Banas et al. BMC Immunology (2017) 18:15
DOI 10.1186/s12865-017-0194-z

BMC Immunology

RESEARCH ARTICLE Open Access

Validation of T-Track® CMV to assess the functionality of cytomegalovirus-reactive cell-mediated immunity in hemodialysis patients

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Abstract

Background: Uncontrolled cytomegalovirus (CMV) replication in immunocompromised solid-organ transplant recipients is a clinically relevant issue and an indication of impaired CMV-specific cell-mediated immunity (CMI). Primary aim of this study was to assess the suitability of the immune monitoring tool T-Track® CMV to determine CMV-reactive CMI in a cohort of hemodialysis patients representative of patients eligible for renal transplantation. Positive and negative agreement of T-Track® CMV with CMV serology was examined in 124 hemodialysis patients, of whom 67 (54%) revealed a positive CMV serostatus. Secondary aim of the study was to evaluate T-Track® CMV performance against two unrelated CMV-specific CMI monitoring assays, QuantiFERON®-CMV and a cocktail of six class I iTAg™ MHC Tetramers.

Results: Positive T-Track® CMV results were obtained in 90% (60/67) of CMV-seropositive hemodialysis patients. In comparison, 73% (45/62) and 77% (40/52) positive agreement with CMV serology was achieved using QuantiFERON®-CMV and iTAg™ MHC Tetramer. Positive T-Track® CMV responses in CMV-seropositive patients were dominated by pp65-reactive cells (58/67 [87%]), while IE-1-responsive cells contributed to an improved (87% to 90%) positive agreement of T-Track® CMV with CMV serology. Interestingly, T-Track® CMV, QuantiFERON®-CMV and iTAg™ MHC Tetramers showed 79% (45/57), 87% (48/55) and 93% (42/45) negative agreement with serology, respectively, and a strong inter-assay variability. Notably, T-Track® CMV was able to detect IE-1-reactive cells in blood samples of patients with a negative CMV serology, suggesting either a previous exposure to CMV that yielded a cellular but no humoral immune response, or TCR cross-reactivity with foreign antigens, both suggesting a possible protective immunity against CMV in these patients.

Conclusion: T-Track® CMV is a highly sensitive assay, enabling the functional assessment of CMV-responsive cells in hemodialysis patients prior to renal transplantation. T-Track® CMV thus represents a valuable immune monitoring tool to identify candidate transplant recipients potentially at increased risk for CMV-related clinical complications.

Keywords: Cytomegalovirus, CMV, IE-1, pp65, Cell-mediated immunity, IFN-γ ELISpot, T-Track® CMV, QuantiFERON®-CMV, iTAg™ MHC Tetramers, Hemodialysis

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Background

Cytomegalovirus (CMV) is a major cause of infectious complications in immunocompromised individuals. Protection against CMV infection or reactivation is normally assured by both the innate and adaptive arms of the immune system [1, 2]. While the humoral and innate responses are essential for the early response to infection [1, 3, 4], cellular immunity is required to control latency and prevent CMV reactivation in latently infected individuals [1]. $CD8^+$ cytotoxic T cells (CTL) and $CD4^+$ T helper (Th) cells are both required to assure efficient immune protection against CMV reactivation [1, 5–8]. Primary infection is dominated by $CD8^+$ T cell response, preferentially targeting CMV immediate early-1 (IE-1) antigen, while long-term recovery is dominated by $CD4^+$ T cell response and a switch of reactivity toward CMV lower matrix phosphoprotein 65 (pp65) [6, 8–11]. The frequency of CMV-specific $CD8^+$ and $CD4^+$ T cells is highly variable, both between healthy CMV-seropositive individuals and during the course of CMV reactivation, and correlates with varying levels of protection [6, 9, 11–13]. Beside changes in T cell frequency, alterations in T cell functionality are associated with impaired response to chronic viral infection [14–17].

Functional impairment of cell-mediated immunity (CMI) in the course of immunosuppression, such as in solid-organ transplant recipients, is a major cause of uncontrolled CMV replication and related clinical complications [18–21]. Treatment regimens with antivirals are costly and associated with harmful side effects. Assessment of CMV-specific immunity may be beneficial to identify patients at increased risk of viral complications, possibly allowing personalized adjustment of antiviral and immunosuppressive therapies.

Various experimental approaches exist to measure CMV-specific CMI. Direct T cell staining with for instance class I iTAg™ MHC Tetramers (Beckman Coulter) allows the quantification of epitope-specific $CD8^+$ cells by flow cytometry [18, 22–24]. The sensitivity of tetramer-based assays strictly depends on the coverage of the patient population by the selected HLA types, and this method cannot assess the functionality of $CD8^+$ cells. Several assays assessing CMV-specific T cell function have been described. Principally, they measure the production of activation markers (e.g. cytokines such as IFN-γ) in response to antigen stimulation, using intracellular cytokine staining followed by flow cytometry [6, 7, 12, 13, 25, 26], ELISA [21, 27–30], or ELISpot [31–33] assays. These approaches differ not only in their read-out format but also in the antigen (peptide vs. protein) used for the *ex vivo* stimulation. Peptide-based immune monitoring tests such as QuantiFERON®-CMV (Qiagen) allow the quantification of IFN-γ produced by epitope-specific $CD8^+$ T cells. Whole blood samples are stimulated with a pool of 22 immunogenic peptides (mapping at IE-1, IE-2, pp28, pp50, pp65 and gB CMV antigens) and covering > 98% of HLA class-I haplotypes. Reactive $CD8^+$ T cells are monitored by quantifying secreted IFN-γ by ELISA [34]. QuantiFERON®-CMV was used in a number of studies to assess the risk of CMV reactivation and related disease following solid-organ transplantation [21, 27–30]. A disadvantage of QuantiFERON®-CMV is that it does not assess CMV-specific $CD4^+$ T cell function and that it often yields indeterminate results that cannot be interpreted [28, 35, 36]. T-Track® CMV is based on the stimulation of freshly isolated peripheral blood mononuclear cells (PBMC) with recombinant urea-formulated (T-activated®) immunodominant CMV IE-1 and pp65 proteins, and the subsequent quantification of antigen-reactive effector cells using an IFN-γ ELISpot assay. T-activated® proteins (*a*proteins) are processed via the exogenous and endogenous antigen processing pathways, resulting in the presentation of naturally-generated peptides by MHC class I and class II molecules, thus enabling the stimulation of a broad spectrum of CMV-protective cells including $CD8^+$ and $CD4^+$ T cells, as well as the bystander activation of NK and NKT-like cells [37, 38]. As such, T-Track® CMV is not restricted to particular HLA types. Performance of T-Track® CMV has been recently characterized [38]. A recent study demonstrated its high sensitivity in evaluating changes in CMV-specific CMI during and after pregnancy [39].

Primary aim of this cross-sectional prospective multicenter study was to evaluate the suitability of T-Track® CMV to assess the functionality of CMV-specific CMI in a cohort of patients on hemodialysis due to end-stage renal failure, and thus being representative of patients prior to renal transplantation. Secondary aim of the study was to compare the performance of T-Track® CMV to that of QuantiFERON®-CMV and iTAg™ MHC Tetramers in terms of positive and negative agreement with CMV serology (gold standard reference).

Methods

Study design and participants

Hemodialysis patients of any gender and race aged at least 18 years were recruited in the study. Patients requiring systemic immunosuppressive treatment within the last 3 months before study inclusion or suffering from chronic or uncontrolled infections (e.g. HIV or chronic hepatitis) were ineligible for study participation. All subjects gave written informed consent. The study was registered and approved according to the rules, at the German Institute of Medical Documentation and Information (DIMDI). Patient enrolment was started only after receiving the exemption of the permit requirement by the BfArM (Federal Institute for Drugs and Medical Devices) and approval by the ethics committee of the University of Regensburg (approval number 11-122-0205). For reasons of transparency and completeness, the study was prospectively registered at clinicaltrials.gov. The authors confirm that all ongoing and related trials for this intervention are registered at clinicaltrials.gov.

Blood collection

Lithium heparinized whole blood was collected during routine withdrawal, prior to the start of the dialysis session. For T-Track® CMV and iTAg™ MHC Tetramer staining, 15 ml blood was collected for further PBMC isolation. For QuantiFERON®-CMV, 0.8 to 1.2 ml whole blood was collected into each of the three assay tubes. CMV serology was performed from 2.6 ml whole blood.

CMV serology

Anti-CMV serological testing was performed using fully automated anti-CMV IgM and IgG tests on the BEP III system (Siemens Healthcare, Eschborn, Germany). CMV IgG-serology was used as primary reference measurement procedure (gold standard method).

CMV-specific cellular immunity assays

T-Track® CMV (Lophius Biosciences GmbH, Regensburg, Germany) was performed according to manufacturer's instructions. Briefly, PBMC were isolated and stimulated individually with T-activated® CMV-specific immediate-early 1 (*a*IE-1) and phosphoprotein pp65 (*a*pp65) proteins for 19 h at 37°C. Staphylococcus enterotoxin B (SEB) and medium served as positive and negative controls for the stimulation, respectively. IFN-γ ELISpot assays were performed following manufacturer's recommendations. IFN-γ-specific spot-forming cells (SFC) were enumerated on a Bioreader® 5000 Pro-Eα (BIO-SYS GmbH, Karben, Germany). Test results were considered positive if the geometric mean of the spots resulting from at least one of the *a*pp65 and *a*IE1 stimulations was ≥ 10 SFC/200,000 PBMC and when the ratio of the geometric means of stimulated and non-stimulated conditions was ≥ 2.5. Positivity rules were calculated as described in the Statistics section.

The QuantiFERON®-CMV assay (Qiagen, Hilden, Germany) was performed according to manufacturer's instructions. Briefly, QuantiFERON®-CMV collection tubes (Nil Control, CMV Antigen and Mitogen Control) were incubated for 16–24 h at 37°C. IFN-γ levels were determined by enzyme-linked immunosorbent assay (ELISA). Calculation of results was achieved using QuantiFERON®-CMV Analysis Software. QuantiFERON®-CMV test results were considered positive when IFN-γ level (IU/mL) in the CMV Antigen-specific assay minus that in the Nil Control was ≥ 0.2, as recommended by the manufacturer. ELISA measurements are accurate up to 10 IU/mL. Values ≥ 10 IU/mL cannot be quantitatively evaluated.

For the CMV-specific tetramer assay, CMV peptide-specific $CD8^+$ T cells were quantified by flow cytometry using a mixture of six class I iTAg™ MHC Tetramers (Beckman Coulter), including: MHC A*0101 Class I Tetramer CMV pp50 (VTEHDTLLY), MHC A*0201 Class I Tetramer CMV pp65 (NLVPMVATV), MHC A*2402 Class I Tetramer CMV pp65 (QYDPVAALF), MHC B*0702 Class I Tetramer CMV pp65 (TPRVTGGGAM), MHC B*0801 Class I Tetramer CMV IE-1 (ELRRKMMYM) and MHC B*3501 Class I Tetramer CMV pp65 (IPSINVHHY). iTAg™ MHC Negative Tetramer PN T01044 (Beckman Coulter) served as negative control. Preselected HLA types are predicted to cover at least 80% of the Caucasian population [40]. Each $1{\times}10^6$ PBMC were stained with 10 μl tetramer mix, 10 μl anti-CD8-FITC (T8-FITC, Beckman Coulter) and 5 μl human CD3 APC-Alexa Fluor® 750 conjugate (Invitrogen/Thermo Fisher Scientific) for 30 min at room temperature protected from light. Cells were washed once in PBS and incubated 45 min at 4 °C protected from light. Dead cells were further stained with SYTOX® RED dead cell stain (Invitrogen/Thermo Fisher Scientific) for 15 min at 4 °C protected from light, prior to flow cytometry analysis. Measurements were performed using a Cytomics FC 500 MPL cytometer (Beckmann Coulter), gating on living and CD3-positive cells. Cell count from the iTAg™ MHC Tetramer Negative staining was subtracted from that of the iTAg™ MHC Tetramer CMV-specific cocktail staining. Data were expressed as the % of CMV-specific tetramer-positive $CD8^+$ T cells relative to total $CD8^+$ T cells. Test results were considered positive, when the proportion of tetramer-positive $CD8^+$ cells was ≥ 0.1% of total $CD8^+$ T cells.

Statistics

Calculations were performed with SAS 9.2 Software and VFP (Variance Function Program) 10.0. Figures were generated using GraphPad Prism. In case of categorically scaled data, absolute and relative frequencies were reported. For continuously scaled data, mean, median, geometric mean, standard deviation, minimum and maximum have been reported. Diagnostic accuracies (sensitivity and specificity) were analyzed from 2 × 2 contingency tables referring bivariate test results to CMV serostatus (reference method). Since the reference standard was not disease but a comparative method, the terms "percent positive agreement" and "percent negative agreement" were used instead of "sensitivity" and "specificity" respectively. The measures are reported with their exact Pearson-Clopper confidence intervals. Kappa (κ) according to Altman and McNemar's test were used to indicate overall agreement and consistency of pairs of methods respectively. Significance was accepted at $p < 0.05$.

The cut-off of T-Track® CMV positivity was determined using z-statistics (α-level = 0.05) on log10-transformed geometric mean values. Values = 0 were replaced by values near detection limit, which was assumed to be 0.5. Intra-assay standard deviation (SD) of ELISpot measurements from the hemodialysis patient cohort ($n = 124$) and from a cohort of healthy donors ($n = 45$; [38]) was calculated. SD was for the unstimulated control, IE-1 stimulation and pp65 stimulation 0.199, 0.240 and 0.220 (hemodialysis patients), and 0.234, 0.192 and 0.136 (healthy donors), respectively. Considering an intra-assay SD of 0.2 and assuming that 4 replicates are measured for each negative control and test samples, a criterion that the ratio of geometric means of stimulated to unstimulated values is at least 2.5 was obtained. In addition, precision profiles were generated from both IE-1- and pp65-specific test results, whereby a coefficient of variation (CV) no higher than 40% was used as a limit of acceptance of assay validity to determine the respective limit of quantitation (LoQ). Precision profiles for IE-1- and pp65-specific T-Track® CMV yielded LoQ values of 7.8 and 8.3 respectively (see Additional file 1). Comparable limits of quantitation were obtained from precision profiles generated from T-Track® CMV assays performed on PBMC from healthy donors [38]. Based on these analyses, a technical cut-off of 10 SFC/200,000 PBMC was chosen. Altogether, T-Track® CMV test results were considered positive if the geometric mean of the spots resulting from at least one of *a*pp65 and *a*IE1 stimulations was ≥ 10 SFC/200,000 PBMC and if the ratio of the geometric means of stimulated to non-stimulated conditions was ≥ 2.5.

Results

Patient characteristics

One hunderd twenty-four hemodialysis patients (68 men, 56 women, mean age 65 ± 13 years) were enrolled in this study. The mean duration of dialysis was 1,913 days (range 21 to 11,640 days). A positive CMV-IgG serostatus was measured in 67/124 (54%) of hemodialysis patients (Table 1). Blood was collected before the start of the dialysis session. Routine blood parameters and inflammation markers are depicted in Table 1.

One hunderd twenty-four T-Track® CMV, 123 QuantiFERON®-CMV and 97 iTAg™ MHC Tetramer measurements were carried out from whole-blood (QuantiFERON®-CMV) or from freshly isolated PBMC (T-Track® CMV, iTAg™ MHC Tetramers). Insufficient amount of blood and/or PBMC did not allow the performance evaluation of all tests for all 124 patients. CMV serology served as a primary reference measurement procedure (gold standard reference) in the performance study.

Table 1 Demographic and blood parameters of hemodialysis patients

Age (years), mean ± SD (range)	65 ± 13 (26; 88)
Gender, N (%)	
Male	68 (54.8%)
Female	56 (45.2%)
CMV serostatus, N (%)	
Positive	67 (54%)
Negative	57 (46%)
Duration of dialysis (days), mean ± SD (range)	1,913 ± 2,079 (21; 11,640)
Blood count, mean ± SD (range)	
Hemoglobin (g/dl)	11.3 ± 1.2 (7.8; 16.1)
Erythrocytes (Tpt/l)	3.7 ± 0.44 (2.5; 5.2)
Leukocytes (pt/nl)	7.5 ± 2.4 (3.0; 17.8)
Thrombocytes (Tsd/μl)	234 ± 65 (84; 426)
Inflammation marker, mean ± SD (range)	
CRP (mg/l)[a]	9.7 ± 17.6 (1.0; 143.0)
Absolute number of PBMC x 10^6 / 15 ml whole blood (mean ± SD (range)	13.5 ± 9.8 (3.2; 87.3)

[a]CRP values were available for 80 out of 124 patients

Performance of T-Track® CMV

Positive and negative agreement of T-Track® CMV with CMV serology was investigated using PBMC samples from 124 hemodialysis patients. 58 of the 67 CMV-seropositive patients showed a positive response to *a*pp65 (Table 2) with a median of 165 spot-forming cells (SFC)/200,000 PBMC and a maximum of 1,040 SFC/200,000 PBMC (Fig. 1a). 33 of the 67 CMV-seropositive patients demonstrated an *a*IE-1-specific response in the T-Track® CMV ELISpot assay, with a median of 9.7 SFC/200,000 PBMC and a maximum of 696 SFC/200,000 PBMC (Table 2 and Fig. 1a). By taking into account the outcome of both *a*IE-1 and *a*pp65 stimulations, T-Track® CMV results were positive in 60 out of 67 CMV-seropositive patients, corresponding to an overall positive agreement with CMV serology of 89.6% (Table 2).

Interestingly, 12 out of 57 (21.1%) study participants who scored negative in a conventional serological assay presented with CMV-reactive effector cells in T-Track® CMV, equivalent to a negative agreement with CMV serology of 78.9% (Table 3). With one exception, positive T-Track® CMV results in CMV-seronegative patients were observed in *a*IE-1-stimulated PBMC samples, and were mostly associated with low spot counts (10.3–23.6 SFC/200,000 PBMC in 9 out 12 patients; Fig. 1a). Three patients however exhibited higher spot counts (93.6–116.5 SFC/200,000 PBMC). In contrast, one *a*pp65-positive test result with a spot count of 94 SFC/200,000 PBMC was observed among the 57 CMV-seronegative patients (Fig. 1a). Of note, this patient also showed a positive test result for *a*IE-1, with 109.2 SFC/200,000 PBMC (Fig. 1a).

Table 2 Positive agreement of T-Track® CMV, QuantiFERON®-CMV and iTAg™ MHC Tetramers with CMV serology in hemodialysis patients

Test	CMV positive serology[a]	CMI+	CMI-	Positive agreement	95% CI
T-Track® CMV	67	60	7	0.896	0.797–0.957
CMV *a*IE-1	67	33	34	0.493	0.368–0.618
CMV *a*pp65	67	58	9	0.866	0.760–0.937
QuantiFERON®-CMV[b]	62	45	17	0.726	0.598–0.831
iTAg™ MHC Tetramers	52	40	12	0.769	0.632–0.875

[a]CMV-serology served as primary reference measurement procedure; [b]calculation of the positive agreement and associated 95% CI do not take into account the 4 indeterminate QuantiFERON®-CMV results out of the 66 CMV-seropositive patients; *CMI*+ positive test result, *CMI*- negative test result, *CI* confidence interval

These observations suggest that T-Track® CMV might have the capacity to detect CMV-reactive cells resulting from a previous CMV infection that was however not sufficient to yield an antibody response.

Performance of QuantiFERON®-CMV and iTAg™ MHC Tetramers

The QuantiFERON®-CMV assay was performed on blood samples from 66 CMV-seropositive and 57 CMV-seronegative hemodialysis patients. QuantiFERON®-CMV was positive (reactive) in 45/66, negative (non-reactive) in 17/66 and indeterminate in 4/66 of CMV-seropositive patients. Conversely, 7/57, 48/57 and 2/57 of CMV-seronegative patients showed positive, negative and indeterminate test results, respectively. Indeterminate results were excluded from subsequent analyses, as a repetition of the QuantiFERON®-CMV assay from fresh blood samples was not possible. Thus, the results of the QuantiFERON®-CMV assay revealed a positive and negative agreement with CMV serology of 72.6% (45/62) and 87.3% (48/55) respectively (Tables 2 and 3; Fig. 1b).

A mixture of six preselected CMV-specific class I iTAg™ MHC Tetramers based on IE-1, pp65 and pp50 epitopes and predicted to cover at least 80% of the Caucasian population was used to quantify the proportion of CMV-specific CTL in freshly isolated PBMC of 52 CMV-seropositive and 45 CMV-seronegative hemodialysis patients. In these experiments, 40/52 (76.9%) of CMV-seropositive patients were test-positive with a median proportion of 0.98% CMV-specific $CD8^+$ T cells / total $CD8^+$ T

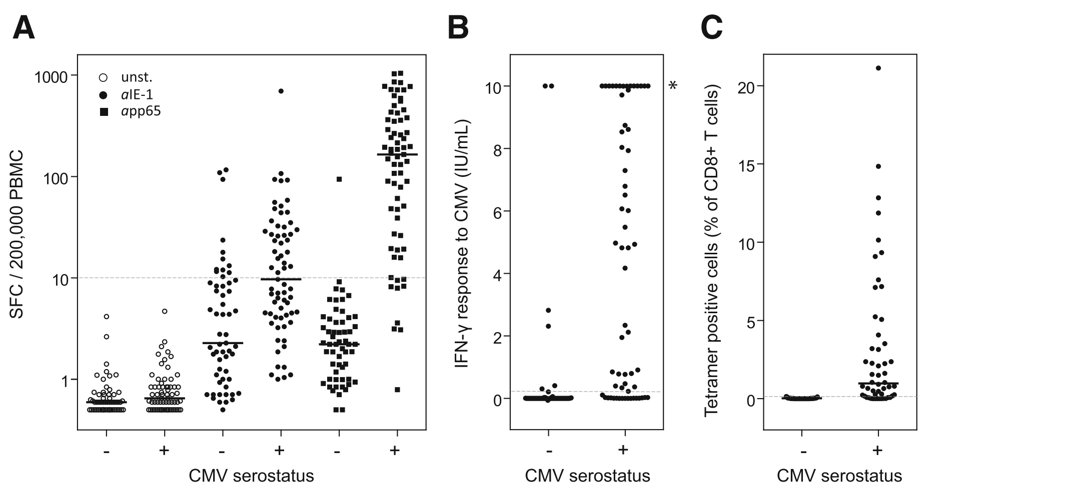

Fig. 1 CMV-specific immunity in hemodialysis patients measured with T-Track® CMV (**a**), QuantiFERON®-CMV (**b**) and iTAg™ MHC Tetramers (**c**). **a** Spot-forming cells (SFC) in IFN-γ ELISpot after in vitro stimulation of PBMC from CMV-seronegative ($n = 57$) and CMV-seropositive ($n = 67$) hemodialysis patients with T-activated® *a*IE-1 and *a*pp65 proteins, or with medium (unst.) as a negative control. SFC levels are presented as log10-transformed values in scatter plots, including median values (horizontal *black* lines). The *horizontal grey dashed line* indicates the positivity cut-off (10 SFC / 200,000 PBMC). **b** $CD8^+$-secreted IFN-γ levels were measured by ELISA following the stimulation of whole blood from CMV-seronegative ($n = 57$) and CMV-seropositive ($n = 66$) hemodialysis patients with HLA class I-specific peptides. Test results were considered positive when IFN-γ levels ≥ 0.2 IU/mL (*grey dashed line*). Indeterminate results (4/66 seropositive and 2/57 seronegative patients) are not represented; therefore the scatter plots represent the results of 62 seropositive and 55 seronegative assays. *, values ≥ 10 IU/mL cannot be quantitatively evaluated; consequently, no median values were depicted. **c** PBMC of CMV-seronegative ($n = 45$) and CMV-seropositive ($n = 52$) hemodialysis patients were stained with a mixture of six iTAg™ MHC class I Tetramers, and CMV peptide-specific $CD8^+$ T cells were quantified by flow cytometry. Test results were considered positive when ≥ 0.1% of total $CD8^+$ T cells were tetramer-positive (*grey dashed line*). The scatter plots show median values (*horizontal black lines*)

Table 3 Negative agreement of T-Track® CMV, QuantiFERON®-CMV and iTAg™ MHC Tetramers with CMV serology in hemodialysis patients

Test	CMV negative serology[a]	CMI-	CMI+	negative agreement	95% CI
T-Track® CMV	57	45	12	0.789	0.661–0.886
CMV *a*IE-1	57	45	12	0.789	0.661–0.886
CMV *a*pp65	57	56	1	0.982	0.906–1.000
QuantiFERON®-CMV[b]	55	48	7	0.873	0.755–0.947
iTAg™ MHC Tetramers	45	42	3	0.933	0.817–0.986

[a]CMV-serology served as primary reference measurement procedure; [b]calculation of the negative agreement and associated 95% CI do not take into account the 2 indeterminate QuantiFERON®-CMV results out of the 57 CMV-seronegative patients; *CMI-* negative test result, *CMI+* positive test result, *CI* confidence interval

cells and a maximum of 21.1% tetramer-positive $CD8^+$ T cells (Table 2 and Fig. 1c). Among 45 CMV-seronegative patients 3 were assay-positive, corresponding to a negative agreement with CMV-serology of 93.3% (Table 3 and Fig. 1c).

Assessment of agreement between the different assays

The results of T-Track® CMV, QuantiFERON®-CMV and iTAg™ MHC Tetramers were compared to assess their level of agreement. Results of T-Track® CMV moderately agreed with that of QuantiFERON®-CMV (κ = 0.445) and of the CMV iTAg™ MHC Tetramers (κ = 0.434) (Table 4). Statistical analysis of the number of discordant results between T-Track® CMV, QuantiFERON®-CMV and iTAg™ MHC Tetramers using the McNemar's test revealed that the consistency in the pairs of methods was statistically different between T-Track® CMV and both QuantiFERON®-CMV ($p = 0.0090$) and iTAg™ MHC Tetramers ($p = 0.0082$) (Table 4).

Notably, 14/17 and 12/12 CMV-seropositive patients with negative results in QuantiFERON®-CMV and iTAg™ MHC Tetramer respectively, were assay-positive in T-Track® CMV. Moreover, 5/5 CMV-seropositive patients with negative results for both QuantiFERON®-CMV and iTAg™ MHC Tetramers showed positive T-Track® CMV results. Conversely, the 7 CMV-seropositive patients with a negative T-Track® CMV result showed either positive or negative results by QuantiFERON®-CMV and iTAg™ MHC Tetramers, revealing inter-assay discordance. Interestingly, 3/4 CMV-seropositive patients with indeterminate QuantiFERON®-CMV results showed a positive T-Track® CMV result, of which 2 were also CMV-Tetramer positive. Finally, among the CMV-seronegative patients, only 1 out of 12 positive T-Track® CMV assays was also positive in QuantiFERON®-CMV while 6/7 and 3/3 positive results in QuantiFERON®-CMV and iTAg™ MHC Tetramers respectively were negative in T-Track® CMV.

Table 4 Assessment of strength (κ) and consistency (McNemar's Test) of agreement of T-Track® CMV results with QuantiFERON®-CMV and iTAg™ MHC Tetramers results

Test 1	Test 2	κ	95% CI	McNemar
T-Track® CMV	QuantiFERON®-CMV	0.445	0.289–0.601	0.009
T-Track® CMV	iTAg™ MHC Tetramers	0.434	0.264–0.604	0.008

According to Altman, kappa (κ) values between 0.4 and 0.6 refer to moderate agreement. Consistency was evaluated by comparing the number of discordant results using the McNemar's Test ($p < 0.05$ was considered statistically significant). Of note, assessment does not take into consideration indeterminate results of the QuantiFERON®-CMV assay. *CI* confidence interval

Discussion

T-Track® CMV represents a novel assay format, which relies on the functional assessment of various CMV protein-reactive effector cells, including $CD4^+$ (Th) cells, $CD8^+$ (CTL), NK and NKT-like cells [37, 38], all of which being described to contribute to the clearance of CMV replication [1, 6–8, 41–44]. In this study, the suitability of T-Track® CMV to measure CMV-specific CMI in a cohort of dialysis patients and its performance against QuantiFERON®-CMV [34] and iTAg™ MHC Tetramer assays [24] were evaluated.

T-Track® CMV revealed a positive agreement with CMV-serology of 90% in hemodialysis patients, higher than that measured with QuantiFERON®-CMV (73%) and a mixture of 6 preselected CMV-specific iTAg™ MHC Tetramers (77%), indicating a higher sensitivity of T-Track® CMV compared to QuantiFERON®-CMV and iTAg™ MHC Tetramers. This difference in positive agreement with CMV-serology is likely due to the difference in format of the three assays. Beside the detection of a broad repertoire of $CD8^+$ T cells as a result of antigen cross-presentation [37], T-Track® CMV is indeed able to assess the functionality of CMV-reactive $CD4^+$ cells but also the bystander activation of IFN-γ-producing NK and NKT-like cells [41, 43–46]. In contrast, QuantiFERON®-CMV and iTAg™ MHC Tetramers are restricted to the detection of selected CMV-specific $CD8^+$ cells. In addition to the assay format, the combination of results of the separate measurement of pp65- and IE-1-responsive effector cells by T-Track® CMV contributes to the increased positive agreement with CMV serology, from 87% with pp65-specific CMI alone to 90% with both pp65- and IE-1-specific CMI. This positive contribution of IE-1 to the sensitivity of T-Track® CMV is in agreement with the demonstration that CMV-seropositive healthy donors do not always exhibit a pp65-specific $CD8^+$ T cell response and that a non-negligible proportion of individuals only show a $CD8^+$ T cell response to IE-1 [47]. Other factors potentially enhancing the sensitivity of T-Track® CMV are the standardization of the assay, which uses a constant number of PBMC (as opposed to whole blood in QuantiFERON®-CMV, possibly resulting in high inter-individual variability), its HLA-type-independence (as opposed especially to the iTAg™ MHC Tetramer assay) and the absence of indeterminate results (as opposed to QuantiFERON®-CMV). In that regard, 4/66 CMV-seropositive and 2/57 CMV-seronegative hemodialysis patients yielded indeterminate results with QuantiFERON®-CMV, which - with a rate of 5% - is lower than what was reported in transplant recipients [28, 35, 36].

Interestingly, the positive agreement of T-Track® CMV with CMV-serology of 90% measured in this cohort of hemodialysis patients was lower than that obtained in CMV-seropositive healthy individuals (97%; [38]). Similarly, the positive agreement of QuantiFERON®-CMV results with CMV-serology in hemodialysis patients (73%) is below the positive agreement of 88% to 97% previously reported in healthy adults [27, 34]. This difference might be explained by a functional impairment of Th cells, CTL, Antigen-presenting cells (APC), NK and NKT cells in patients with end-stage renal failure undergoing hemodialysis [48–52]. A reduced CMV-CMI prior to renal transplantation might be associated with an increased risk of CMV reactivation following transplantation. In support to this proposition, several studies reported an association between impaired CMV-specific CMI pre-transplantation and increased risk for CMV viremia post-transplantation [29, 31, 53]. The high positive agreement of T-Track® CMV with CMV serology observed in this cohort of hemodialysis patients therefore emphasizes the suitability and clinical relevance of T-Track® CMV for patients eligible for renal transplantation.

Although both CMV pp65 and IE-1 antigens contain multiple $CD4^+$ and $CD8^+$ T cell epitopes presented by different HLA alleles [40], the number of reactive effector cells responding to stimulation with *a*pp65 was substantially higher than that responding to *a*IE-1. This difference might result in part from the dynamics of pp65- and IE-1-reactive $CD4^+$ and $CD8^+$ T cells in the course of the immune response to CMV infection, long-term seroconversion being dominated by pp65- over IE-1-reactive T cells [8–11, 53, 54]. Moreover, mechanisms of immune evasion involving CMV-encoded unique short (US) proteins and resulting in the inhibition of the MHC-I-dependent antigen presentation pathway appear to be responsible for impaired IE-1 antigen processing and presentation, and thus in the low frequency of IE-1-reactive $CD8^+$ T cells [55–57]. On the other hand, differential antigen uptake, processing and presentation by APC, possibly influenced by pp65 and IE-1 intrinsic properties [54, 58, 59], might explain inter-individual differences in the frequency of CMV antigen-specific T cells. Accordingly, comparable $CD8^+$ T cell response to IE-1 and pp65 has also been described in some CMV-seropositive healthy donors [47, 60]. The clinical significance of the differential responses to different antigens using T-Track® CMV needs to be elucidated in future studies.

12/57 CMV-seronegative patients revealed positive T-Track® CMV results, corresponding to a negative agreement of T-Track® CMV with CMV serology of 79%. Positive test results were mainly attributed to *a*IE-1 stimulation, and negative agreement raised to 98% when considering the results of *a*pp65 stimulation alone. IE-1-induced spot counts were close to T-Track® CMV positivity threshold in 9/12 patients and only 3 CMV-seronegative patients showed higher IE-1-induced spot counts. We can reasonably rule out false negative CMV serology test results in these patients, as repetition of CMV serology 6 months upon completion of the study in 9 patients who were still available, confirmed their negative serostatus for IgG and IgM (data not shown). Comparatively, 7/55 QuantiFERON®-CMV and 3/45 CMV iTAg™ MHC Tetramer measurements also revealed positive test results in seronegative dialysis patients. Interestingly, with one exception, the 12, 7 and 3 CMV seronegative patients with positive test results in T-Track® CMV, QuantiFERON®-CMV and CMV iTAg™ MHC Tetramers, respectively, were different. This inter-assay variability contributes to the moderate agreement ($\kappa = \sim 0.4$) observed between T-Track® CMV and the 2 alternative assays, and is in agreement with previous studies reporting discordant results between IFN-γ ELISpot and QuantiFERON®-CMV [61, 62]. This inter-assay variability likely reflects differences in the ability of antigen stimulants in each assay to activate distinct subsets of CMV-reactive T cells. Urea formulation of T-activated® CMV antigens increases protein uptake and promotes antigen processing and presentation in the context of both MHC-I (cross-presentation) and MHC-II [37]. T-Track® CMV is thus able to activate a broad range of $CD8^+$ and $CD4^+$ T cells, encompassing a larger T cell repertoire than QuantiFERON®-CMV or iTAg™ MHC-I Tetramers, and possibly explaining the higher number of CMV-seronegative patients with positive T-Track® CMV test results.

Interestingly, detection of CMV-reactive effector T cells within CMV-seronegative individuals has been described by others, both in healthy individuals and in transplant recipients, at frequencies of 2–11% among healthy donors and up to 30% in renal transplant recipients [13, 63–65]. With 21% CMV-seronegative hemodialysis patients with positive T-Track® CMV results, our data are in concordance with these published studies. Although Sester et al.

questioned the accuracy of serologic testing, in particular in case of borderline immunoglobulin titers [65], Loeth et al. elegantly demonstrated in their study on healthy individuals, that the frequency of 11% seronegative donors with pp65-specific $CD4^+$ and $CD8^+$ response was neither due to wrong serological assignment, nor to immune cross-reactivity with the closely related herpes virus HHV6, nor to *in vitro* priming. Instead, their demonstration that a large proportion of seronegative donors could mount a strong pp65-specific $CD4^+$ (and to a lesser extent $CD8^+$) response upon *in vitro* stimulation, led the authors to suggest that these individuals were previously exposed to CMV but failed to mount a humoral immune response [63]. We cannot exclude at this point this possibility nor that TCR cross-reactivity with closely related herpes viruses [66, 67] or with environmental antigens [68, 69] is responsible for the detection of CMV-reactive cells in CMV-seronegative hemodialysis patients. On the other hand, we can reasonably exclude the possibility that signals detected by IFN-γ ELISpot following 19 h of antigen stimulation originate from CMV-specific naïve T cells present in the PBMC population [70–72]. Indeed, although antigen-specific naïve T cells can be primed and expanded in vitro and in vivo [69, 72–78], the vast majority of antigen-stimulated naïve T cells produce no IFN-γ and do not divide for the first ~3 days of stimulation [69, 76, 79–82]. The higher proportion of CMV-reactive cells in seronegative dialysis patients in T-Track® CMV compared to QuantiFERON®-CMV and iTAg™ MHC Tetramers supports the hypothesis that IE-1-specific $CD4^+$ T cells (and possibly $CD8^+$ T cells through cross-presentation) contribute to the detected signals. On the other hand, the increased proportion of IE-1-reactive cells over pp65-reactive cells in CMV-seronegative patients supports the idea of a recent exposure to CMV, as response to primary infection is usually dominated by IE-1-reactive (predominantly $CD8^+$) effector cells [6, 8–11]. Additional experiments will be necessary to address these propositions. Whatever the mechanism involved in the generation of CMV-reactive effector cells in CMV-seronegative patients, these observations raise the attractive possibility that these individuals might have a protective immunity against CMV infection. Clearly, further investigations are needed to address this possibility. Altogether, our data suggest that T-Track® CMV exhibits a performance superior to that of QuantiFERON®-CMV and of iTAg™ MHC Tetramers, and possibly also superior to that of CMV-IgG serology, for the detection of possible immunity against CMV.

Conclusions

T-Track® CMV represents a highly standardized and sensitive assay suitable for the monitoring of CMV-specific cell-mediated immunity in end-stage renal failure patients, representative of patients prior to renal transplantation. Further validation of T-Track® CMV in multi-center clinical studies on kidney and allogeneic stem cell transplant patients is currently on-going, to evaluate its use for the risk assessment and prediction of CMV-related clinical complications in transplant recipients. In these situations, monitoring of CMV-specific CMI could help physicians better define patient populations that would benefit from prophylactic antiviral therapy, and assist the decision as to when to withdraw prophylaxis safely. Reducing prophylactic antiviral treatment would be beneficial in limiting both treatment-related nephrotoxic side effects and costs.

Additional file

Additional file 1: Precision profiles of the specific response to IE-1 (A) and pp65 (B) in T-Track® CMV. A coefficient of variation (CV) no higher than 40% was used as a limit of acceptance of assay validity to determine the respective limit of quantitation (LoQ). LoQ values determined at CV = 40% for IE-1 (A) and pp65 (B) in the hemodialysis study ($n = 124$) using T-Track® CMV was 7.8 and 8.3 (SFC / 200,000 PBMC) respectively. Comparable LoQ values were obtained from T-Track® CMV assays performed on PBMC from 45 healthy donors [38]. Based on these analyses, a technical cut-off of 10 SFC / 200,000 PBMC was chosen. (PDF 334 kb)

Abbreviations

APC: Antigen-presenting cell; *a*protein: T-activated® protein; CMI: Cell-mediated immunity; CMV: Cytomegalovirus; CTL: Cytotoxic T lymphocyte; CV: Coefficient of variation; ELISA: Enzyme-linked immunosorbent assay; ELISpot: Enzyme-linked immunospot; HIV: Human immunodeficiency virus; HLA: Human leukocyte antigen; IE-1: Immediate early-1 protein; IFN-γ: Interferon gamma; LoQ: Limit of quantitation; NK: Natural killer cell; NKT: Natural killer T cell; PBMC: Peripheral blood mononuclear cell; pp65: 65 kDa lower matrix phosphoprotein; SD: Standard deviation; SFC: Spot-forming cell; Th: T helper cell

Acknowledgements

The authors are grateful to Theresa Spindler, Tamara Lugner, Charlotte Tonar and Astrid Starke for their expertise and technical assistance in laboratory testing as well as in data collection and analysis. We thank Dr. Thomas Keller (ACOMED Statistik) for statistical evaluation. T-Track® CMV tests were provided by Lophius Biosciences GmbH, Regensburg.

Funding

This project was funded in part by the Bayerische Forschungsstiftung Grants AZ 924-10 (to L.D.) and AZ 1070-13 (ForBiMed project D5 to R.W.) and by institutional funds of the University of Regensburg (ReForM C Project 03-082 to B.K.K., later transferred to B.B.). The funding body had no role in the design of the study and in collection, analysis and interpretation of data and in writing the manuscript.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional file.

Authors' contributions

LD, BB and BKK designed the study. JB supervised the study. BB, BKK, CB, GL, BK and JL obtained patient samples and collected the data. SB, MS and JK performed the assays and analyzed the data. RW supervised the interpretation and representation of data. AR and HB drafted the manuscript and figures. All authors edited and/or approved the final version.

Competing interests
SB, JB, HB, LD, AR and MS are or were employees of Lophius Biosciences GmbH at the time of the study. LD is co-founder and Chief Scientific Officer of Lophius Biosciences GmbH. RW is Chairman of the Board of Lophius Biosciences GmbH. RW, SB and LD are shareholders of Lophius Biosciences GmbH. The participating clinical and dialysis centers have received research funding from Lophius Biosciences GmbH for this study.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The study was conducted in accordance with the rules of the German Institute of Medical Documentation and Information (DIMDI). Patient enrolment was started after receiving the exemption of the permit requirement by the BfArM (Federal Institute for Drugs and Medical Devices) and approval by the ethics committee of the University of Regensburg (approval number 11-122-0205). All subjects gave written informed consent. The study was prospectively registered at clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT02630537).

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Received: 13 August 2016 Accepted: 10 February 2017
Published online: 07 March 2017

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