/CMV pp65$_{495–503}$ tetramer$^+$ CTL conferred maximal lysis. In contrast, unstimulated PBMC of the same donor did not show lysis even with 3000 PBMC (corresponding to approximately 1 CMVpp65$_{495–503}$ tetramer$^+$ CTL *ex vivo*) per well, after 72 h incubation time. Therefore, unspecific lysis or intra-assay priming of specific CTL was not observed. Next we analyzed peptide-specific lysis. PBMC stimulated twice with autologous dendritic cells that were pulsed with the IMP$_{58–66}$ peptide were used as effector cells. These cell populations eventually contained 5.2% HLA-A2/IMP$_{58–66}$ tetramer$^+$ CTL. CFSE-labelled

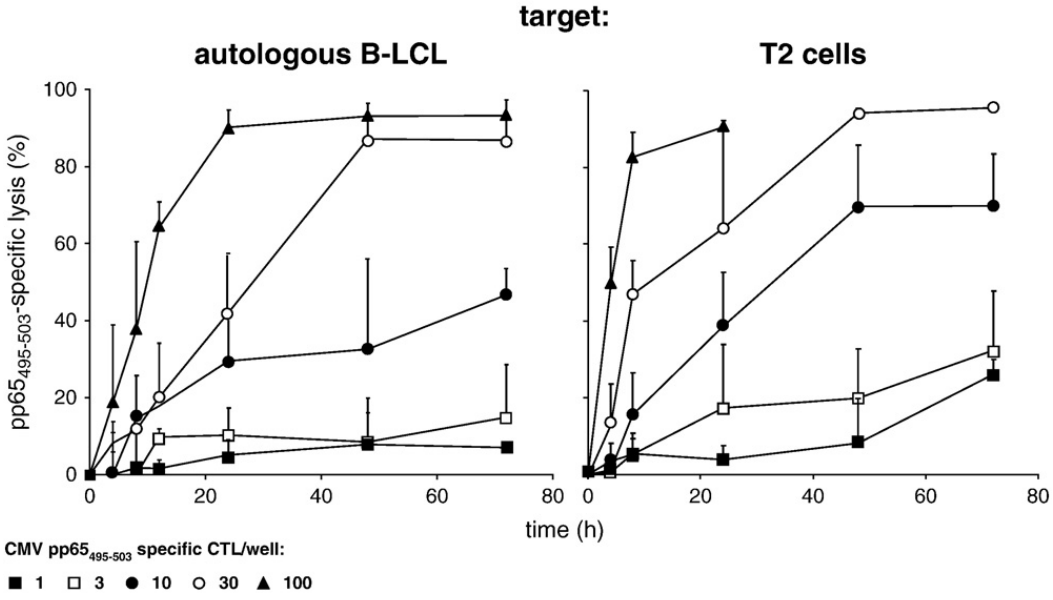

Fig. 2. Kinetic of specific lysis of autologous B-LCL and T2 target cells. Autologous B-LCL (A) or T2 cells (B) were labelled with CFSE or Far Red to be used as target or control cells, respectively. Cells were loaded with CMV pp65$_{495–503}$ as specific or HIV pol$_{510–518}$ as control peptide. Specific and control target cells were mixed with T cell lines containing the indicated numbers of HLA-A2/CMV pp65$_{495–503}$-tetramer$^+$ CD8$^+$ T cells. Target and effector cells were incubated for 4 h to 72 h and epitope-specific lysis was assessed by flow cytometry. Representative for three independent donors. Mean values ± SD of triplicate determinations.

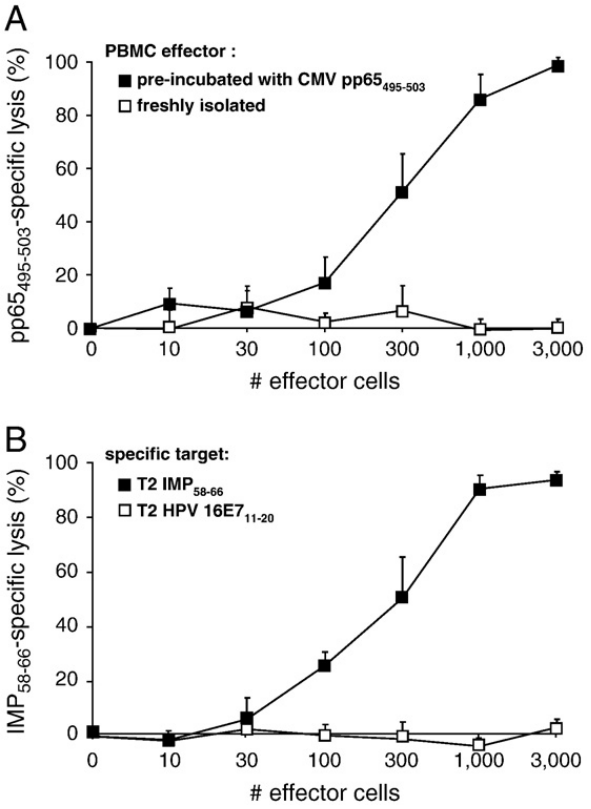

Fig. 3. Specificity of the VITAL-FR assay. (A) PBMC were sensitized for 14 days by autologous dendritic cells pulsed with the CMV $pp65_{495–503}$ peptide. T2 cells labelled with CFSE or Far Red were loaded with CMV $pp65_{495–503}$ as specific or HIV $pol_{510–518}$ as control peptide. (A) Pre-sensitized and freshly isolated PBMC of the same donor were used as effector T cell populations and results acquired after 72 h. (B) PBMC were pre-sensitized for 14 days with $IMP_{58–66}$ pulsed autologous dendritic cells. T2 target cells were labelled with CFSE and loaded with $IMP_{58–66}$ or with HPV16 $E7_{11–20}$ peptides. Far Red-stained control target cells were pulsed with the HIV $pol_{510–518}$ peptide. Specific lysis was determined by flow cytometry after 72 h. Representative of two independent experiments. Mean values ± SD of triplicate determinations.

T2 cells were loaded with the $IMP_{58–66}$ peptide, and Far Red-labelled T2 cells were loaded with HIV $pol_{510–518}$ and used in a VITAL-FR assay. About 100 prestimulated effector cells caused a distinct specific lysis of the peptide-matched target cells (Fig. 3B). To further control for epitope specificity, a second set of target cells using CFSE/HPV16 $E7_{11–20}$ as an unrelated epitope and Far Red/HIV $pol_{510–518}$ again as a control epitope was tested, with the same $IMP_{58–66}$-specific effector cell population. T2 cells loaded with the irrelevant HLA-A2-restricted peptide HPV16 $E7_{11–20}$ as potential target cells did not show any relative reduction to HIV $pol_{510–518}$ loaded T2 control target cells even with 3000 PBMC added. These findings confirmed the high sensitivity and specificity of the VITAL-FR assay even if using HLA-A2 matched allogeneic target cells.

3.3. Standardized HLA-A2$^+$ allogeneic target cells allow for highly sensitive, effector T cell dose-dependent assessment of CTL function

So far we had demonstrated suitability of autologous B-LCL and T2 cells as generic target cells in the VITAL-FR assay. Another potentially generic target cell type could be K562 cells transfected to express individual HLA alleles. Therefore we tested K562-A2 ectopically expressing HLA-A*0201 (Fig. 4A). Parental K562 and HLA-A2 transfected K562 cells were pulsed with the CMV $pp65_{495–503}$ peptide and incubated with a CMV $pp65_{495–503}$ peptide pre-activated T cell line having a tetramer-reactive CTL frequency of 7%. Peptide-loaded K562-A2 cells were significantly lysed by addition of 10 CMV $pp65_{495–503}$-specific CTL. Parental K562 used as control target cells were not lysed even with increasing CTL numbers demonstrating high specificity of the lytic activity for HLA restriction. This also demonstrates that activity of natural killer cells did not compromise the result of the VITAL-FR assay even after three days of incubation.

In order to validate the quality of autologous B-LCL, K562-A2, and T2 as target cells we directly compared their specific lysis in the presence of CMV $pp65_{495–503}$-prestimulated CTL in a 72 h assay (Fig. 4B). T2 cells were significantly better lysed at lower effector cell numbers than K562-A2 or B-LCL. In most

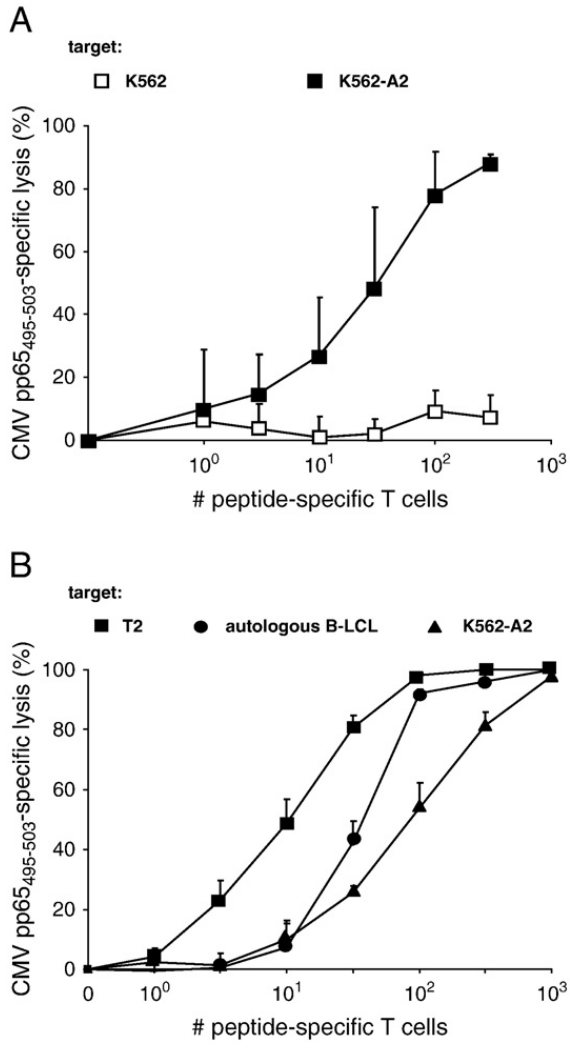

Fig. 4. Sensitivity of the VITAL-FR assay using autologous B-LCL, T2, and K562-A2 target cells. Specific target cells were labelled with CFSE and loaded with CMV $pp65_{495–503}$ while control target cells were labelled with Far Red and the HIV $pol_{510–518}$ peptide. Target cells and titrated numbers of effector cells were incubated for 72 h. (A) HLA negative K562 and HLA-A2$^+$ K562-A2 cells were used as target cells. Effector T cells contained 5.2% HLA-A2/CMV $pp65_{495–503}$ tetramer$^+$ CD8$^+$ T cells. (B) HLA-A2$^+$ K562-A2, B-LCL, and T2 cells were directly compared as target cells. Effector T cells contained 9.9% HLA-A2/CMV $pp65_{495–503}$ tetramer$^+$ CD8$^+$ T cells. One representative of four independent donors is shown. Mean values ± SD of triplicate determinations.

experiments significant lysis of K562-A2 and B-LCL was mediated by about 10–30 tetramer-reactive CTL, while only 2–3 CTL were needed for T2 target cells. Therefore, T2 cells and K562-A2 cells were suitable as generic target cells for quantification of HLA-A2-restricted peptide-specific CTL function. Using these cell lines may avoid time-consuming preparation of autologous B-LCL. In these experiments T2 cells, although allogeneic and lacking endogenous peptide processing again proved to be highly sensitive target cells for epitope-specific CTL-mediated lysis in the optimized VITAL-FR assay.

3.4. The VITAL-FR-assay is 10- to 30-fold more sensitive than the standard CRA in detection of peptide-specific CTL and requires 100-fold less effector cell numbers

In order to validate the sensitivity we directly compared the detection limits of the VITAL-FR assay with the standard CRA. Peptide-specific T cell lines previously stimulated with CMV pp65$_{495–503}$ or with IMP$_{58–66}$ were tested with T2 target cells in parallel in both assays. As low as 1–10 CTL specific for the respective peptide were sufficient to show significant CMV pp65$_{495–503}$- or IMP$_{58–66}$-specific lysis by the VITAL-FR assay, whereas 30–100 CTL were required for comparable lysis as detectable by CRA (Fig. 5A). Peptide-specific CTL numbers required to reach half maximal T2 target cell lysis were calculated for CRA and for VITAL-FR assays (Fig. 5B) at a median of 325 peptide-specific CTL required in CRA, while median numbers of only 7 specific CTL were required for VITAL-FR assay. This difference was statistically highly significant. Lower target cell numbers of 300 supported the sensitivity of the VITAL-FR assay (Supplementary Fig. 2). However for practical reasons the use of 1000 target cells proofed to be more reliable. Therefore, the high sensitivity of the VITAL-FR was caused by low numbers of target cells (Supplementary Fig. 2) in combination with prolonged assay incubation time. This allowed us to use 30–100 fold less effector cells to achieve comparable results as by standard CRA.

3.5. The standardized VITAL-FR assay reproducibly detects three peptide-specific, functional CTL

By limiting dilution analysis, we assessed the lowest number of peptide-specific CTL necessary to confer specific lysis as detected by our optimized VITAL-FR assay. PBMC were prestimulated in the presence of IMP$_{58–66}$ or CMV pp65$_{495–503}$ for 7 days and the proportion of CTL specific for the given peptide in the context of HLA-A2 was determined by tetramer staining (Table 1). Titrated numbers of these effector cells were used in VITAL-FR assays with HLA-A2$^+$ T2 cells as target cells. When the ratio R3/R2 between the median of the control samples and the experimental sample was higher than the threefold variation coefficient of the control samples,

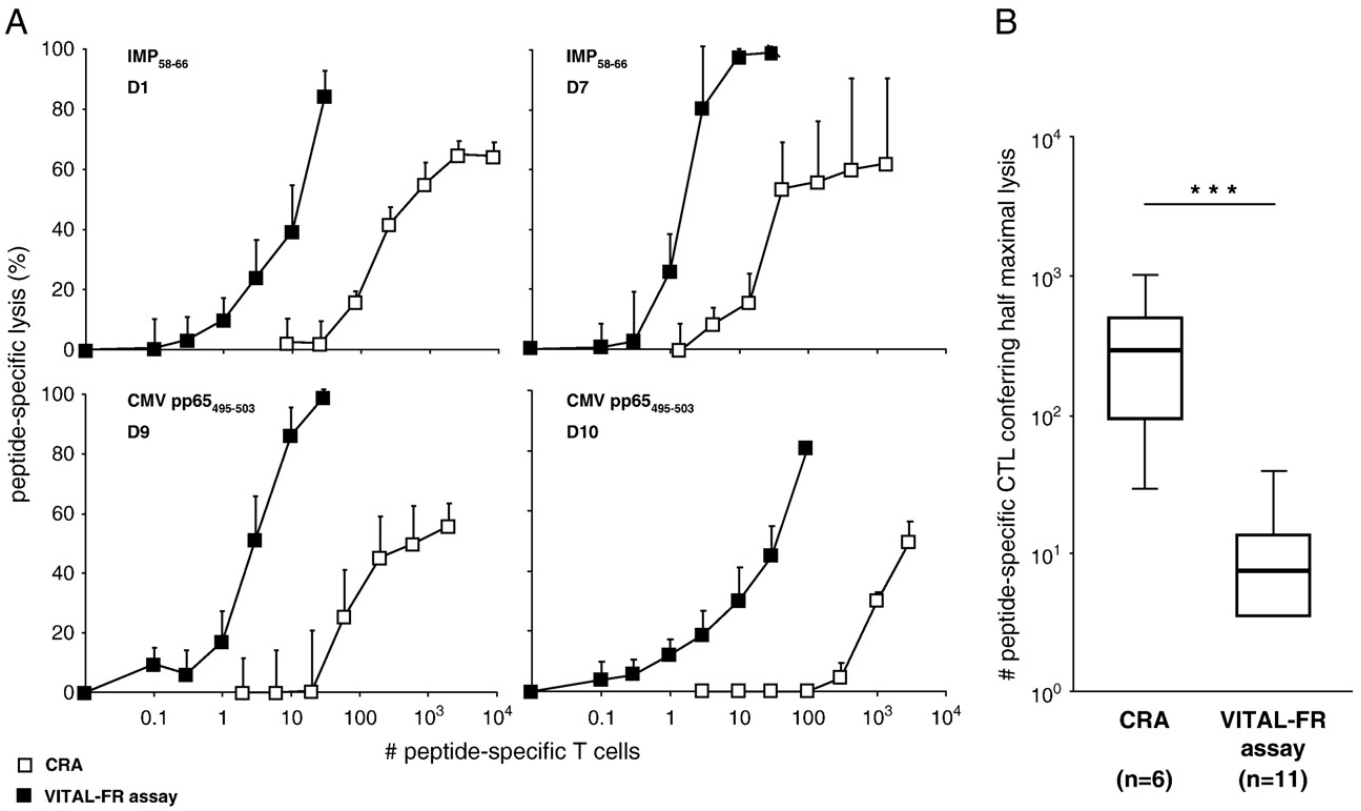

Fig. 5. Comparison of the VITAL-FR assay with the standard CRA. (A) PBMC were pre-sensitized with IMP$_{58–66}$ (upper row) or CMV pp65$_{495–503}$ loaded autologous dendritic cells (lower row) for 7 days. T cell lines contained 8.5% (D1) or 1.4% (D7) HLA-A2/IMP$_{58–66}$-tetramer$^+$ CTL, and 1.9% (D9) or 3.1% (D10) HLA-A2/CMV pp65$_{495–503}$-tetramer-reactive T cells. For the VITAL-FR assay, T2 cells were labelled with CFSE and loaded with IMP$_{58–66}$ or CMV pp65$_{495–503}$ to be used as specific target cells. Control target cells were labelled with Far Red and loaded with the HIV pol$_{510–518}$ peptide. Assays were incubated for 72 h and assessed by flow cytometry. For the CRA, T2 cells were labelled with ^{51}chromium and loaded with peptides as above. CRA were incubated for 4 h. Specific lysis was calculated in relation to the respective reactions with the control peptide. Representative for 8 independent donors. Mean values ± SD of triplicate determinations. (B) VITAL-FR assays and CRA performed in parallel, according to A, and analyzed for half maximal lysis. Median and percentiles of 8 independent donors were evaluated, $^{***}p<0.001$.

individual reactions were considered positive in terms of peptide-specific cytotoxic activity. Summarizing the results of 10 different donors, in 60 of 84 experimental wells only 1–3 IMP_{58-66}- or CMV $pp65_{495-503}$-specific CTL were necessary to mediate significant target cell lysis (Table 1).

After demonstration of such high sensitivity for recall responses to highly immunogenic peptide epitopes, we finally tested if the VITAL-FR assay was also able to detect specific HLA-A2/peptide-specific CTL of unknown low frequencies. PBMC of healthy donors were restimulated over three weeks in the presence of the respective autologous dendritic cells pulsed with the HIV $pol_{510-518}$ peptide before the resulting T cell populations were compared in VITAL-FR and CRA assays (Fig. 6). In one of the three donors a significant CTL response starting at 3×10^3 effector cells was detected by the VITAL-FR assay amounting 80% of lysis at 1×10^4 effector cells. Again, CRA was approximately 10-fold less sensitive and even at higher effector cell numbers tested maximal lysis did not exceed 30%. Thus, initial peptide-specific CTL preactivation and the overall sensitivity of our VITAL-FR assay reproducibly detected functional CTL at low initial frequencies of CTL specific for minimal epitopes in a defined HLA background.

4. Discussion

Direct experimental assessment of CTL function from human tissue samples is often challenged by limited numbers of effector cells that can be recovered. We here describe a highly sensitive method to quantify epitope-specific CTL by their cytolytic activity based on the VITAL assay (Hermans et al., 2004). The high sensitivity of our assay system that reproducibly detected as few as three epitope-specific CTL resulted from a combination of several features on different levels of the procedure. Since the major contribution came from the use of Far Red as second fluorescent dye for target cell distinction we termed our modified approach VITAL-FR assay. Low toxicity of Far Red was proven by lack of induction of caspase 3 cleavage as marker of apoptosis induction (He et al., 2005). This certainly contributes to its long-term staining potential (Wang et al., 2005). Long-term stability and high staining intensity of CFSE and Far Red allowed reducing the numbers for each target cell population to 10^3 cells subsequently requiring fewer CTL. It also allowed extension of *in-vitro* incubation for up to 72 h. In addition, with a peak emission at a wavelength >650 nm Far Red (Brehm et al., 2005) more precisely discriminates from CFSE (517 nm) (Ingulli, 2007) as compared to CMTMR (565 nm) (Hermans et al., 2004).

To establish specificity and sensitivity of the experimental settings of the VITAL-FR assay, we related cell-mediated lysis to the number of epitope-specific CTL for strong viral antigenic epitopes in the context of a defined MHC molecule. These CTL are readily assessed by flow cytometry after staining with fluorescent multimeric MHC/peptide complexes (Altman et al., 1996). We used highly conserved immunodominant epitopes of antigens of viral infections with high prevalence in the population, allowing for reliable expansion of specific memory CTL. Initially, we chose the HLA-A*0201-restricted nonameric peptide epitopes from the influenza A virus matrix protein 1 and from CMV pp65 (Moss and Khan, 2004; Rimmelzwaan et al., 2009). The HLA-A2 allele is common in the Caucasian population with a frequency of about 45% (Kiewe et al., 2008). Thus, specific CTL expanded reliably in short term *in-vitro* culture from PBMC of healthy HLA-$A2^+$ donors (12 out of 16 for IMP_{58-66}; 6 out of 7 for CMV $pp65_{495-503}$). Responses to the HLA-A2-restricted peptide HIV $pol_{510-518}$ have a low prevalence of 0.1% in the German population (UNAIDS, 2008) and therefore was considered a suitable negative control.

One important aspect contributing to the high sensitivity of the VITAL-FR assay is the incubation time. Co-incubation of effector and target cells in assays like ELISpot, intracellular FACS staining, CRA, or the VITAL assay run for 4–20 h (Karawajew et al., 1994; Hermans et al., 2004; Black et al., 2006). According to our kinetics of co-incubation of effector and target cell populations, the sensitivity of the cytotoxic readout using the VITAL-FR assay was clearly a function of the incubation time. At high effector cell numbers, however, we sometimes observed a coordinate decline of both control and specific target cells that might be due to unspecific bystander killing mediated by undirected FasL expression and MHC-

Table 1
Sensitivity of the VITAL-FR assay by limiting dilution analysis.

#HLA-A2 tetramer $^+$ T cells/well	Peptide: IMP_{58-66}								Peptide: CMV $pp65_{495-503}$	
	D1 [a]	D2	D3	D4	D5	D6	D7	D8	D9	D10
0	−/10 [b]	−/5	−/5	−/10	−/10	−/10	−/10	−/10	−/10	−/4
0.1	−/10	−/5	−/5	−/10	−/10	−/10	−/10	−/10	−/10	−/4
0.3	−/10	−/5	−/5	−/10	−/10	1/10	2/10	−/10	−/10	−/4
1.0	−/10	1/5	−/5	6/10	1/10	4/10	6/10	1/10	4/10	−/4
3.0	3/10	3/5	4/5	9/10	6/10	10/10	10/10	5/10	8/10	2/4
10.0	8/10	5/5	5/5	10/10	10/10	10/10	10/10	10/10	10/10	3/4
30.0	10/10	5/5	5/5	10/10	10/10	10/10	10/10	10/10	10/10	4/4
100.0	10/10	5/5	5/5	10/10	10/10	10/10	10/10	10/10	10/10	4/4
HLA-A2 tetramer$^+$ CTL frequency in initial T cell line (%)	10.4	1.0	1.2	2.3	0.8	9.5	2.4	1.6	3.2	3.0

PBMC were preincubated with IMP_{58-66} or $pp65_{495-503}$ for 14 days. Tetramer-reactive CTL were determined by flow cytometry and titrated into VITAL-FR assays. Cut off for positive reaction was threefold higher lysis values for experimental wells than the standard deviation of control wells.

[a] Different PBMC donors.

[b] Number of reactive wells per total wells tested.

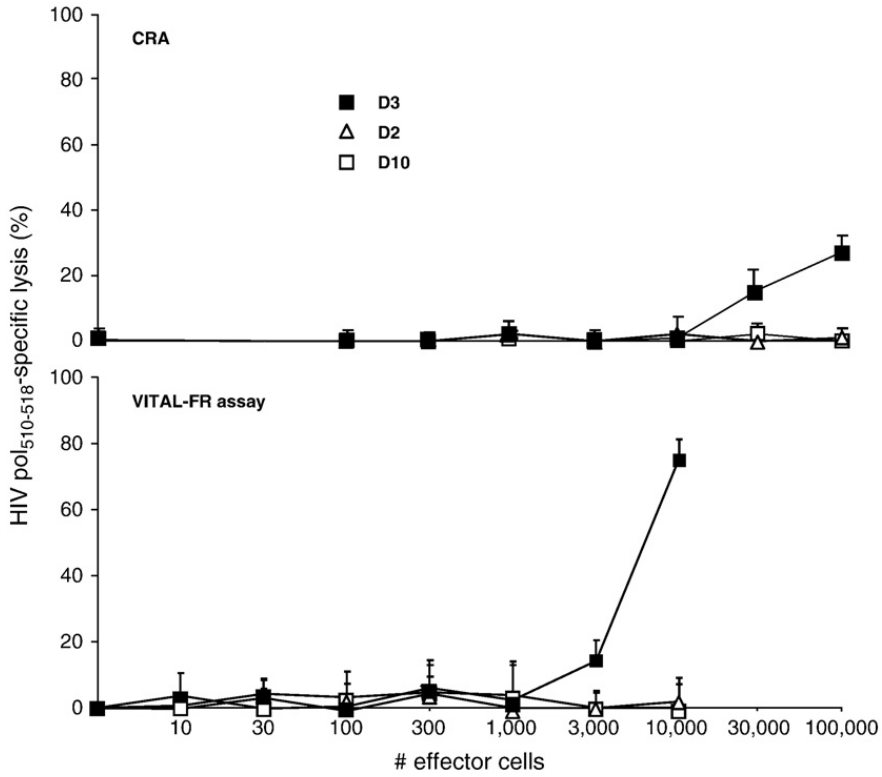

Fig. 6. Detection of HIV $pol_{510-518}$ specific CTL by the VITAL-FR assay. PBMC were preincubated with HIV $pol_{510-518}$ peptide-loaded autologous dendritic cells for 21 days with weekly restimulation. T2 cells labelled with CFSE and loaded with HIV $pol_{510-518}$ were used as specific target cells in the VITAL-FR assay and Far Red-labelled cells loaded with the HPV16 $E7_{11-20}$ peptide served as controls. Assays were incubated for 72 h and assessed by flow cytometry. For standard CRA, ^{51}chromium-labelled T2 cells were loaded with HIV $pol_{510-518}$ for specific target cells or with HPV16 $E7_{11-20}$ peptide for control target cells. Assays were incubated for 4 h. Specific lysis was calculated in relation to the respective reactions with the control peptide. Mean values ± SD of triplicate determinations.

independent co-killing (Kuwano and Arai, 1996). Therefore we suggest to include titrations of the T cells in the assay.

Under optimal conditions after 72 h, the VITAL-FR assay detected specific lysis by 1–10 epitope-specific CTL identified by tetramer reactivity. However, it has been shown that not all phenotypically tetramer-reactive CTL are functional (Zehbe et al., 2007). Therefore, we may have even underestimated the sensitivity for detection of functional cytolytic CTLs.

In the VITAL assay, 25% target cell lysis required 500 effector CTL of a $CD8^+$ T cell clone specific for the cancer/testis antigen NY-ESO-1 (Hermans et al., 2004). We wanted to compare the sensitivity of the modified VITAL-FR assay to the CRA, a well established and classical assay to assess cytotoxicity. Taken into account the importance of the incubation time for the VITAL-FR assay, the main limitation of CRA lies in a high background release of ^{51}chromium due to spontaneous release and radiolysis after longer incubation periods (Dunkley et al., 1974). Consequently, CRA requires 10–30 fold higher numbers of specific CTL to detect significant target cell lysis as compared to the VITAL-FR assay. Also the endpoint of the assay, the levels of maximal lysis achievable were lower in CRA and the half maximal target cell lysis in the VITAL-FR assay was reached at 100-fold lower numbers of epitope-specific CTL than in CRA. It is also noteworthy that the CRA calculates control and specific target cell lysis from two independent wells while in the VITAL-FR assay both target cell populations are exposed to the very same CTL, reducing CTL numbers needed by half. In addition to the intrinsic higher sensitivity of the VITAL-FR assay system, this methodical difference supports that a total of 5×10^4 effector cells suffice to achieve results comparable to 5×10^6 CTL for CRA.

In an effort to define different generic target cells we compared autologous B-LCL, T2, and ectopically HLA-A2-expressing K562 cells. In general, autologous target cells such as B-LCL bind and present all relevant peptides by MHC/HLA molecules of a given donor (Purner et al., 1994). As compared to the generic target cells, B-LCL generally showed lower sensitivity for cytolysis that could result from immunosuppressive or Th2-polarizing cytokines like IL-10, IL-6, tumor necrosis factor-α, tumor necrosis factor-β and transforming growth factor-β1 released by B-LCL (Rochford et al., 1997). In T2 cells mutations in the Transporter Associated in Protein Presentation prevent the presentation of endogenously processed peptides competing with externally loaded HLA-A2 restricted peptides (Hosken and Bevan, 1990). The overall high specific lysis of peptide-loaded T2 cells might also be due to high density, homogenous distribution and stabilisation of the HLA complex after incubation with peptides (Baas et al., 1992). In contrast, the K562-A2 cell line fully capable of antigen processing and ectopic expression of any individual recombinant HLA allele may offer several possibilities like HLA libraries for epitope screening or use of complete antigen as source for naturally processed peptides (Britten et al., 2002). However, like for autologous $HLA\text{-}A2^+$ B-LCL, the ability of K562-A2 to present internally processed antigenic peptides might interfere with externally loaded peptides and

might be responsible for the reduced sensitivity compared to T2 cells.

The high sensitivity and the detection limit of our VITAL-FR assay were further documented by limiting dilution analysis. When pre-activated CTL were used with peptide-loaded T2 cells as target cells, the VITAL-FR assay reliably detected as few as three epitope-specific functional CTL. This was highly reproducible in 10 different blood donors and with different target epitopes.

Our experimental strategy relied on very strong viral epitopes inducing high CTL numbers. We also proved that by VITAL-FR functional CTL specific for the HIV $pol_{510-518}$ peptide in PBMC of healthy donors were detected at effector cell numbers that were about 10–30 fold lower as compared to the standard CRA performed in parallel. T cell responsiveness to HLA-A2 associated HIV $pol_{510-518}$ was described in HIV-1-infected patients and in persons who had no serologic and virologic signs of HIV infection but were at high risk for recent sexual exposure to HIV (Herr et al., 1998). However, our protocol does not allow for discrimination, whether these specific CTL were primed *in-vitro* (Lubong Sabado et al., 2009) or present *in vivo* despite of sero-negativity of the blood donor (Herr et al., 1998; Pinto et al., 1995).

Taken together, with the VITAL-FR assay we provide a very sensitive convenient flow cytometry-based system to assess CTL function from low cell numbers with suspended generic target cell populations. Although due to several additional factors, its sensitivity basically depends on the characteristics of Far Red in combination with CFSE as fluorescent dyes. Our findings underline the suitability of the VITAL-FR assay for detection of rare specific CTL or few T cells isolated from very restricted samples e.g. from tumor infiltrating lymphocytes. CTL assays for immunomonitoring like ELISpot or major histocompatibility complex multimers identifying epitope-specific CTL by measuring surrogate parameters made a major impact on immunological research (Malyguine et al., 2007; Brooimans et al., 2008). Measuring specific CTL numbers by those techniques, however, does not provide information on their lytic functionality. The sensitive functional analyses enabled by the VITAL-FR assay may allow analysis of sample types beforehand too small to be considered for CTL detection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jim.2010.06.005.

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