



Impaired detection of *Mycobacterium tuberculosis* immunity in patients using high levels of immunosuppressive drugs

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ABSTRACT: We have previously shown, in renal transplant recipients on maintenance immunosuppression, that a whole-blood assay was superior in detecting immunity towards purified protein derivative (PPD) compared with skin testing. As blood tests may have limitations during high-dose immunosuppression therapy, the present study was aimed at characterising the effect of high immunosuppressive drug levels on PPD-specific T-cell immunity.

PPD-reactive CD4 T-cells from 13 renal transplant recipients were longitudinally quantified by the induction of cytokines using flow cytometry. To further address the effect of high and low maintenance immunosuppression, drug effects were studied *in vitro* and in 49 age-matched lung transplant recipients and 49 renal transplant recipients.

Maintenance immunosuppression after renal transplantation did not affect PPD-specific T-cell detection (median T-cell frequencies 0.55% before and 0.46% >12 months after transplantation), whereas specific T-cell frequencies were significantly lower 3 months after transplantation (0.15%; $p=0.0002$). Likewise, high-level maintenance immunosuppression after lung transplantation was associated with a significantly lower prevalence in PPD-specific T-cell reactivity compared with renal transplant recipients (16.7% versus 52.1%; $p=0.0005$). In line with the observations made *in vivo*, calcineurin inhibitors analysed *in vitro* led to a dose-dependent decrease in antigen-specific T-cell reactivity.

The flow cytometric assay is not adversely affected by low drug doses. In contrast, decreased levels of PPD-specific T-cells early after transplantation and low prevalence of PPD-reactivity in lung transplant recipients suggest a reduced sensitivity of *in vitro* testing during high-level immunosuppression.

KEYWORDS: Flow cytometry, immunosuppression, interferon- γ release assay, T-cells, transplantation, tuberculosis

The World Health Organization has estimated that more than 2 billion humans are currently infected with *Mycobacterium tuberculosis* complex, and tuberculosis is among the most frequent causes of death from infection, accounting for around 1.6 million deaths annually [1]. Due to systemic immunosuppression, tuberculosis may represent an important cause of morbidity and mortality among transplant recipients. The frequency of tuberculosis approaches up to 6% in developed countries and may reach up to ~15% in areas of high endemicity [2, 3]. Even in low-prevalence countries, the incidence of tuberculosis among transplant recipients is higher compared with the general population and its attendant mortality was reported to be 23% [2].

As with other persistent pathogens, *M. tuberculosis* is largely controlled by the cellular arm of immunity. A latent tuberculosis infection may be defined as the presence of a specific cellular immune response without evidence of active disease. The risk of progression from latent infection to active tuberculosis varies greatly. In the first years following infection, the risk is 2–5% [4] and cumulates to 10–15% over a lifetime [5]. In transplant recipients, reactivation risk is even higher, as drug-mediated immunosuppression accounts for a considerable decrease in cellular immune function. Thus, although the effect of immunosuppressive drugs on anti-mycobacterial T-cell immunity has not been studied so far, progressive impairment in cellular immunity may contribute to the increased incidence of

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TABLE 1 Patient characteristics and immunosuppressive drug regimens in transplant recipients depending on graft type and time after transplantation

	Renal longitudinal		p-value	Renal cross-sectional	Lung cross-sectional	p-value
Subjects (males/females) n	13 (8/5)			49 (26/23)	49 (26/23)	
Time after transplantation	3 months	12 months		>12 months (5.7±3.7 yrs)	>12 months (4.2±2.5 yrs)	
Age yrs	55.1±8.3			48.4±14.1	48.4±14.3	
Triple/double drug regimen	13/0	2/11	<0.0001	5/44	49/0	<0.0001
Calcineurin inhibitor						
Cyclosporine A ng·mL ⁻¹	167.9±20.7	104.1±6.7	0.004	109.1±26.0	176.4±90.5	<0.0001
Subjects n	4	4		28	35	
Tacrolimus ng·mL ⁻¹	11.4±2.6	7.6±2.6	0.04	7.8±1.9	8.9±3.6	NS
Subjects n	9	9		21	14	
Azathioprine mg·day⁻¹	50–150	50–150		50–150	50–150	
Methylprednisolone[#]/prednisolone[†] mg·day⁻¹	8–20	≤4		≤4	7.5	
Mycophenolate mofetil g·day⁻¹	NA	≤2		≤2	≤2	

Data are presented as mean ± SD (actual doses for cyclosporine A and tacrolimus, intended doses for other drugs) unless otherwise stated. NA: not applicable; NS: nonsignificant. #: renal transplant recipients; †: lung transplant recipients.

reactivation from latent infection [6]. In addition, primary infections due to exogenous contact or due to graft transmission may further increase the risk for infectious complications in immunocompromised patients [7, 8].

Screening tests for evidence of latent tuberculosis infection in clinical practice are largely based on functional analysis of T-cells reactive towards mycobacterial antigens by skin testing or *in vitro* interferon (IFN)- γ release assays (IGRAs), in which either tuberculin (as purified protein derivative (PPD)) or *M. tuberculosis*-specific antigens, such as ESAT-6 or CFP-10, are most widely used as stimuli [9]. Obviously, as the readout parameters used in these tests, such as delayed type hypersensitivity reaction in the skin test or IFN- γ induction in IGRA, are direct targets of immunosuppressive drugs, this may negatively affect test sensitivity. Consequently, with increasing dosage of immunosuppressive drugs, skin testing is unreliable, often resulting in falsely negative results [10]. In contrast, *in vitro* tests seem to be of higher sensitivity as compared to skin testing. Promising studies comparing IGRA and skin testing have already been performed in immunocompetent individuals [11] and patients with uremic immunodeficiency prior to transplantation [12–15]. We have recently shown that maintenance immunosuppressive drug levels in long-term transplant recipients do not adversely affect test results of a flow cytometry-based assay that detects intracellularly accumulated IFN- γ after stimulation with both PPD as well as with *M. tuberculosis*-specific antigens [16]. However, the effect of increasing dosages of immunosuppressive drugs on the detectability of *M. tuberculosis*-specific T-cells *in vitro* has not yet been studied in detail. Thus, the objective of this study was to characterise the effect of high levels of immunosuppressive drugs on the detection of PPD-specific T-cells *in vitro* in patients after solid organ transplantation.

MATERIALS AND METHODS

Study participants

The study was conducted in a clinical routine setting in Germany (a low-prevalence country) among 49 long-term lung transplant recipients and 49 age-matched long-term renal transplant recipients in our outpatient clinic (University of the Saarland, Homburg, Germany). Demographic and clinical characteristics of all patients are shown in table 1. All transplant patients were of Caucasian origin, were transplanted for ≥ 12 months, had stable graft function, and no signs or symptoms of active tuberculosis during the study period. Information on bacille Calmette–Guérin (BCG) vaccination status was not consistently available. One lung transplant recipient reported a treated tuberculosis infection during childhood. Four lung and six renal transplant patients were born in a country with 25–299 tuberculosis cases per 100,000 population; three lung and two renal transplant recipients had an occupational risk, *i.e.* healthcare workers. Moreover, 13 renal transplant recipients were studied prospectively before, as well as 3 and 12 months after, transplantation. Initial immunosuppression comprised tacrolimus (n=9) or cyclosporine A (n=4), methylprednisolone, azathioprine and daclizumab (1 mg per kilogramme body weight on day 0 and after 2, 4, 6 and 8 weeks). Patients did not receive any isoniazid prophylaxis. Blood was drawn in the morning before the intake of drugs. To address the effect of calcineurin inhibitors (cyclosporine A and tacrolimus) on PPD- and ESAT-6/CFP-10-specific T-cell reactivity *in vitro*, four individuals reactive towards PPD and ESAT-6/CFP-10 were studied (median (range) age 70.9 (53.7–85.6) yrs). To analyse the effect of trough and peak levels of immunosuppressive drugs on T-cell function *ex vivo*, blood from eight renal transplant recipients on a cyclosporine A-based drug regimen (median (range) 7.8 (1.4–15.7) yrs after transplantation) was analysed before intake of immunosuppressive drugs and 2 h thereafter. The study

was approved by the local ethics committee (reference number 142/02; University of the Saarland) and all individuals gave oral informed consent.

Quantification of antigen-specific CD4 T-cells within whole blood

Specific stimulation of CD4 T-cells was performed directly from heparinised whole blood for a total of 6 h, as previously described [12, 16, 17]. Cells were stimulated with 222 IU·mL⁻¹ PPD (Tuberkulin-GT-1000; Chiron-Behring, Marburg, Germany) and, where indicated, with a mixture of recombinant ESAT-6 and CFP-10 (10 µg·mL⁻¹ each; Lionex, Braunschweig, Germany). As negative controls, cells were stimulated with diluent (Chiron-Behring), stimulation with 2.5 µg·mL⁻¹ *Staphylococcus aureus* enterotoxin B (SEB) was used as positive control. Stimulation in all samples including negative and positive controls was performed in the presence of 1 µg·mL⁻¹ anti-CD28 and anti-CD49d (Becton Dickinson, Heidelberg, Germany), respectively. Staining was done as previously described [12, 16, 17] using anti-CD4, anti-IFN-γ, and anti-CD69 (Becton Dickinson). At least 15,000 CD4 T-cells were analysed on a FACSCalibur (Becton Dickinson) using Cellquest-Pro 4.0.2. The percentage of specific T-cells was calculated by subtracting the frequency obtained by the respective control stimulation. The lower limit of detection is 0.05%, as previously established [18]. In the present study, we used PPD as a proxy for a specific T-cell response in the setting of a mycobacterial infection, as it gives a higher frequency of reacting T-cells compared with the *M. tuberculosis*-specific antigens ESAT-6 and CFP-10 [12, 16, 17]. Although this approach is less specific for *M. tuberculosis*, we considered that important when studying the effects of immunosuppressive drugs, as this better allowed addressing dynamic decreases in T-cell frequencies and reactivities with increasing immunosuppression.

Quantification of the suppressive effect of calcineurin inhibitors *in vitro*

To analyse the effect of calcineurin inhibitors on PPD- and ESAT-6/CFP-10-specific CD4 T-cell reactivity *in vitro*, whole blood from four immunocompetent individuals was preincubated with increasing doses of cyclosporine A and tacrolimus for 2 h and subsequently stimulated with PPD or a mixture of recombinant ESAT-6/CFP-10 and processed as described above. Staining was performed as described above using anti-CD4, anti-CD69, anti-IFN-γ and anti-interleukin (IL)-2 (clone MQ1-17H12).

Statistical analysis

Statistical analysis was performed using Prism v4.01 software (Graphpad, San Diego, CA, USA). The paired Friedman test was used to compare differences in PPD-reactive T-cell frequencies before, and 3 and 12 months after, transplantation. The nonparametric paired Wilcoxon test was used to compare drug levels in patients analysed 3 and 12 months after transplantation and differences in cyclosporine levels and T-cell frequencies in patients at trough and peak levels of cyclosporine A. The Mann–Whitney test was used to analyse differences in calcineurin inhibitor levels between lung and renal transplant recipients. The Fisher's exact test was used to analyse differences between long-term renal and lung transplant recipients with respect to the prevalence of PPD reactivity and the number of immunosuppressive drugs. The

Mann–Whitney test was applied to analyse differences in PPD-reactive T-cell frequencies between long-term renal and lung transplant recipients. Unless stated otherwise, mean ± SD is used to describe the distribution of a given parameter within the study population. If normality cannot be assumed, median (range) is given.

RESULTS

PPD-specific CD4 T-cell frequencies significantly decreased within the first 3 months after renal transplantation

In order to address the effect of different doses of immunosuppressive drugs after transplantation, PPD-specific T-cells were longitudinally quantified in 13 patients before, as well as 3 and 12 months after, renal transplantation. As expected, both the number of drugs as well as actual levels of calcineurin inhibitors were significantly different at 3 and 12 months after transplantation (table 1). The frequency of PPD-specific CD4 T-cells was determined after a 6-h stimulation and is given as the percentage of CD69 and IFN-γ-positive CD4 T-cells (fig. 1a–c). In general, the diluent failed to induce any specific cytokines (data not shown). Dot plots from a representative patient is shown in figure 1a–c. Interestingly, PPD-specific T-cell frequencies showed a significant decrease from an initial 0.55 (0.07–3.27)% to 0.15 (0.04–2.63)% in the first 3 months after transplantation (fig. 1d; *p*<0.0001). Thereafter, specific T-cell frequencies regained pre-transplant levels when immunosuppressive drugs were tapered to maintenance dosages (0.46 (0.06–3.25)% 12 months after transplantation).

Lower frequencies and prevalence of PPD-specific CD4 T-cells in patients on high levels of maintenance immunosuppression

To further characterise the effect of high doses of immunosuppressive drugs on PPD-specific immunity, PPD-specific T-cell frequencies were cross-sectionally analysed in two cohorts of long-term renal and lung transplant recipients, respectively (*n*=49 patients in each group). These two groups of solid organ transplant recipients were matched for age and sex, but differ in the number and dosage of immunosuppressive drugs. Specific risk factors did not differ between lung and renal transplant recipients (see Materials and methods section). Differences in immunosuppressive drug regimens and doses were evident from the fact that the majority of renal transplant recipients received two drugs only (*n*=44), whereas all lung transplant recipients were receiving a triple drug regimen with higher intended dosages (table 1). Moreover, when quantifying actual levels of calcineurin inhibitors, cyclosporine A levels were significantly higher in lung transplant patients compared with renal transplant recipients. Tacrolimus levels were also higher, but this difference did not reach statistical significance (table 1).

Interestingly, frequencies of PPD-specific CD4 T-cells were significantly higher in long-term renal transplant recipients compared with patients after lung transplantation (0.06 (0–3.25)% versus 0.004 (0–0.28)%; *p*<0.0001) (fig. 2). Moreover, a significantly higher percentage of renal transplant recipients had PPD-specific CD4 T-cells above detection limit (26 (53.1%) out of 49) compared with patients after lung transplantation (eight (16.3%) out of 49; *p*=0.0002, Fisher's exact test). An overall lack of T-cell reactivity as a cause of this low percentage

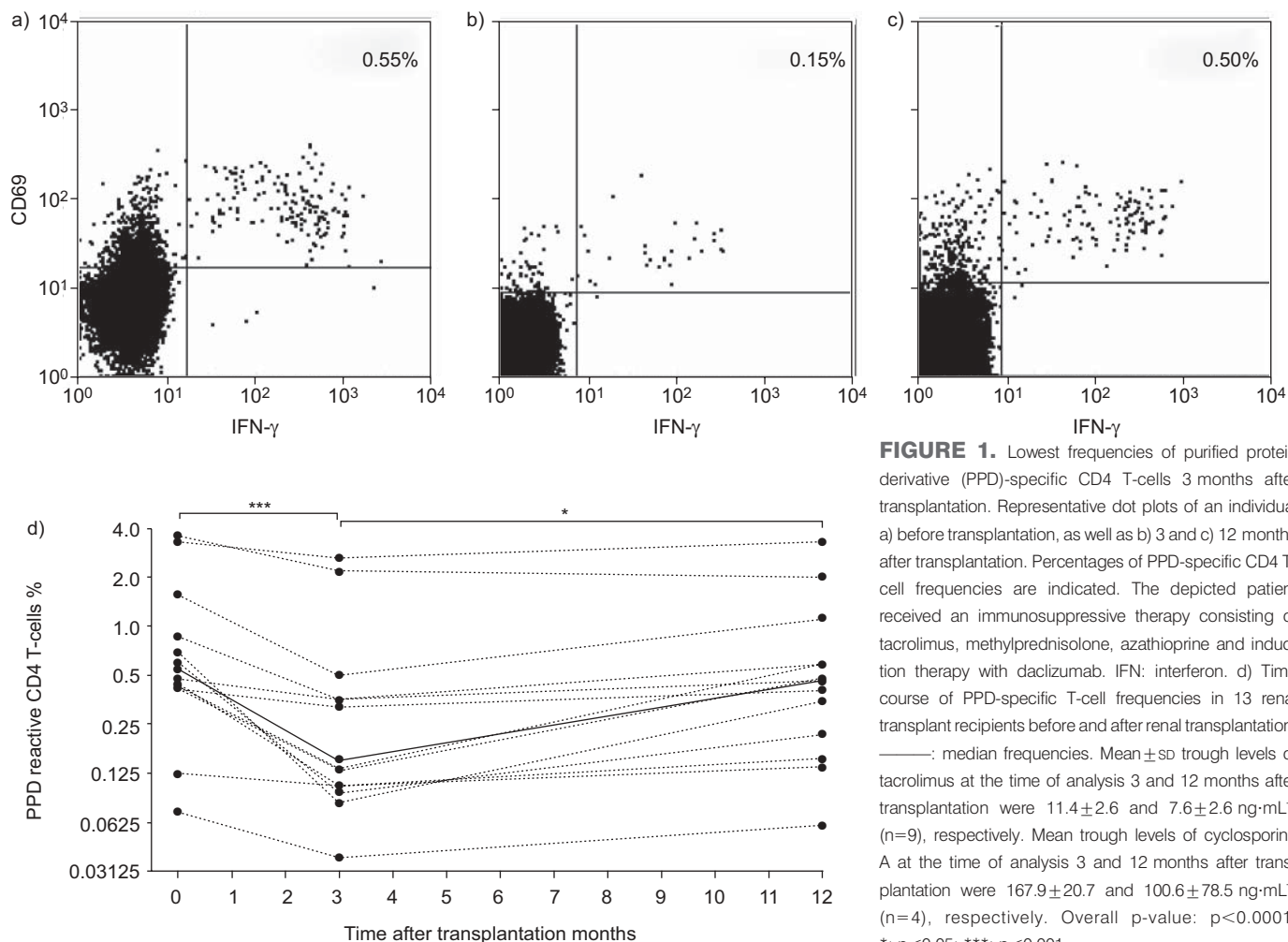


FIGURE 1. Lowest frequencies of purified protein derivative (PPD)-specific CD4 T-cells 3 months after transplantation. Representative dot plots of an individual a) before transplantation, as well as b) 3 and c) 12 months after transplantation. Percentages of PPD-specific CD4 T-cell frequencies are indicated. The depicted patient received an immunosuppressive therapy consisting of tacrolimus, methylprednisolone, azathioprine and induction therapy with daclizumab. IFN: interferon. d) Time course of PPD-specific T-cell frequencies in 13 renal transplant recipients before and after renal transplantation. —: median frequencies. Mean \pm SD trough levels of tacrolimus at the time of analysis 3 and 12 months after transplantation were 11.4 ± 2.6 and 7.6 ± 2.6 ng·mL⁻¹ (n=9), respectively. Mean trough levels of cyclosporine A at the time of analysis 3 and 12 months after transplantation were 167.9 ± 20.7 and 100.6 ± 78.5 ng·mL⁻¹ (n=4), respectively. Overall p-value: $p < 0.0001$. *: $p < 0.05$; ***: $p < 0.001$.

of PPD-positive individuals in lung transplant recipients was excluded by the fact that all tested patients readily reacted towards the polyclonal stimulus SEB (n=46, frequencies 3.44 (0.08–26.7)%; data not shown). Among patients with PPD-specific CD4 T-cells above the detection limit, the median frequencies of PPD-specific CD4 T-cells were 0.27 (0.06–3.25)% in renal and 0.15 (0.06–0.28)% in lung transplant recipients. Taken together, both the longitudinal and the cross-sectional analyses suggest that the detection of immunity towards *M. tuberculosis* is significantly impaired upon receipt of high levels of immunosuppressive drugs.

Differences in risk factor distribution or BCG vaccination practices may represent potential variables that may confound results observed in the two groups. However, even when comparing renal and lung transplant recipients with and without risk factors, respectively, PPD-reactive T-cell frequencies remained significantly higher in renal transplant patients (data not shown). The BCG vaccination status was frequently not remembered or documented by patients. Thus, results were analysed according to age groups, as BCG vaccinations had been recommended in West Germany between 1945 and 1975 [19]. PPD-reactive CD4 T-cell frequencies were below detection limit in nine patients born after 1975 (five lung and

four renal transplant recipients). In either lung or renal transplant patients, the frequency of PPD-reactive CD4 T-cells was comparable among patients born between 1945 and 1975 and patients born before 1945. However, in line with higher immunosuppression, in both age groups, the frequency of PPD-reactive CD4 T-cells was significantly lower in lung compared with renal transplant recipients ($p = 0.0001$ for lung *versus* kidney transplant recipients in both age groups).

Calcineurin inhibitors dose-dependently affected antigen-specific CD4 T-cell function

Among the immunosuppressive drugs that are used after transplantation, calcineurin inhibitors exert direct suppressive activity on activated T-cells and affect cytokine induction, which is used as a readout system for *in vitro* assays. To directly assess the effect of the two calcineurin inhibitors cyclosporine A and tacrolimus on PPD-specific CD4 T-cell reactivity, whole blood from four nonimmunosuppressed individuals was incubated with increasing doses of cyclosporine A and tacrolimus. As shown in figure 3a–d, there was a dose-dependent decrease in PPD-specific CD4 T-cell reactivity both with respect to the production of IFN- γ and IL-2. Interestingly, the decrease not only affected the percentage of PPD-specific CD4 T-cells (fig. 3a and b) but also the cytokine

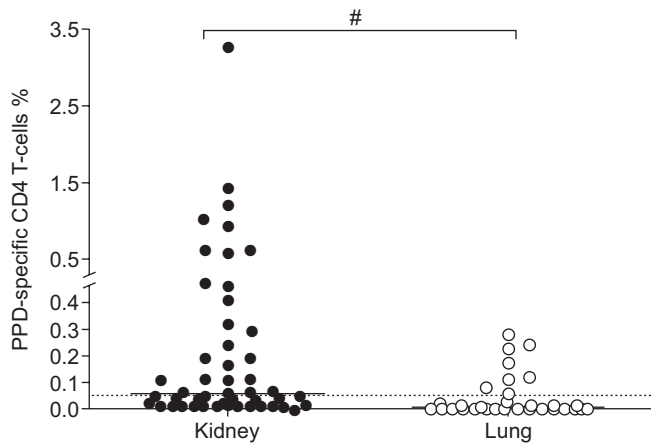


FIGURE 2. Lower frequencies of purified protein derivative (PPD)-specific CD4 T-cells in long-term lung transplant recipients. PPD-specific CD4 T-cells were quantified in age-matched long-term renal and lung transplant recipients using flow cytometry (49 patients in each group). One lung transplant recipient with a history of treated childhood tuberculosis had 0.12% PPD-reactive CD4 T-cells. —: median;: detection limit. #: $p < 0.0001$.

production on the single-cell basis (mean fluorescence intensity of cytokines; fig. 3c and d). Moreover, the effect of calcineurin inhibitors was studied in parallel on T-cell reactivity towards the *M. tuberculosis*-specific antigens ESAT-6 and CFP-10 (fig. 3e–h). As with PPD-reactive T-cells, both ESAT-6/CFP-10-specific T-cell frequencies (fig. 3e and f) and cytokine production on the single-cell level (fig. 3g and h) were reduced with increasing doses of drugs, although the suppressive effect appeared to be less pronounced compared with cells reactive towards PPD. This dose–response curve illustrates that calcineurin inhibitor concentrations that are typically found in long-term renal transplant recipients do not overtly affect antigen-specific CD4 T-cell reactivity, whereas high trough or peak drug levels applied in lung transplant recipients may well affect specific T-cell responses. In order to directly address the relevance of these findings for the transplant-recipient *in vivo*, antigen-specific T-cell reactivity from eight renal transplant recipients were quantified before intake of a cyclosporine A-based drug regimen and 2 h thereafter. Stimulation was carried out directly from whole blood using SEB. As shown in figure 4a, median (range) trough levels were 106 (74–122) $\text{ng}\cdot\text{mL}^{-1}$ and peak levels increased to 459 (313–712) $\text{ng}\cdot\text{mL}^{-1}$ ($p=0.008$). As expected, peak levels were associated with a significantly lower frequency of antigen-reactive CD4 T-cells (6.25 (1.18–18.84)% versus 7.78 (2.63–20.67)% SEB-reactive CD4 T-cells at peak and trough levels, respectively; $p=0.008$) (fig. 4b). This corresponds to a decrease by up to 55.1% (mean \pm SD $24.7 \pm 15.8\%$).

DISCUSSION

In recent years, IGRAs have been developed that have the potential to eventually replace skin testing in routine clinical practice [11, 20]. Among the main advantages are that IGRAs have been shown to be of higher specificity, owing to the use of *M. tuberculosis*-specific antigens, and of potentially increased sensitivity, owing to optimised *in vitro* culture conditions [11]. Up to now, however, limited data exist on the sensitivity of

IGRAs in patients on immunosuppressive drug therapy, in particular in patients receiving high-level immunosuppressive drugs. We have previously shown, by the use of a flow cytometric whole blood assay based on intracellular IFN- γ staining after specific stimulation with PPD or with the *M. tuberculosis*-specific antigens ESAT-6 and/or CFP-10, that test results are not adversely affected by moderate extent of immunodeficiency, such as uremia-associated immunodeficiency in haemodialysis patients [12], by steroid therapy in patients with rheumatoid arthritis [17] or by maintenance immunosuppression applied in long-term renal transplant recipients [16]. We now show that the detection of PPD-specific T-cells may be impaired when patients receive high levels of immunosuppressive drugs, such as in the first months after transplantation or in lung transplant recipients that receive high levels of maintenance immunosuppression in the long term. A direct causal link between increasing levels of immunosuppressive drugs and decreasing T-cell reactivity is further supported by lower T-cell reactivity in the presence of peak drug levels and by a dose-dependent decrease in T-cell reactivity upon addition of calcineurin inhibitors to whole blood of immunocompetent individuals *in vitro*. Unfortunately, the pre-transplant infection status of our patients was not determined on a routine clinical basis. Therefore, one cannot formally distinguish true negative responders from patients that are negative due to high levels of immunosuppressive drugs. However, we did not find any imbalance in potential tuberculosis risk factors or biasing effects of BCG vaccination policies in the patients that we analysed. To best exclude other potential confounding effects of early transplant surgery on antigen-specific T-cell reactivity (*i.e.* transient cytokine release, rejection, blood transfusion and anaesthesia), patients were analysed ≥ 3 months after transplantation in a clinically stable state. Together, these findings may have important general implications for the interpretation of IGRAs tests in clinical routine, as results may become falsely negative when patients receive high levels of immunosuppressive drugs.

The flow cytometric assay used in this study is particularly suited to the simultaneous assessment of the impact of immunosuppressive drug therapy on both the quantity and reactivity of antigen-specific T-cells on a single cell basis. Its use from whole blood and the short stimulation time of only 6 h closely reflects the situation found *in vivo*, in that all drugs are present in clinically relevant concentrations and results are unaffected by prolonged *in vitro* culture. We show that reduced frequencies of antigen-specific T-cells and an impaired ability to produce cytokines such as IFN- γ or IL-2 are associated with higher levels of immunosuppressive drugs. From a practical point of view, these data emphasise the particular importance of performing *in vitro*-testing on blood drawn prior to intake of immunosuppressive drugs, as T-cell frequencies may be reduced by $>50\%$ when analysed at peak levels. Moreover, as the amount of drugs *in vivo* continuously cycles from trough to peak levels within 12 h [21, 22], the dose–response curve shown in this study gives a good estimate of the overall suppressive strength that acts daily on specific T-cells in the individual patient. Accordingly, PPD-specific immunocompetence is only moderately affected in the immunosuppressive drug range that exists in long-term renal transplant recipients (from trough levels of 100 $\text{ng}\cdot\text{mL}^{-1}$

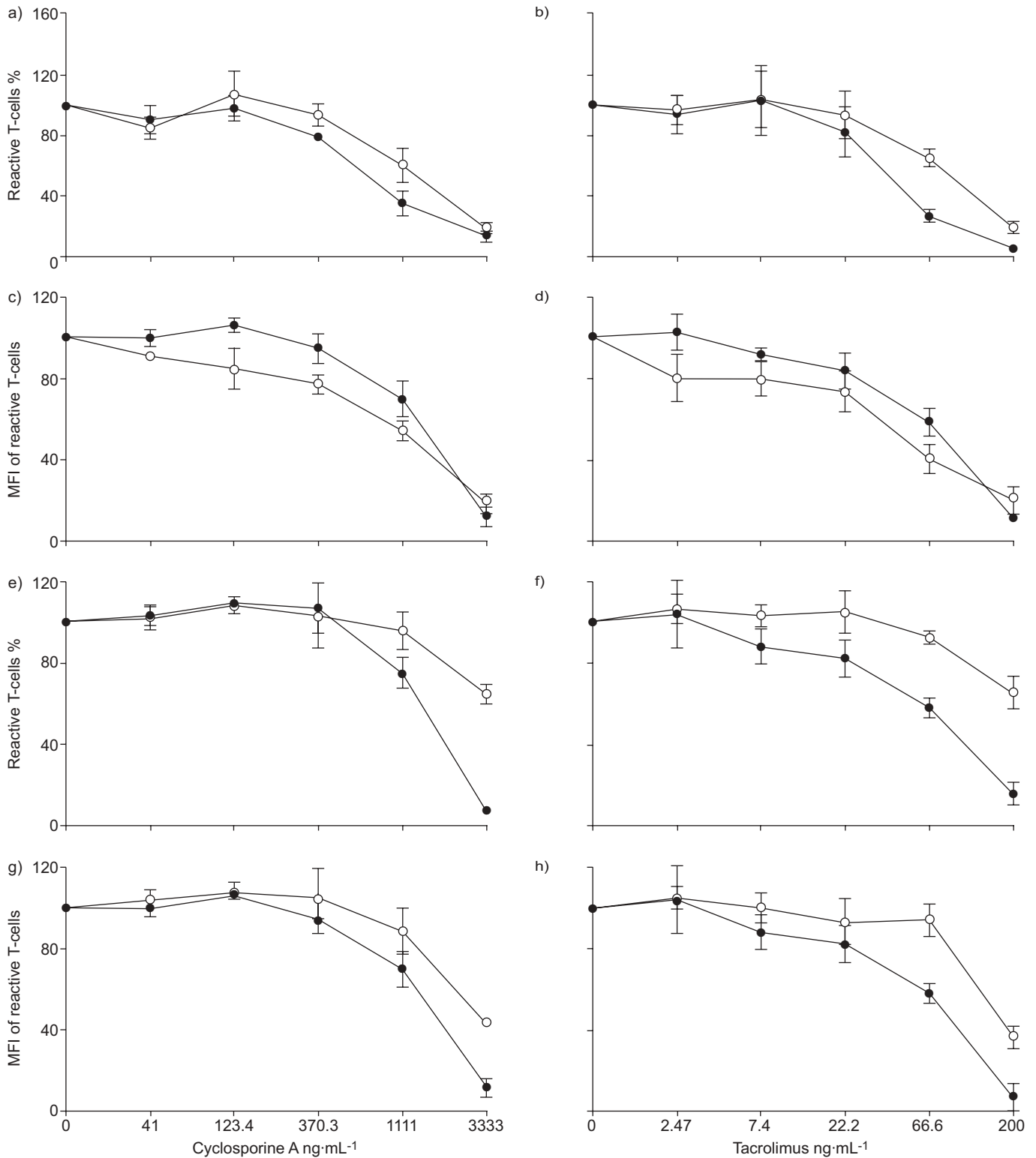


FIGURE 3. Dose-dependent decrease in purified protein derivative (PPD)- and ESAT-6/CFP-10-specific CD4 T-cell reactivity upon incubation with increasing doses of calcineurin inhibitors. Whole blood from four nonimmunosuppressed individuals was preincubated with various concentrations of cyclosporine A (a, c, e and g) or tacrolimus (b, d, f and h) and the percentage of interferon (IFN)- γ (\circ) and interleukin (IL)-2 (\bullet)-producing cells was quantified after 6 h of stimulation with PPD (a and b) or ESAT-6/CFP-10 (e and f). Moreover, the production of IFN- γ and IL-2 on the single cell level was determined after stimulation with PPD (c and d) and ESAT-6/CFP-10 (g and h). Respective values are normalised to values without addition of calcineurin inhibitors. Basal production of IFN- γ per single cell was higher after ESAT-6/CFP-10 stimulation (mean fluorescence intensity (MFI) $20,842 \pm 5,937$) compared with PPD as a stimulus (MFI $13,909 \pm 5,072$ and data not shown).

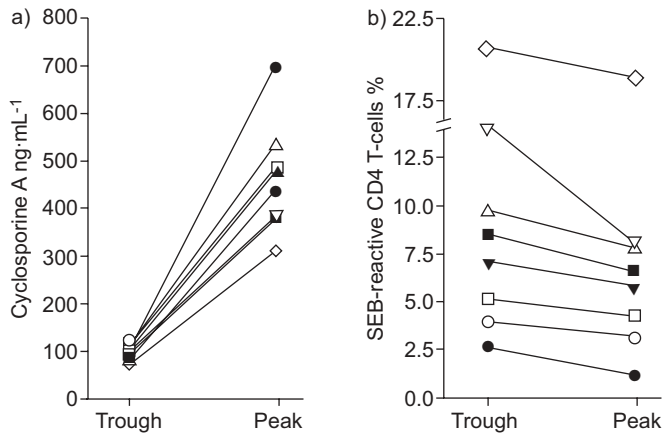


FIGURE 4. Decrease in antigen-reactive T-cell frequencies in the presence of peak levels of cyclosporine A. Blood from eight renal transplant recipients was drawn before intake of a cyclosporine A-based drug regimen and 2 h thereafter, and stimulated with *Staphylococcus aureus* enterotoxin B (SEB) for 6 h. a) Cyclosporine A levels significantly increased ($p=0.008$) and b) SEB-reactive CD4 T-cells concomitantly decreased by up to 55.1% ($p=0.008$; mean \pm SD $24.7 \pm 15.8\%$). Each different symbol in panel a) and b) refers to data from one particular patient.

cyclosporine A to peak levels of ~ 600 ng·mL⁻¹ [22]), whereas the overall suppressive effect is much stronger in lung transplant recipients, where drug levels range between 150 and $>1,500$ ng·mL⁻¹ [21]. This effect may further be enhanced by immunosuppressants other than calcineurin inhibitors that may, in addition, reduce the frequencies and/or reactivity of specific T-cells. Apart from these directly measurable effects of immunosuppressive drugs on T-cell reactivity, prolonged drug therapy may, in addition, lead to a progressive functional exhaustion and/or physical reduction of *M. tuberculosis*-specific T-cells over time. This is supported by the fact that: 1) PPD-reactive T-cell frequencies are lower after 3 months compared with the first days after transplant (data not shown), although immunosuppressive drugs have already been tapered; and 2) the prevalence of a positive PPD-reactive CD4 T-cell response is significantly lower in long-term lung transplant recipients compared with patients who had undergone renal transplantation. Together, these data emphasise that immunosuppressive drugs exert both direct and indirect suppressive activity. While direct effects may have immediate consequences for *in vitro* test results, indirect effects of prolonged high-level drug treatment on the reduction in numbers and functionality of antigen-specific T-cells may have important implications for the individual's immunocompetence towards *M. tuberculosis* in the long term.

Our findings of low levels of PPD-specific T-cells in the first months after transplantation and in long-term lung transplant recipients are similar to our previous findings for T-cells specific for cytomegalovirus (CMV), another clinically relevant persistent pathogen [18, 23, 24]. In the setting of CMV, we have even shown that low levels of CMV-specific CD4 T-cells and their functional exhaustion are directly associated with impaired CMV control and an increased incidence of CMV-related clinical complications [18, 23, 24]. Thus, these data indicate that a drop of antigen-specific T-cells is a direct correlate of impaired pathogen control. As with CMV infection, an impaired

immunocompetence towards *M. tuberculosis* in transplant recipients may contribute to more frequent reactivation events [6]. Although not formally proven, this may be of particular relevance for the increased incidence of tuberculosis observed in high-prevalence countries [3, 8]. Ironically, patients with highest levels of immunosuppressive drugs are more vulnerable towards infectious complications but are more likely to escape detection by the recommended tests. From a practical point of view, the results of this study indicate that the management of tuberculosis in transplant recipients should involve a careful evaluation of evidence for *M. tuberculosis* contact prior to transplantation. In the presence of clinical signs of infection after transplant, a decrease of *M. tuberculosis*-specific immunity in a patient with detectable immunity prior to transplantation does not necessarily imply test failure, but may be considered as a meaningful measure to identify patients at risk for infectious complications. The measurable benefit of such a monitoring strategy should be determined in large trials and will certainly depend on the overall prevalence of tuberculosis in a given country. In the setting of contact investigations, our data further indicate that caution should be applied when interpreting negative test results of highly immunocompromised patients.

Up to now, two IFN- γ -based assays are commercially available, the enzyme-linked immunosorbent spot (ELISPOT)-based T.SPOT.TB (Oxford Immunotec, Abingdon, UK) and the enzyme-linked immunosorbent assay (ELISA)-based QuantiFERON (Cellestis, Carnegie, Australia) assay. Both tests share similarities with the flow cytometric approach, in that they use the antigen-specific induction of IFN- γ as a readout system to determine evidence of *M. tuberculosis* contact. We have previously shown for stimuli such as PPD, ESAT-6 and CFP-10 that results of the flow cytometric approach significantly correlate to those of the ELISPOT assay [16]. At present, there is inadequate evidence on the value of the two commercial IGRAs for use in immunocompromised individuals [20, 25]. In line with results from flow cytometry [12, 16, 17], there are case reports indicating that ELISPOT- or ELISA-based IGRAs are of higher sensitivity compared with skin testing during moderate immunosuppression, such as monotherapy with steroids [26] or azathioprine [27], or in haematological diseases [28]. However, there are also reports that show a higher rate of indeterminate results in patients on immunosuppressive drug therapy compared with immunocompetent individuals [15, 29–31]. While the overall intensity of immunosuppression was not clearly apparent in these studies, these results may provide further evidence that increasing dosages of immunosuppressive drugs may negatively affect test results.

Limitations of the present study include the fact that we did not know the pre-transplant infection or BCG vaccination status of our patients. Moreover, PPD was used as a proxy for an infection with *M. tuberculosis*, although we cannot exclude that immunosuppressive drugs may differentially affect T-cell responses in a patient with true latent infection compared with a BCG-vaccinated individual. However, based on the similarity of assay principles with respect to the detection of IFN- γ as readout system, results from the present study are likely to be extrapolated to the commercially available IGRAs such as the T.SPOT.TB or the QuantiFERON assay. Whether our findings using PPD may also apply to other, more specific, antigens,

such as ESAT-6 or CFP-10, will have to be clarified in future studies. From a conceptual point of view, the use of single antigens such as ESAT-6 or CFP-10 has the disadvantage of yielding specific T-cell frequencies that are ~10-fold lower than respective frequencies obtained after stimulation with a complex mixture of ~200 antigens such as PPD [12, 16, 17]. Therefore, it seems conceivable that such a low T-cell response may more easily drop below the detection limit in transplant recipients after prolonged immunosuppressive drug therapy. Interestingly, however, despite lower net frequencies, our data indicate that highly immunogenic antigens, such as ESAT-6 or CFP-10, may be advantageous over PPD, owing to their higher cytokine production on the single-cell level (fig. 3 and data not shown). As a consequence, this may result in a less pronounced effect of immunosuppressive drugs on ESAT-6/CFP-10-specific T-cell reactivity compared with that after stimulation with PPD. While our *in vitro* data on the effect of calcineurin inhibitors lend support to that hypothesis, the sensitivity of an ESAT-6/CFP-10-based flow cytometric approach and of the commercially available ESAT-6/CFP-10-based IGRAs will have to be assessed in future for patients taking high levels of immunosuppressive drugs. Based on the present study and recent meta-analyses [20, 25], we have now launched a large European clinical multicentre study among patients with various levels of immunosuppression within the Tuberculosis Network European Trials (TBNET) group in order to comparatively evaluate the use and limitations of the commercially available IGRAs and skin testing in a head-to-head manner.

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STATEMENT OF INTEREST

A statement of interest for M. Singh can be found at www.erj.ersjournals.com/misc/statements.dtl

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