




ORIGINAL ARTICLE

Clinical validation of a novel enzyme-linked immunosorbent spot assay-based *in vitro* diagnostic assay to monitor cytomegalovirus-specific cell-mediated immunity in kidney transplant recipients: a multicenter, longitudinal, prospective, observational study

Bernhard Banas¹, Dominik Steubl², Lutz Renders², Dominik Chittka¹, Miriam C. Banas¹, Thomas Wekerle³, Martina Koch⁴, Oliver Witzke⁵, Anja Mühlfeld⁶, Claudia Sommerer⁷ , Antje Habicht⁸ , Christian Hugo⁹, Thomas Hünig¹⁰, Monika Lindemann¹¹, Traudel Schmidt¹², Anne Raschle¹² , Sascha Barabas¹², Ludwig Deml¹², Ralf Wagner^{12,13}, Bernhard K. Krämer¹⁴ & Bernd Krüger¹⁴

1 Department of Nephrology, University Medical Center Regensburg, Regensburg, Germany

2 Department of Nephrology, Klinikum rechts der Isar, Technical University Munich, Munich, Germany

3 Department of Surgery, Medical University of Vienna, Vienna, Austria

4 Transplantation Immunology Research Group, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

5 Departments of Nephrology and of Infectious Disease, University Hospital Essen, Essen, Germany

6 Uniklinik RWTH Aachen, Aachen, Germany

7 Division of Nephrology, University Hospital Heidelberg, Heidelberg, Germany

8 Transplantation Center, Ludwig-Maximilians-University Medical Center Munich, Munich, Germany

9 Department of Nephrology, Carl Gustav Carus University Medical Center Dresden, Dresden, Germany

10 Institute of Virology and Immunobiology, University Medical Center Würzburg, Würzburg, Germany

11 Institute for Transfusion Medicine, University Hospital Essen, Essen, Germany

SUMMARY

Impaired cytomegalovirus (CMV)-specific cell-mediated immunity (CMV-CMI) is a major cause of CMV reactivation and associated complications in solid-organ transplantation. Reliably assessing CMV-CMI is desirable to individually adjust antiviral and immunosuppressive therapy. This study aimed to evaluate the suitability of T-Track[®] CMV, a novel IFN- γ ELISpot assay based on the stimulation of peripheral blood mononuclear cells with pp65 and IE-1 CMV proteins, to monitor CMV-CMI following kidney transplantation. A prospective longitudinal multicenter study was conducted in 86 intermediate-risk renal transplant recipients. CMV-CMI, CMV viral load, and clinical complications were monitored over 6 months post-transplantation. Ninety-five percent and 88–92% ELISpot assays were positive pre- and post-transplantation, respectively. CMV-specific response was reduced following immunosuppressive treatment and increased in patients with graft rejection, indicating the ability of the ELISpot assay to monitor patients' immunosuppressive state. Interestingly, median pp65-specific response was ninefold higher in patients with self-clearing viral load compared to antivirally treated patients prior to first viral load detection ($P < 0.001$), suggesting that reactivity to pp65 represents a potential immunocompetence marker. Altogether, T-Track[®] CMV is a highly sensitive IFN- γ ELISpot assay, suitable for the immunomonitoring of CMV-seropositive renal transplant recipients, and with a potential use for the risk assessment of CMV-related clinical complications (ClinicalTrials.gov Identifier: NCT02083042).

Transplant International 2018; 31: 436–450

Key words

CMV-specific cell-mediated immunity, cytomegalovirus, IFN- γ ELISpot, immunomonitoring, *in vitro* diagnostic, kidney or renal transplantation

Received: 12 July 2017; Revision requested: 17 August 2017; Accepted: 19 December 2017; Published Online 16 January 2018

12 Lophius Biosciences, Regensburg,
Germany

13 Institute of Clinical Microbiology
and Hygiene, University Medical
Center Regensburg, Regensburg,
Germany

14 Vth Department of Medicine,
University Medical Center
Mannheim, Mannheim, Germany

Correspondence

Bernhard Banas MD, Department of Nephrology, University Medical Center Regensburg,
Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany.

Tel.: +49(0)941 944 7301;

fax: +49(0)941 944 7302;

e-mail: bernhard.banas@ukr.de

Introduction

Cytomegalovirus (CMV) infection is one of the most common complications after kidney transplantation [1–3]. In immunocompetent individuals, CMV-specific cell-mediated immunity (CMI) (thereafter referred to as CMV-CMI) is usually able to control primary and latent CMV infection [4–12]. Impairment of CMV-CMI in immunocompromised patients, such as renal transplant recipients under immunosuppressive medication, is a major cause for CMV reactivation and related complications [6,13–18]. Antiviral drugs are also associated with non-negligible side effects (e.g., nephrotoxicity and bone marrow suppression) and are expensive. Reliable assessment of CMV-CMI and of its functional impairment might help to individually adjust antiviral and immunosuppressive therapy, and thus improve patient care.

A number of assays have been described to monitor CMV-CMI, from CMV epitope-specific CD8⁺ staining [19,20] to functional assays monitoring the *ex vivo* response of CMV-specific effector cells [9,11,12,21–23]. Several studies investigated the ability of these assays to predict the risk of CMV replication and/or CMV disease after kidney transplantation [2,6,17,21,24–35]. Identifying a consensus between these studies is made difficult by the disparity in capability of the respective CMV-CMI monitoring assays (e.g., the nature of the stimulating antigens and of the monitored CMV-reactive effector cells) and by the absence of standardization in case of in-house assays.

T-Track® CMV is a novel, highly sensitive, and standardized IFN- γ enzyme-linked immunosorbent spot (ELISpot) assay measuring the response of a large spectrum of clinically relevant CMV-reactive effector cells (including T helper (Th) cells, cytotoxic T lymphocytes (CTL), as well as natural killer (NK) and natural killer T (NKT)-like cells via bystander activation) to immediate early-1 (IE-1) and phosphoprotein 65 (pp65) antigens [36,37]. Two recent studies validated its ability to monitor CMV-CMI in non-transplanted subjects [38,39]. Primary aim of this

prospective multicenter study was to determine the suitability and sensitivity of this novel ELISpot assay for the monitoring of CMV-CMI in intermediate-risk (D–/R+, D+/R+) renal transplant recipients. Secondary aim was to investigate a possible association between IFN- γ ELISpot test results and occurrence of clinical complications post-transplantation.

Patients and methods

Study design and participants

A prospective, longitudinal, observational, multicenter study was conducted in a cohort of 96 intermediate-risk (D–/R+, D+/R+) renal transplant recipients over 6 months post-transplantation. Patients receiving a standard immunosuppressive regimen (Table 1) and scheduled for preemptive antiviral therapy were eligible for study participation. Patients were scheduled for one pretransplantation (visit 1) and seven post-transplantation (visits 2–8) visits at 3-week intervals. Unscheduled visits took place in case of suspicion of CMV-related complications (study flowchart shown in Table S1). Additional information, such as ineligibility, study aims, definitions, and ethics committee approvals, may be found in Supporting Information (Appendix S1).

CMV-CMI measurement

Blood collection, peripheral blood mononuclear cell (PBMC) isolation, and T-Track® CMV assays (Lophius Biosciences GmbH, Regensburg, Germany) were performed as previously described [37,39], and as detailed in Supporting Information (Appendix S1).

Viral load measurement

Cytomegalovirus load in blood was evaluated either by quantitative PCR (CMV DNAemia) or by immunocytochemistry (pp65 antigenemia), as detailed in Supporting Information (Appendix S1).

Table 1. Patient characteristics; *N* = 86 (100%).

Gender, <i>N</i> (%)	
Male	59 (68.60%)
Female	27 (31.40%)
Age in years, mean \pm SD (range)	53.7 \pm 13.6 (20–78)
CMV serostatus, <i>N</i> (%)	
D+/R+	38 (44.19%)
D–/R+	48 (55.81%)
Induction therapy (Basiliximab), <i>N</i> (%)	
Yes	53 (61.63%)
No or not documented	33 (38.37%)
Immunosuppressive regimen, <i>N</i> (%)	
CNI & MMF/MPA & steroid	67 (77.91%)
CNI & MMF/MPA & steroid & other	1 (1.16%)
MMF/MPA & steroid	14 (16.28%)
CNI & steroid & mTOR inhibitor	2 (2.33%)
CNI & mTOR inhibitor	1 (1.16%)
CNI & steroid & other	1 (1.16%)
Patients with end-organ CMV disease, <i>N</i> (%)	0 (0.00%)
Patients with CMV syndrome, <i>N</i> (%)	4 (4.65%)
Patients with at least one recorded CMV event*, <i>N</i> (%)	28 (32.56%)
Time to onset of CMV event* in days, median (range)	48 (14–145)
Patients with at least one recorded CMV viral load (VL > 0), <i>N</i> (%)	49 (56.98%)
Time to onset of first CMV VL in days, median (range)	41 (14–145)
Patients with infections other than CMV, <i>N</i> (%)	
Any infection	36 (41.86%)
BKV	9 (10.47%)
Urinary tract infection	19 (22.09%)
Bacteria	10 (11.63%)
Fungi	2 (2.33%)
EBV	0 (0.00%)
Other	5 (5.81%)
Delayed graft function	10 (11.63%)
Graft rejection (Banff '09)	13 (15.12%)
T cell-mediated rejection (TCMR; scores: IA, 2; IIA, 5; IIB, 1)	8 (9.30%)†
Borderline changes (suspicious for TCMR)	4 (4.65%)†
Antibody-mediated rejection (ABMR)	1 (1.16%)
Unknown	1 (1.16%)
Time to onset of graft rejection in days, median (range)	34.5 (3–140)
Graft loss	1 (1.16%)

D/R, donor/recipient; D–, CMV-seronegative graft donor; D+, CMV-seropositive graft donor; R+, CMV-seropositive graft recipient; VL, viral load; EBV, Epstein–Barr virus; MMF/MPA, mycophenolate mofetil/mycophenolic acid; CMV, cytomegalovirus; BKV, BK virus; CNI, calcineurin inhibitor; mTOR, mammalian target of rapamycin.

*Defined as CMV viral load requiring treatment (also designated as “CMV complications”), as per investigator’s assessment.

†One patient experienced three rejection episodes (one TCMR and two borderline changes), thus contributing to both categories.

Other variables

Cytomegalovirus-related clinical complications, that is CMV syndrome and end-organ CMV disease [3,40,41], were documented together with occurrence of opportunistic infections (BKV, urinary tract infection, bacteria, fungi, EBV, other), graft dysfunction (rejection or loss), and death. Diagnosis of graft rejection was

biopsy-based and followed the Banff classification of renal allograft pathology [42].

Statistical analysis

Calculations were performed with SAS 9.4 Software, and figures were generated using GRAPHPAD PRISM, as detailed in Supporting Information (Appendix S1). Differences

in IE-1- and pp65-specific spot-forming cell (SFC) distributions between groups with independent samples were tested using the nonparametric two-sided Mann–Whitney U (MWU) test. Comparison of groups with paired samples was performed using the nonparametric two-sided Wilcoxon signed-rank test. In case of comparison of groups with multiple measurements per patient, analysis of variance (ANOVA) on ranks tests was conducted, as indicated. Two-sided *P*-values <0.05 were considered statistically significant.

Results

Patient characteristics

Altogether, 96 CMV-seropositive patients with end-stage renal failure were enrolled in the study prior to or following kidney transplantation. Ten of the 96 intermediate-risk (D−/R+, D+/R+) renal transplant recipients had no ELISpot data acquired, as illustrated in the study flow diagram (Fig. S2). Blood from the remaining 86 patients was collected to conduct IFN- γ ELISpot assays at a minimum of one scheduled visit. Circulating CMV viral load (VL) was determined at most 3-week interval post-transplantation visits. An overview of IE-1- and pp65-specific ELISpot results and of VL levels for the 86 individual patients is presented in Fig. S1. Patients' demographics, immunosuppressive regimen, transplantation characteristics, and recorded complications are summarized in Table 1. Of 86 patients, 49 manifested at least one positive VL and 28 experienced at least one CMV complication (positive VL requiring treatment as per investigator's assessment). In four patients, a CMV syndrome was diagnosed, but no end-organ CMV disease occurred. Thirty-eight patients experienced infections other than CMV, and 13 patients showed at least one graft rejection (Table 1).

IFN- γ ELISpot measures CMV-CMI with high sensitivity before and after renal transplantation

The suitability of T-Track® CMV ELISpot assay to monitor CMV-CMI in renal transplant recipients under immunosuppressive therapy was addressed by analyzing IE-1- and pp65-specific SFC levels at all documented visits (Fig. 1). SFC levels were highly variable between patients, ranging from 0.1 to 866 SFC/200 000 cells in IE-1-stimulated conditions and from 0.1 to 1059 SFC/200 000 cells in pp65-stimulated conditions. As previously shown [37–39], median SFC levels were overall lower in IE-1 than in pp65 stimulations. Highest median SFC count was observed pretransplantation (visit 1) for

both IE-1 and pp65 stimulations (25 and 206 SFC/200 000 cells, respectively), prior to the start of immunosuppressive therapy. Lowest median SFC count was observed at visit 2 (median day 20 after transplantation) for IE-1-stimulated conditions (9 SFC/200 000 cells) and at visit 4 (median day 63 after transplantation) for pp65-stimulated conditions (114 SFC/200 000 cells) (Fig. 1). Similar patterns were observed in the whole cohort (*n* = 86; including missing visits) and in the limited set of patients with all eight completed visits (*n* = 7; data not shown), indicating no major bias due to missing visits.

Analysis of qualitative (positive versus negative) test results revealed a proportion of 95% positive tests before transplantation and of 88% to 92% positive tests post-transplantation (Table 2). As previously described [37–39], pp65-specific measurements contributed to the majority of positive test results while IE-1-specific measurements improved the proportion of positive assays by approximately 2–6% points (Table 2). Analysis of ELISpot test results in relation to recipient's HLA class I antigens, notably as to whether associated with the presentation of immunodominant IE-1 and/or pp65 epitopes (Tables S2 and S3), demonstrated comparable frequencies of positive test results, regardless of HLA antigens (Table 3).

Altogether, the IFN- γ ELISpot assay can measure CMV-CMI before and after kidney transplantation with high sensitivity and in a HLA-I antigen-independent manner.

CMV-CMI is reduced following treatment with immunosuppressive agents

Treatment with immunosuppressive agents is expected to impair CMV-CMI. Accordingly, median IE-1- and pp65-specific SFC levels decreased by approximately 60% and 40%, respectively (Fig. 1), and the percentage of IFN- γ ELISpot-positive test results diminished by 4 percentage points, from 95% to 91% (Table 2) between visit 1 and visit 2.

To further assess the effect of immunosuppressive therapy on IFN- γ ELISpot test results, post-transplantation IE-1- and pp65-specific SFC levels were compared before and after treatment with high-dose steroid. Median IE-1- and pp65-specific SFC were globally reduced by approximately 60% and 40%, respectively, at visits following treatment with ≥ 1 mg/kg prednisolone equivalent (Fig. 2a). Analysis of after-to-before ratios of paired samples confirmed a decrease in SFC levels, consistently detected in 19 of 27 (70%) and 20 of 26 (77%) of IE-1 and pp65 stimulations, respectively (Fig. 2b). A reduction (30–40%) in SFC levels was even

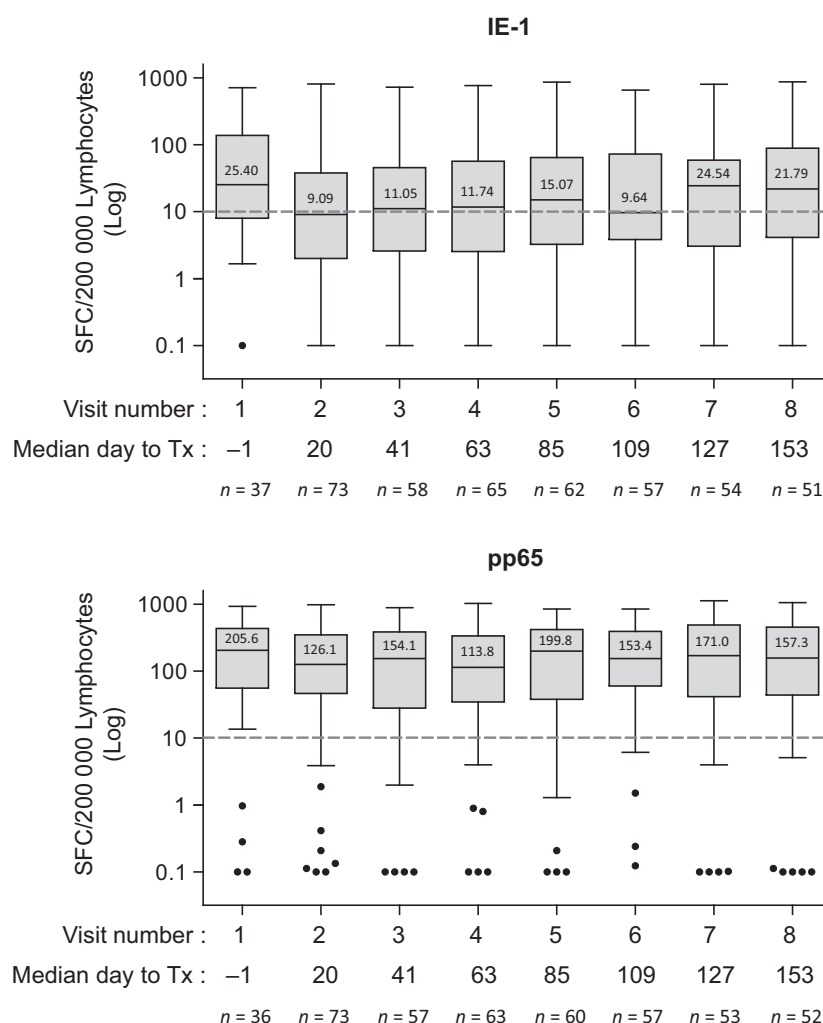


Figure 1 IFN- γ ELISpot can measure IE-1- and pp65-specific cell-mediated immunity (CMI) prior to (visit 1) and following (visits 2–8) kidney transplantation. IE-1- and pp65-specific ELISpot results are expressed as the number of spot-forming cells (SFC) per 200 000 lymphocytes and are shown for all documented visits, both prior to (visit 1) and after (visits 2–8) kidney transplantation (Tx) and start of immunosuppressive regimen. The dashed line indicates the positivity threshold of the T-Track[®] cytomegalovirus (CMV) assay (10 SFC/200 000 cells), according to the manufacturer's instructions [37]. T-Track[®] CMV IFN- γ ELISpot can measure CMV-specific cell-mediated immunity (CMI) both before and after kidney transplantation. Highest median SFC level for both IE-1- and pp65-specific CMI was observed prior to transplantation (25.4 and 205.6, respectively). At the first visit post-transplantation (visit 2; 13 to 30 days post-transplantation) IE-1- and pp65-specific median SFC was reduced by 64% and 39%, respectively, in comparison with the pretransplantation visit. Median SFC levels slightly increased thereafter, yet remained below the pretransplantation level. The corresponding data expressed as percentage (%) of positive test results are shown in Table 2. Indicated n values represent the number of measurements (not exceeding one per patient per visit).

detectable in patients under high-dose (≥ 10 mg) steroid compared to patients under low-dose (< 10 mg) steroid (Fig. 2c). These observations indicate that the ELISpot assay can monitor a functional impairment in CMV-CMI induced by immunosuppressive agents.

CMV-CMI is increased following occurrence of CMV events

Cytomegalovirus-CMI in CMV-seropositive immunocompetent subjects is expected to be boosted following

a new encounter with CMV or following CMV reactivation, via the *in vivo* stimulation of CMV-reactive memory T cells and of the innate arm of cellular immunity [4,6–8,36,43–47]. To determine whether activation of cell-mediated immunity in immunosuppressed CMV-seropositive renal transplant recipients can be monitored by T-Track[®] CMV, IE-1- and pp65-specific SFC levels were examined at post-transplantation visits affected by CMV complications (i.e., positive viral load requiring treatment) and at visits following CMV complications. Median IE-1-specific

Table 2. Frequency of T-Track® CMV-positive test results per visit.

Visit number	Time to transplantation in days, median (range)	Number of patients	IE-1-specific SFC, N (%)	pp65-specific SFC, N (%)	T-Track® CMV*, N (%)
1	−1 (−3 to 0)	42	25/37 (67.6%)	33/37 (89.2%)	35/37 (94.6%)
2	20 (13–30)	83	37/74 (50.0%)	66/74 (89.2%)	68/75 (90.7%)
3	41 (21–51)	74	33/59 (55.9%)	51/60 (85.0%)	55/61 (90.2%)
4	63 (42–76)	72	35/66 (53.0%)	55/64 (85.9%)	59/67 (88.1%)
5	85 (63–113)	71	38/64 (59.4%)	57/63 (90.5%)	59/64 (92.2%)
6	109 (89–120)	67	28/57 (49.1%)	51/57 (89.5%)	53/58 (91.4%)
7	127 (102–147)	63	35/55 (63.6%)	47/54 (87.0%)	49/55 (89.1%)
8	153 (126–172)	60	32/52 (61.5%)	45/53 (84.9%)	48/53 (90.6%)

CMV, cytomegalovirus.

*According to manufacturer's instruction (IE-1- and/or pp65-specific positive test results).

Table 3. T-Track® CMV-positive test results in individuals with HLA class I antigens presenting immunodominant CMV IE-1- and/or pp65-specific epitopes ($n = 86$), N (%).

	Presence of the respective HLA antigens*		
	Yes (%)	No (%)	Missing (%)
IE-1-specific SFC	46/67 (68.7)	12/16 (75.0)	3/3 (100)
pp65-specific SFC	75/78 (96.2)	4/5 (80.0)	3/3 (100)
T-Track® CMV†	76/79 (96.2)	4/4 (100)	3/3 (100)

CMV, cytomegalovirus.

*Defined as at least one epitope for IE-1 (IE-1-specific SFC), for pp65 (pp65-specific SFC), and for IE-1 and/or pp65 (T-Track® CMV) (see Tables S2 and S3).

†Performed according to manufacturer's instructions.

SFC was approximately 4 times higher at visits documented with CMV complications (44 vs. 12 SFC/200 000 cells; MWU $P = 0.007$; ANOVA on ranks $P = 0.038$), while median pp65-specific SFC levels were comparable at visits with and without CMV events (116 vs. 151 SFC/200 000 cells, respectively; MWU $P = 0.497$) (Fig. 3a). On the other hand, IE-1- and pp65-specific test results were increased in 11 of 14 (79%) and 12 of 14 (86%) of paired samples, respectively, following CMV complications, with median ratios after-to-before CMV complication of 1.8 (Wilcoxon signed-rank test, $P = 0.009$) and 2.4 (Wilcoxon signed-rank test, $P = 0.007$), for IE-1 and pp65, respectively (Fig. 3b). These observations suggest that the IFN- γ ELISpot assay can monitor changes in CMV-CMI consecutive to manifestations of CMV complications, upon *ex vivo* stimulation of PBMC with IE-1 and pp65 antigens.

Elevated CMV-CMI is associated with occurrence of graft rejection

Cytomegalovirus infection has been associated with increased allograft rejection following solid-organ transplantation (SOT) [48–51]. In this cohort, 15 rejection episodes (in 13 patients) were documented (Table 1). Of 15 rejection events, 14 were not associated with detection of positive VL at the visit prior to or at visit of rejection (data not shown), suggesting no association between CMV infection and graft rejection. Another cause for graft rejection is under-immunosuppression, for instance due to strong baseline immunity and/or poor responsiveness to immunosuppressive therapy [52]. According to the Banff classification [42], eight cases of T-cell-mediated rejections (TCMR) and five borderline changes (defined as suspicious for TCMR) were documented (Table 1), suggesting an association

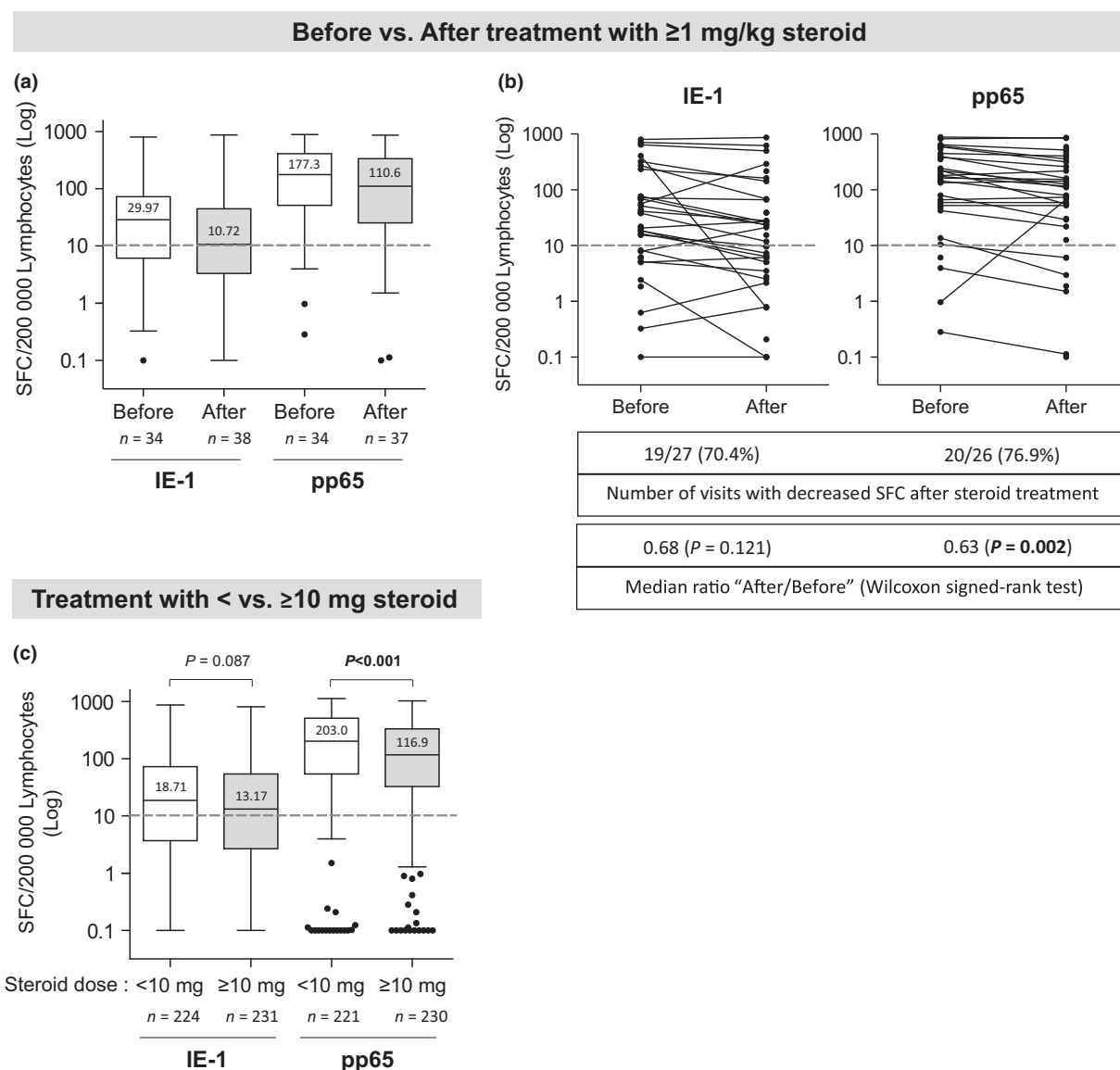


Figure 2 IE-1- and pp65-specific cell-mediated immunity (CMI) is reduced upon high-dose steroid treatment. The effect of high-dose steroid treatment on IE-1- and pp65-specific ELISpot results post-transplantation (visits 2-8) was analyzed by comparing spot-forming cell (SFC) levels before and after treatment with ≥ 1 mg/kg steroid (a, b) and by comparing SFC levels at visits affected by treatment with $<$ and ≥ 10 mg steroid (c). IE-1- and pp65-specific ELISpot results at any visits before and after high-dose steroid treatment are represented as box plots (a) as well as spaghetti plots of paired visits (b). In (b), "After/Before" median ratios of IE-1- and pp65-specific CMI were tested against the hypothetical value of 1.0 using the nonparametric paired Wilcoxon signed-rank test. In (c), differences in IE-1- and pp65-specific CMI between visits under lower and higher steroid doses were tested using the nonparametric Mann-Whitney U test. However, P values should be considered with caution given the bias introduced by the existence of multiple visits with high-dose steroid per patient. Indicated n values represent the number of measurements (a, c) and of paired measurements (b).

of graft rejection with cellular immunity. Interestingly, median IE-1- and pp65-specific SFC counts were approximately sixfold and threefold higher, respectively, at visits with future (within the next 6 weeks) graft rejection compared to those with no future graft rejection (Fig. 4a; $n = 8$). Moreover, among the 13 patients who experienced graft rejections, those with TCMR consistently showed higher IE-1- and pp65-specific CMI

at any visit prior to and including the visit with documented TCMR, compared to visits with other types of rejection (Fig. 4b). IE-1- and pp65-specific CMI reached high median SFC counts (330 and 619 SFC/200 000 cells, respectively) in association with future TCMR (Fig. 4b). Notably, the analysis of paired IE-1- and pp65-specific results revealed a higher proportion (14/18 or 78%) of double-positive test results in association

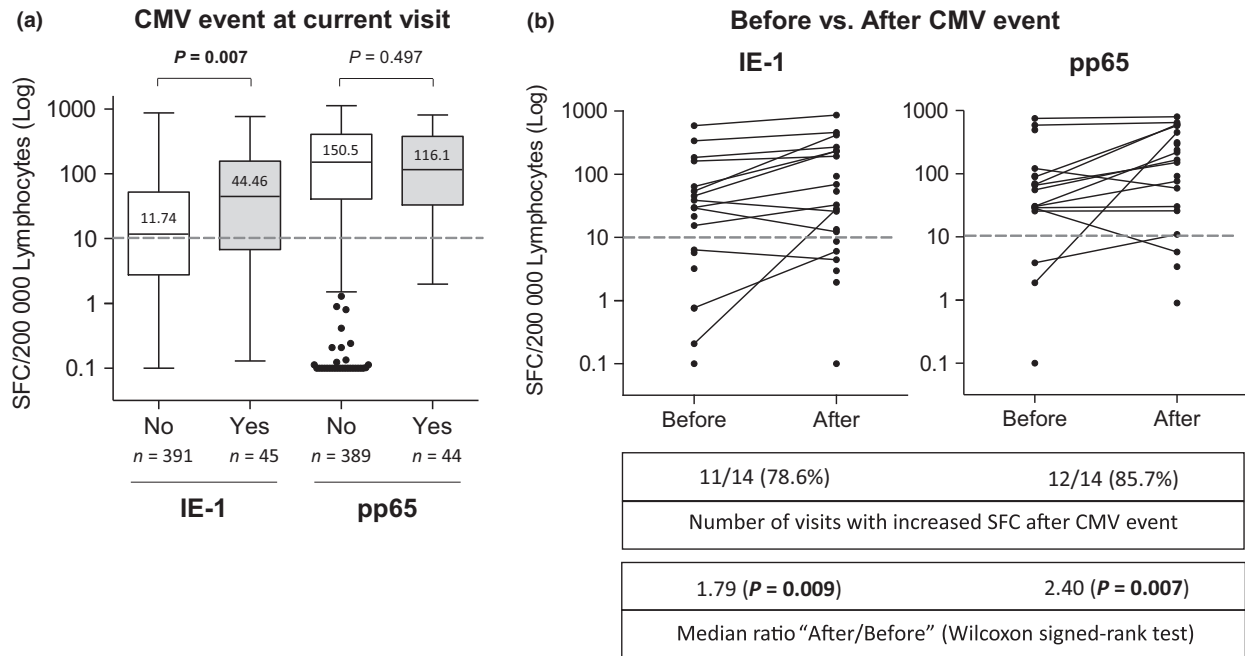


Figure 3 IE-1- and pp65-specific cell-mediated immunity (CMI) is increased following occurrence of cytomegalovirus (CMV) complications. (a) IE-1- and pp65-specific CMI post-transplantation at visits affected by CMV events. N values represent the number of measurements. Differences in IE-1- and pp65-specific CMI between groups with (Yes) and without (No) CMV complication were tested using the nonparametric Mann–Whitney U test. However, p values should be taken with caution given the bias introduced by the consideration of multiple visits affected by a CMV event per patient. An ANOVA on ranks statistical test taking into account multiple measurements resulted in $P = 0.038$ and $P = 0.725$ for IE-1- and pp65-specific CMI in "No" versus "Yes," respectively. Median IE-1-specific CMI was 3.8 times higher at visits with CMV complications (MWU $P = 0.007$; ANOVA on ranks $P = 0.038$). (b) IE-1- and pp65-specific CMI at paired "Before" and "After" visits with documented CMV events. N values in the table indicate the number of paired measurements. "After/Before" median ratios of IE-1- and pp65-specific CMI were tested against the hypothetical value of 1.0 using the nonparametric paired Wilcoxon signed-rank test ($P = 0.009$ and $P = 0.007$, respectively). Of note, 28 patients experienced CMV complications (Table 1), documented as one event in 27 of 28 patients and as two events in one of 28 patients.

with future TCMR, compared to TCMR-independent rejection (1/6 or 17%) (Fig. S3). Altogether, these observations indicate that the IFN- γ ELISpot detects elevated CMV-CMI in association with allograft rejection, and more particularly T-cell-mediated rejection.

Reduced pp65-specific CMI is associated with occurrence of opportunistic infections

Opportunistic infections (OI) are a common cause of clinical complications following SOT [3,49]. Among them, BK virus (BKV) infection, responsible for BKV nephritis, is a frequent cause of impaired renal function in kidney transplant recipients [53]. OI mainly occur as a consequence of immunosuppressive therapy (over-immunosuppression) [54]. In addition, CMV infection itself is associated with increased OI incidence [3,49,54]. In this study, 38 patients were subject to infections other than CMV (51 events), and nine patients manifested one episode of BKV infection (Table 1). Thirty-

four of 51 (67%) infection episodes took place in patients with positive CMV VL. However, the time frame of occurrence of other versus CMV infections was distinct (11/34 or 32% within -1 to $+1$ week of CMV infection). These findings indicate a general susceptibility to infection rather than a close association with CMV infection. No association with CMV infection was observed in case of BKV alone (five of nine or 56% BKV infections in patients with CMV infection), maybe due to the small case number. Interestingly, clearance of BKV infection is known to be strongly dependent on cellular immunity [53,55,56]. The analysis of pp65-specific reactivity after kidney transplantation in relation to the occurrence of BKV and of any infection at current or at future (within the next 6 weeks) visits revealed that median pp65-specific SFC values were decreased by 50–80% in association with BKV and any infections (ranging from 158–170 to 28–91 SFC/200 000 cells in the absence and presence, respectively, of current or future infection) (Fig. 5).

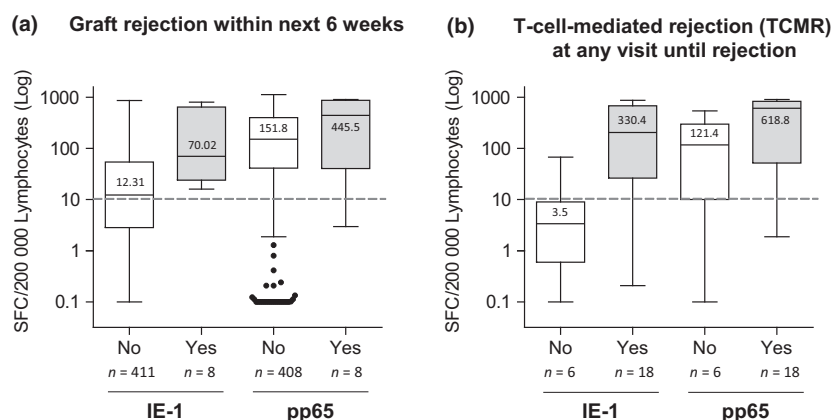


Figure 4 Increased IE-1- and pp65-specific cell-mediated immunity (CMI) is associated with future occurrence of graft rejection. (a) IE-1- and pp65-specific CMI was evaluated at post-transplantation visits not affected by graft rejection with respect to occurrence (Yes) or no occurrence (No) of graft rejection within the next 6 weeks. Of note, 13 patients were affected by graft rejection (Table 1), corresponding to 15 documented graft rejection events (due to one patient with three rejections). This analysis considers IFN- γ ELISpot results post-transplantation (visits 2–8) and is thus missing graft rejection events taking place at visit 2, thus explaining the low number of measurements ($n = 8$) in the “Yes” group. Consequently, no statistical comparison of groups with and without future graft rejection was performed. Median IE-1- and pp65-specific CMI is 5.7-fold and 2.9-fold higher in case of graft rejection within the next 6 weeks. (b) Occurrence of T-cell-mediated rejection (TCMR) was associated with increased IE-1- and pp65-specific CMI compared to graft rejection classified under other Banff criteria [42]. IE-1- and pp65-specific CMI was assessed at all post-transplantation visits preceding and including graft rejection by TCMR (Yes) or by other rejection criteria (No). Of note, eight of 13 patients with graft rejection were documented with TCMR. This analysis includes multiple measurements per patient and a low number of measurements ($n = 6$ in the “No” group); therefore, no statistical comparison of groups was performed. The corresponding paired IE-1- and pp65-specific IFN- γ ELISpot results investigating the proportion of double-positive test results in the “No” and “Yes” groups are shown in Fig. S3.

Elevated pp65-specific CMI predicts self-clearance of CMV reactivation (*post hoc* analysis)

In terms of patient management, one expects from a diagnostic assay to assist clinicians in decision-making for the treatment of patients at risk of developing CMV disease. Due to the absence of documented CMV disease in this cohort, *post hoc* analyses were conducted, investigating CMV-CMI in relation to CMV reactivation (existence of positive VL). Patients with positive VL and who did not receive antiviral treatment (self-limiting CMV reactivation) were extracted from the initial “no CMV event” group, thus defining three groups: (i) patients with VL = 0 (untreated), (ii) patients with VL > 0, untreated (self-limiting CMV reactivation), and (iii) patients with VL > 0 and antivirally treated (i.e., “CMV event” group). From the full analysis set ($n = 86$), five patients from the “untreated” category but who incidentally received antiviral prophylaxis (Fig. S2) and two patients with no documented VL measurements were excluded (*post hoc* analysis on $n = 79$ patients).

As a general comment, the mean age (\pm SD) of patients without versus with CMV infection post-transplantation was 48.5 (\pm 12.8) vs. 57.6 (\pm 6.5) years (MWU; $P = 0.022$) and correlated with higher median SFC

counts (18.33 vs. 36.52 for IE-1 and 177.3 vs. 351.4 for pp65) pretransplant, although the difference was not statistically significant (MWU; $P = 0.339$ for IE-1 and $P = 0.285$ for pp65). This suggests that the higher SFC count pretransplantation might be due to CMV-triggered age-related T-cell inflation.

Interestingly, two of two (100%) patients with self-cleared VL post-transplantation had double-positive (IE-1 and pp65) ELISpot test results pretransplantation, compared to 12 of 21 (57%) and eight of 11 (73%) in the no and treated VL groups, respectively (Fig. S4a). Post-transplantation, median IE-1-specific SFC was 5–6 times higher among patients with treated VL (“CMV event”) compared to the two other groups (MWU $P < 0.001$; ANOVA on ranks $P = 0.021$ between untreated and treated VL), despite broad SFC distribution in all three groups (Fig. S4b). This difference indicates significant changes in the response of IE-1-reactive effector cells in patients with higher VL. Remarkably, median pp65-specific CMI was highest in patients with self-cleared VL, both before and after transplantation (Fig. S4a,b). Similarly, median pp65-specific SFC among patients with self-limiting VL was approximately two-fold higher at visits with no VL detection (457 SFC/200 000 cells) compared to visits with positive VL (196 SFC/200 000 cells) (Fig. 6a). In contrast, median IE-1-

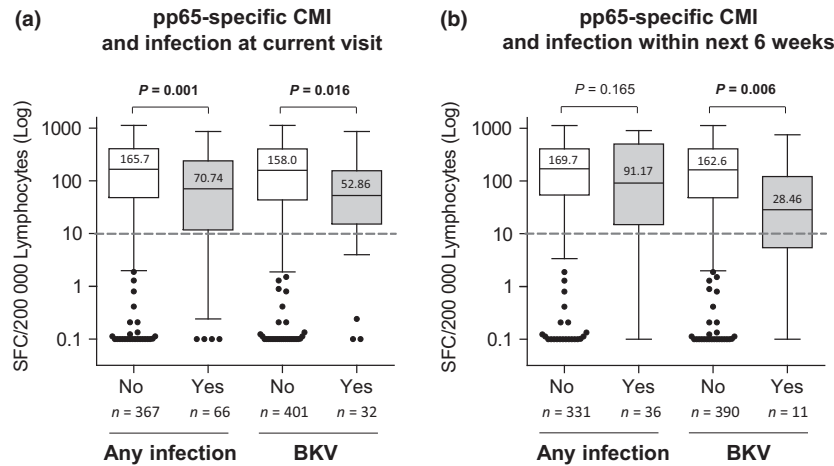


Figure 5 Decreased pp65-specific cell-mediated immunity (CMI) is associated with occurrence of opportunistic infections. pp65-specific CMI at post-transplantation visits was analyzed in relation to occurrence of either any infection other than cytomegalovirus (CMV) or BK virus (BKV) infection. pp65-CMI is presented at visits affected by infection (a) and at visits not affected by infection with respect to occurrence (Yes) or no occurrence (No) of infection within the next 6 weeks (b). N values represent the number of measurements. As several visits were affected by infection, these analyses take into consideration multiple measurements per patients. Differences in pp65-specific CMI between “Yes” and “No” groups were tested using the nonparametric Mann–Whitney U (MWU) test. *P* values should be considered with caution given the potential bias resulting from the consideration of several measurements per patient. ANOVA on ranks tests taking into account multiple measurements yielded *P* values >0.05. Occurrence of any infection other than CMV or of BKV infection at current visit or in the future was consistently associated with a 46.3–82.5% decrease in median pp65-specific CMI.

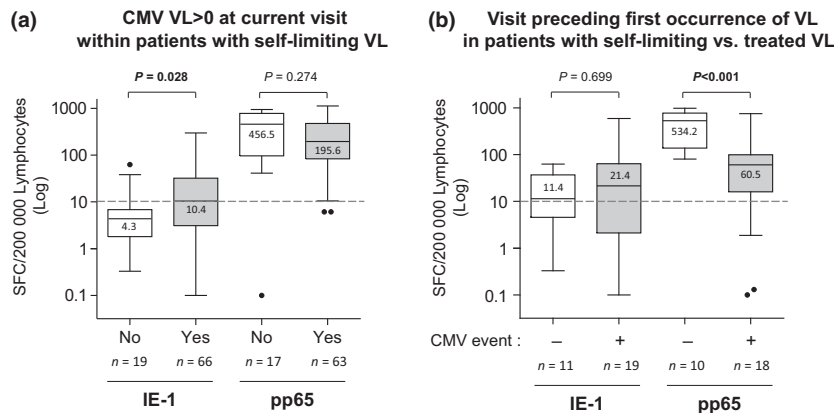


Figure 6 pp65-specific cell-mediated immunity (CMI) prior to first detection of viral load is higher in patients with self-limiting cell-mediated immunity (CMV) reactivation (*post hoc* analysis; *n* = 79 patients). (a) IE-1- and pp65-specific CMI among untreated patients with positive viral load (VL) (defined as patients with “self-limiting” CMV reactivation), at any visit post-transplantation with documented positive VL (“Yes” versus “No”). N values represent the number of measurements. This analysis includes several visits per patients, possibly introducing a bias in the group comparison using a MWU test. An ANOVA on ranks test taking into account multiple measurements resulted in *P* = 0.031 and *P* = 0.625 for IE-1- and pp65-specific CMI in “No” versus “Yes,” respectively. (b) IE-1- and pp65-specific CMI at visit preceding first detection of VL in untreated patients (“self-limiting VL”; white boxes) versus antivirally treated patients (“CMV event” definition; gray boxes). This analysis considers no more than one measurement per patient and is therefore devoid of the bias associated with the consideration of multiple measurements per patient. Groups were compared using a MWU test. Median pp65-specific CMI was 8.8 times higher in patients with self-limiting VL at the visit preceding first occurrence of VL (*P* < 0.001). Additionally, median pp65-specific SFC inversely correlated with VL levels (Fig. S5). These observations suggest that pp65-specific CMI might be a marker of immunocompetence.

specific SFC in patients with self-limiting VL was low at both visits without and with positive VL (4.3 and 10.4 SFC/200 000 cells, respectively), while respective SFC distributions were significantly different (range 0.3–63 and 0.1–298 SFC/200 000 cells, respectively; MWU

P = 0.028; ANOVA on ranks *P* = 0.031) (Fig. 6a). Thus, while IE-1-specific IFN- γ ELISpot can monitor changes in the activation of IE-1-reactive cells following CMV replication, IE-1-induced SFC level does not correlate with CMV replication.

Finally, the potential prognostic value of the ELISpot assay was evaluated by assessing IE-1- and pp65-specific SFC distributions at the visit preceding the first detection of positive VL among patients with self-limiting VL versus those treated with antivirals ("CMV event" group). As before, IE-1-specific SFC distribution was wide and skewed in both groups of patients with CMV reactivation prior to first detection of VL (Fig. 6b). In addition, IE-1-specific SFC levels in these two groups did not correlate with VL levels (Fig. S5), indicating that IE-1-specific test results prior to detection of CMV replication reflect broad changes in the response of IE-1-reactive cells but is not predictive of CMV reactivation. Strikingly however, pp65-specific SFC levels were ninefold higher in patients with self-controlled VL (median 534 SFC/200 000 cells, range 80–982; $n = 10$) compared to patients with treated VL (median 60 SFC/200 000 cells, range 0.1–751; $n = 18$) (MWU $P < 0.001$) prior to first detection of VL (Fig. 6b). In addition, pp65-specific SFC levels were inversely correlated to VL levels (Fig. S5). Remarkably, the proportion of patients showing a pp65-specific CMI above the arbitrary value of 100 SFC/200 000 lymphocytes (Fig. S5; red dashed line) was 9/10 (90%) among patients with self-controlled CMV reactivation (VL < center-specific limit), 3/8 (38%) among treated patients with VL < center-specific limit, and 1/10 (10%) among treated patients with VL > center-specific limit (Fig. S5).

Discussion

This study describes the IFN- γ ELISpot T-Track[®] CMV as a highly sensitive and suitable assay to monitor CMV-CMI in immunocompromised renal transplant recipients. This high proportion of positive test results is likely due to the stimulation of a broad spectrum of CMV-specific effector cells by T-activated[®] antigens, combined with the consideration of both pp65- and IE-1-specific responses [36,37,39,57].

The immunosuppressive state is determinant in defining clinical outcomes in SOT recipients. This "net state of immunosuppression" [54] is the result of the balance between overimmunosuppression, resulting in infections such as CMV, and underimmunosuppression, associated with increased risk of graft rejection or loss. Proper evaluation and monitoring of patients' immunosuppressive state are therefore critical. IFN- γ ELISpot SFC levels were consistently reduced following immunosuppressive therapy, demonstrating the assay's ability to monitor the response of patients to

immunosuppression. On the other hand, IFN- γ ELISpot test results were increased in most patients following CMV reactivation, as expected from the reactivation of CMV-specific memory T cells. Increased pp65-specific CMI after but not at visits affected by CMV events, as opposed to IE-1-specific CMI, might reflect the kinetics of CMV reactivation, IE-1 being an immediate-early gene and acknowledged marker of early CMV reactivation as opposed to the late antigen pp65 [10,58,59]. It might additionally reflect the dynamics of IE-1- and pp65-specific CD8⁺ and CD4⁺ T-cell responses during CMV infection and reactivation [6–8,10]. That most patients were responders in the ELISpot assay correlated nicely with the absence of clinical outcome in this cohort, further suggesting that the majority of patients still presented a protective immunity against CMV. Likewise, this finding indicates that the IFN- γ ELISpot assay might identify nonresponders as patients potentially over-immunosuppressed. Conversely, ELISpot test results were increased prior to graft rejection. As 13 of 15 (87%) of graft rejection episodes were attributed to or suspicious for TCMR, the ELISpot assay might be able to monitor strong cellular immunity in potentially under-immunosuppressed patients. The absence of standardized immunosuppressive therapy in this study did not allow to directly address whether immunosuppressant doses correlated to the occurrence of infections and graft rejections. Finally, changes in CMV-CMI in relation to occurrence of opportunistic infections were similar to those detected in association with CMV-related complications, indicating that IFN- γ ELISpot results, notably in response to pp65, might not only reflect CMV-CMI but more globally the patient's immunocompetence status and susceptibility to infection.

Altogether, these analyses demonstrate that T-Track[®] CMV is a sensitive assay enabling the monitoring of the immune status of renal transplant recipients. Additional studies will be necessary to determine whether it might assist clinicians in their decision to adjust immunosuppressive therapy. Interestingly, a randomized, prospective study is currently assessing the ability of measuring CMV-specific T cells to steer immunosuppressive therapy in pediatric kidney transplant recipients [60].

An essential goal of a CMV-CMI monitoring assay is to enable risk stratification of future CMV-related complications in order to improve clinical management of patients. Due to the absence of documented CMV disease in this cohort, a clinical cutoff could not be defined. Instead, ELISpot results were analyzed in terms

of prediction of spontaneous clearance of CMV viral load (VL) among patients with CMV reactivation. It should be emphasized that the comparison of patients with self-cleared VL to those with treated VL was in part biased by the fact that a number of treated patients with low VL might have undergone self-clearance in the absence of treatment (Patient 037 in Fig. S1 might be one such example). Nevertheless, we observed significantly higher pp65-specific SFC counts in patients with self-limiting VL ($n = 10$) compared to antivirally treated patients ($n = 18$) at the visit preceding the first detection of VL ($P < 0.001$), supporting the proposition that pp65-specific SFC is a marker of immunocompetence. Interestingly, 90% (9/10) of patients with self-cleared VL had pp65-specific test results above 100 SFC/200 000 lymphocytes at the visit preceding first detection of VL, as opposed to 22% (4/18) among antivirally treated patients. Additional studies should be performed, including high-risk renal transplant recipients, to confirm this observation and identify a potential clinical cutoff of protection against CMV-related clinical complications. Supporting the proposition that pp65-specific IFN- γ ELISpot results mirror CMV-CMI, pp65-specific SFC prior to first detection of VL negatively correlated with VL level, in agreement with reports of an inverse correlation between CMV-CMI and CMV viremia in renal transplant recipients [13,15,21].

As opposed to pp65, IE-1-specific SFC levels post-transplantation did not correlate with VL levels and did not mirror CMV-CMI, although significant changes in IE-1-specific ELISpot test results were observed following CMV reactivation. Accordingly, while pp65-specific SFC levels remained relatively stable over time at the level of individual patients, IE-1-specific SFC showed greater variability (e.g., patients 020, 058, 082, or 096; Fig. S1). These broad changes likely reflect the dynamics and kinetics of response of IE-1-responsive effector cells characteristic of CMV infection and/or reactivation [6–8,10,11,28,58,59,61], and might explain the lack of predictive value of the response to IE-1 antigen noted in this study. Further investigations are necessary to determine whether monitoring CMV-CMI more frequently might reveal a predictive value of IE-1-induced ELISpot results.

On the other hand, pretransplantation IE-1-specific response, together with pp65-specific response, might have some predictive value for self-cleared viremia post-transplantation, based on our observation that both patients with self-limited VL whom were tested in ELISpot before transplantation showed double-positive

T-Track[®] CMV test results. This would confirm studies suggesting that pretransplantation CMV-CMI can predict the risk of occurrence of CMV complications after kidney transplantation [24–27]. A survey of a larger cohort of paired pre- and post-transplantation IFN- γ ELISpot assays should address this proposition. A predictive value of both IE-1- and pp65-specific test results pretransplantation combined with a predictive value of pp65-specific test results post-transplantation, together with CMV viral load measurement, would considerably improve risk stratification of renal transplant recipients and guide decision-making of antiviral treatment.

In conclusion, this study confirmed T-Track[®] CMV as a valuable immune-monitoring tool to assess CMV-CMI but also the general immunosuppressive state of CMV-seropositive patients, especially in the setting of renal transplantation. Importantly, this study identified pp65-specific CMI as a potential immunocompetence marker. This work also confirmed the value of IE-1-specific CMI measurement for assay performance, contributing to a gain of up to 6 percent points in positive test results. In combination with CMV viral load monitoring, this optimized IFN- γ ELISpot assay might therefore greatly improve the risk assessment of immune-related clinical complications in CMV-seropositive patients and assist clinicians in their decision to steer antiviral therapy following SOT.

Authorship

LD, BB, BKK, BK and RW: designed the study. TS, LD and RW: organized the logistics, coordinated and supervised the study. BB, BK, DS, LR, DC, MCB, TW, MK, OW, AM, CS, AH and CH: obtained patient samples and collected the data. SB, ML, TH, MK and TW: performed the ELISpot assays. SB: analyzed the ELISpot data. RW, LD, TS and AR: supervised the interpretation and representation of results. AR: drafted the manuscript and figures, with input from TS, BB, LD, RW, BKK and BK. All authors: contributed to the revision and approval of the manuscript.

Funding

This work was supported in part by the Bayerische Forschungsförderung Grant AZ 924-10 (to LD) and by institutional funds of the University of Regensburg (ReForM C Project 03-082; to BKK, later transferred to BB). The funding body had no role in the design of the

study, in the collection, analysis, and interpretation of data, and in the writing of the manuscript.

Conflict of interest

RW, SB, LD, TS, and AR are or were employees of Lophius Biosciences GmbH. LD is cofounder 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 measurement centers have received research funding from Lophius Biosciences GmbH for this study.

Acknowledgements

We are indebted to all study participants for their contribution. The authors gratefully acknowledge the members of the respective nephrology and measurement centers, as well as Anja Svenson, Hanna Bendfeldt, and Alcedis GmbH (Giessen, Germany) for their expertise and assistance in data collection, data management, study coordination, and data preliminary analysis. We thank Thomas Keller and Stephan Weber (ACOMED Statistik, Leipzig, Germany) for their statistical evaluation. T-Track[®] CMV tests were provided by Lophius Biosciences GmbH, Regensburg, Germany.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Single patient data of T-Track[®] cytomegalovirus (CMV) results and viral load measurements over time (relative to transplantation) ($n = 86$).

Figure S2. Study flow diagram.

Figure S3. Patients with T cell-mediated rejection (TCMR) show an increased proportion of double-positive IE-1- and pp65-specific IFN- γ ELISpot results.

Figure S4. Patient group description based on cytomegalovirus (CMV) reactivation and antiviral treatment (*post hoc* analysis; $n = 79$ patients).

Figure S5. pp65-specific cell-mediated immunity (CMI) prior to first detection of viral load is higher in patients with self-limiting cytomegalovirus (CMV) reactivation (*post hoc* analysis; $n = 79$ patients).

Table S1. Study flow chart.

Table S2. HLA class I antigens presenting immunodominant cytomegalovirus (CMV) IE-1- and pp65-specific epitopes.

Table S3. Number of individuals with HLA class I antigens presenting immunodominant cytomegalovirus (CMV) IE-1- and pp65-specific epitopes.

Appendix S1. Patients and methods.

REFERENCES

- Egli A, Humar A, Kumar D. State-of-the-art monitoring of cytomegalovirus-specific cell-mediated immunity after organ transplant: a primer for the clinician. *Clin Infect Dis* 2012; **55**: 1678.
- Lúcia M, Crespo E, Cruzado JM, Grinyó JM, Bestard O. Human CMV-specific T-cell responses in kidney transplantation; toward changing current risk-stratification paradigm. *Transpl Int* 2014; **27**: 643.
- Kotton CN, Kumar D, Caliendo AM, et al. Updated international consensus guidelines on the management of cytomegalovirus in solid-organ transplantation. *Transplantation* 2013; **96**: 333.
- Hanley PJ, Bollard CM. Controlling cytomegalovirus: helping the immune system take the lead. *Viruses* 2014; **6**: 2242.
- Crough T, Khanna R. Immunobiology of human cytomegalovirus: from bench to bedside. *Clin Microbiol Rev* 2009; **22**: 76.
- Sester M, Sester U, Gärtner B, et al. Levels of virus-specific CD4 T cells correlate with cytomegalovirus control and predict virus-induced disease after renal transplantation. *Transplantation* 2001; **71**: 1287.
- Sester M, Sester U, Gärtner BC, Girndt M, Meyerhans A, Köhler H. Dominance of virus-specific CD8 T cells in human primary cytomegalovirus infection. *J Am Soc Nephrol JASN* 2002; **13**: 2577.
- Sester M, Sester U, Gärtner B, et al. Sustained high frequencies of specific CD4 T cells restricted to a single persistent virus. *J Virol* 2002; **76**: 3748.
- Gamadia LE, Remmerswaal EBM, Weel JF, Bemelman F, van Lier RAW, Ten Berge IJM. Primary immune responses to human CMV: a critical role for IFN-gamma-producing CD4⁺ T cells in protection against CMV disease. *Blood* 2003; **101**: 2686.
- Sacre K, Carcelain G, Cassoux N, et al. Repertoire, diversity, and differentiation of specific CD8 T cells are associated with immune protection against human cytomegalovirus disease. *J Exp Med* 2005; **201**: 1999.
- Dunn HS, Haney DJ, Ghanekar SA, Stepick-Biek P, Lewis DB, Maecker HT. Dynamics of CD4 and CD8 T cell responses to cytomegalovirus in healthy human donors. *J Infect Dis* 2002; **186**: 15.
- Sinclair E, Black D, Epling CL, et al. CMV antigen-specific CD4⁺ and CD8⁺ T cell IFN γ expression and proliferation responses in healthy CMV-seropositive individuals. *Viral Immunol* 2004; **17**: 445.
- Reusser P, Cathomas G, Attenhofer R, Tamm M, Thiel G. Cytomegalovirus (CMV)-specific T cell immunity after renal transplantation mediates protection from CMV disease by limiting the systemic virus load. *J Infect Dis* 1999; **180**: 247.
- Crough T, Fazou C, Weiss J, et al. Symptomatic and asymptomatic viral recrudescence in solid-organ transplant recipients and its relationship with the antigen-specific CD8(+) T-cell response. *J Virol* 2007; **81**: 11538.
- Egli A, Binet I, Binggeli S, et al. Cytomegalovirus-specific T-cell respon-

- ses and viral replication in kidney transplant recipients. *J Transl Med* 2008; **6**: 29.
16. Nickel P, Bold G, Presber F, *et al.* High levels of CMV-IE-1-specific memory T cells are associated with less alloimmunity and improved renal allograft function. *Transpl Immunol* 2009; **20**: 238.
 17. Sester U, Gärtner BC, Wilkens H, *et al.* Differences in CMV-specific T-cell levels and long-term susceptibility to CMV infection after kidney, heart and lung transplantation. *Am J Transplant* 2005; **5**: 1483.
 18. Lisboa LF, Kumar D, Wilson LE, Humar A. Clinical utility of cytomegalovirus cell-mediated immunity in transplant recipients with cytomegalovirus viremia. *Transplantation* 2012; **93**: 195.
 19. Klenerman P, Cerundolo V, Dunbar PR. Tracking T cells with tetramers: new tales from new tools. *Nat Rev Immunol* 2002; **2**: 263.
 20. Gratama JW, Boeckh M, Nakamura R, *et al.* Immune monitoring with iTag MHC Tetramers for prediction of recurrent or persistent cytomegalovirus infection or disease in allogeneic hematopoietic stem cell transplant recipients: a prospective multicenter study. *Blood* 2010; **116**: 1655.
 21. Abate D, Saldan A, Mengoli C, *et al.* Comparison of cytomegalovirus (CMV) enzyme-linked immunosorbent spot and CMV quantiferon gamma interferon-releasing assays in assessing risk of CMV infection in kidney transplant recipients. *J Clin Microbiol* 2013; **51**: 2501.
 22. Schmitt A, Keilholz U, Scheibenbogen C. Evaluation of the interferon-gamma ELISPOT-assay for quantification of peptide specific T lymphocytes from peripheral blood. *J Immunol Methods* 1997; **210**: 167.
 23. Walker S, Fazou C, Crough T, *et al.* Ex vivo monitoring of human cytomegalovirus-specific CD8+ T-cell responses using QuantiFERON-CMV. *Transpl Infect Dis* 2007; **9**: 165.
 24. Cantisán S, Lara R, Montejó M, *et al.* Pretransplant interferon- γ secretion by CMV-specific CD8+ T cells informs the risk of CMV replication after transplantation. *Am J Transplant* 2013; **13**: 738.
 25. Bestard O, Lucia M, Crespo E, *et al.* Pretransplant immediately early-1-specific T cell responses provide protection for CMV infection after kidney transplantation. *Am J Transplant* 2013; **13**: 1793.
 26. López-Oliva MO, Martínez V, Buitrago A, *et al.* Pretransplant CD8 T-cell response to IE-1 discriminates seropositive kidney recipients at risk of developing CMV infection posttransplant. *Transplantation* 2014; **97**: 839.
 27. Rittà M, Costa C, Sidoti F, *et al.* Pretransplant assessment of CMV-specific immune response by Elispot assay in kidney transplant recipients. *New Microbiol* 2015; **38**: 329.
 28. Gerna G, Lilleri D, Fornara C, *et al.* Monitoring of human cytomegalovirus-specific CD4 and CD8 T-cell immunity in patients receiving solid organ transplantation. *Am J Transplant* 2006; **6**: 2356.
 29. Manuel O, Husain S, Kumar D, *et al.* Assessment of cytomegalovirus-specific cell-mediated immunity for the prediction of cytomegalovirus disease in high-risk solid-organ transplant recipients: a multicenter cohort study. *Clin Infect Dis* 2013; **56**: 817.
 30. Costa C, Balloco C, Sidoti F, *et al.* Evaluation of CMV-specific cellular immune response by ELISPOT assay in kidney transplant patients. *J Clin Virol* 2014; **61**: 523.
 31. Abate D, Saldan A, Fison M, *et al.* Evaluation of cytomegalovirus (CMV)-specific T cell immune reconstitution revealed that baseline antiviral immunity, prophylaxis, or preemptive therapy but not antithymocyte globulin treatment contribute to CMV-specific T cell reconstitution in kidney transplant recipients. *J Infect Dis* 2010; **202**: 585.
 32. Lúcia M, Crespo E, Melilli E, *et al.* Preformed frequencies of cytomegalovirus (CMV)-specific memory T and B cells identify protected CMV-sensitized individuals among seronegative kidney transplant recipients. *Clin Infect Dis* 2014; **59**: 1537.
 33. Kim S-H, Lee H-J, Kim S-M, *et al.* Diagnostic usefulness of cytomegalovirus (CMV)-specific T cell immunity in predicting CMV infection after kidney transplantation: a pilot proof-of-concept study. *Infect Chemother* 2015; **47**: 105.
 34. Favi E, Santangelo R, Iesari S, *et al.* Enzyme-linked immunospot assay as a complementary method to assess and monitor cytomegalovirus infection in kidney transplant recipients on preemptive antiviral therapy: a single-center experience. *Transplant Proc* 2017; **49**: 1766.
 35. Schachtner T, Stein M, Reinke P. CMV-specific T cell monitoring offers superior risk stratification of CMV-seronegative kidney transplant recipients of a CMV-seropositive donor. *Transplantation* 2017; **101**: e315.
 36. Barabas S, Gary R, Bauer T, *et al.* Urea-mediated cross-presentation of soluble Epstein-Barr virus BZLF1 protein. *PLoS Pathog* 2008; **4**: e1000198.
 37. Barabas S, Spindler T, Kiener R, *et al.* An optimized IFN- γ ELISPOT assay for the sensitive and standardized monitoring of CMV protein-reactive effector cells of cell-mediated immunity. *BMC Immunol* 2017; **18**: 14.
 38. Reuschel E, Barabas S, Zeman F, *et al.* Functional impairment of CMV-reactive cellular immunity during pregnancy. *J Med Virol* 2017; **89**: 324.
 39. Banas B, Böger CA, Lückhoff G, *et al.* Validation of T-Track® CMV to assess the functionality of cytomegalovirus-reactive cell-mediated immunity in hemodialysis patients. *BMC Immunol* 2017; **18**: 15.
 40. Ljungman P, Griffiths P, Paya C. Definitions of cytomegalovirus infection and disease in transplant recipients. *Clin Infect Dis* 2002; **34**: 1094.
 41. Ljungman P, Boeckh M, Hirsch HH, *et al.* Definitions of cytomegalovirus infection and disease in transplant patients for use in clinical trials. *Clin Infect Dis* 2017; **64**: 87.
 42. Sis B, Mengel M, Haas M, *et al.* Banff '09 meeting report: antibody mediated graft deterioration and implementation of Banff working groups. *Am J Transplant* 2010; **10**: 464.
 43. Rossini G, Cerboni C, Santoni A, *et al.* Interplay between human cytomegalovirus and intrinsic/innate host responses: a complex bidirectional relationship. *Mediators Inflamm* 2012; **2012**: 607276.
 44. Sylwester AW, Mitchell BL, Edgar JB, *et al.* Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *J Exp Med* 2005; **202**: 673.
 45. van Dommelen SLH, Tabarias HA, Smyth MJ, Degli-Esposti MA. Activation of natural killer (NK) T cells during murine cytomegalovirus infection enhances the antiviral response mediated by NK cells. *J Virol* 2003; **77**: 1877.
 46. Kamath AT, Sheasby CE, Tough DF. Dendritic cells and NK cells stimulate bystander T cell activation in response to TLR agonists through secretion of IFN-alpha beta and IFN-gamma. *J Immunol* 2005; **174**: 767.
 47. Min-Oo G, Lanier LL. Cytomegalovirus generates long-lived antigen-specific NK cells with diminished bystander activation to heterologous infection. *J Exp Med* 2014; **211**: 2669.
 48. Aiello FB, Calabrese F, Rigotti P, *et al.* Acute rejection and graft survival in renal transplanted patients with viral diseases. *Mod Pathol* 2004; **17**: 189.
 49. Eid AJ, Razonable RR. New developments in the management of cytomegalovirus infection after solid organ transplantation. *Drugs* 2010; **70**: 965.

50. Costa C. Cytomegalovirus and acute rejection in kidney transplantation. *Nephro-Urol Mon* 2011; **3**: 237.
51. Reischig T, Jindra P, Svecová M, Kormunda S, Opatrný K, Treska V. The impact of cytomegalovirus disease and asymptomatic infection on acute renal allograft rejection. *J Clin Virol* 2006; **36**: 146.
52. Moreau A, Varey E, Anegón I, Cuturi M-C. Effector mechanisms of rejection. *Cold Spring Harb Perspect Med* 2013; **3**: a015461.
53. Dall A, Hariharan S. BK virus nephritis after renal transplantation. *Clin J Am Soc Nephrol CJASN* 2008; **3**(Suppl. 2): S68.
54. Fishman JA. Opportunistic infections—coming to the limits of immunosuppression? *Cold Spring Harb Perspect Med* 2013; **3**: a015669.
55. Comoli P, Azzi A, Maccario R, et al. Polyomavirus BK-specific immunity after kidney transplantation. *Transplantation* 2004; **78**: 1229.
56. Prosser SE, Orentas RJ, Jurgens L, Cohen EP, Hariharan S. Recovery of BK virus large T-antigen-specific cellular immune response correlates with resolution of bk virus nephritis. *Transplantation* 2008; **85**: 185.
57. Kern F, Surel IP, Faulhaber N, et al. Target structures of the CD8(+)-T-cell response to human cytomegalovirus: the 72-kilodalton major immediate-early protein revisited. *J Virol* 1999; **73**: 8179.
58. Hummel M, Abecassis MM. A model for reactivation of CMV from latency. *J Clin Virol* 2002; **25**(Suppl. 2): S123.
59. Scholz M, Doerr HW, Cinatl J. Inhibition of cytomegalovirus immediate early gene expression: a therapeutic option? *Antiviral Res* 2001; **49**: 129.
60. Ahlenstiel-Grunow T, Koch A, Großhennig A, et al. A multicenter, randomized, open-labeled study to steer immunosuppressive and antiviral therapy by measurement of virus (CMV, ADV, HSV)-specific T cells in addition to determination of trough levels of immunosuppressants in pediatric kidney allograft recipients (IVIST01-trial): study protocol for a randomized controlled trial. *Trials* 2014; **15**: 324.
61. Widmann T, Sester U, Gärtner BC, et al. Levels of CMV specific CD4 T cells are dynamic and correlate with CMV viremia after allogeneic stem cell transplantation. *PLoS One* 2008; **3**: e3634.