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Impaired detection of *M. tuberculosis* immunity upon high levels of immunosuppressive drugs

Urban Sester[1], Heinrike Wilkens[2], Kai van Bentum[1], Mahavir Singh[3], Gerhard W. Sybrecht[2], Hans-Joachim Schäfers[4], and Martina Sester[1]

[1]Department of Internal Medicine IV, [2]Department of Internal Medicine V, [3]Helmholtz Center for Infection Research, and Lionex GmbH, Inhoffenstraße 7, D-38124 Braunschweig, Germany, [4]Department of thoracic and cardiovascular surgery, University of the Saarland, D-66421 Homburg, Germany
Address correspondence to: Martina Sester, PhD; Department of Internal Medicine IV; University of the Saarland; D-66421 Homburg, Germany; phone +49-6841-1623557, FAX +49-6841-1621347; email: martina.sester@uks.eu

Short title: TB immunity on immunosuppression

Abstract

We previously showed in renal transplant-recipients on maintenance-immunosuppression that a whole-blood assay was superior in detecting immunity towards purified-protein-derivative (PPD) compared to skin-testing. As blood-tests may have limitations upon high-dose immunosuppression, the present study was aimed at characterizing 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- 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 three 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 to renal transplant-recipients (16.7% vs. 52.1%, p=0.0005). In line with observations in vivo, calcineurin-inhibitors analyzed 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-dosages. 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 upon high-level immunosuppression.

Key words: CD4 T cells, flow-cytometry, immunosuppression, T cells, tuberculosis, transplantation, interferon-γ release assay, *M. tuberculosis*

Introduction

The World Health Organization (WHO) estimated that over 2 billion humans are currently infected with *M. tuberculosis* complex and tuberculosis is among the most frequent causes of death from infection that accounts 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 approximately 15% in areas of high endemicity [2, 3]. Even in low-prevalence countries, the incidence of tuberculosis is higher as compared to 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 approximately 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 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 immunocompromized 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 (IGRA), where 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 detectablity of *M. tuberculosis* specific T-cells in vitro has not yet been studied in detail. Thus, the objective of this study was to characterize the effect of high levels of immunosuppressive drugs on the detection of PPD-specific T-cells in vitro in patients after solid organ transplantation.

Material and Methods

Study participants

The study was conducted in a clinical routine setting in Germany (low prevalence-country) among 49 long-term lung transplant-recipients and 49 age-matched long-term renal transplant-recipients in our out-patient clinic. Demographic and clinical characteristics of all patients are shown in table 1. All transplant-patients were of caucasian origin, were transplanted for at least 12 months, had stable graft function, and no signs or symptoms of active tuberculosis during the study period. Information on BCG vaccination status was not consistently available. One lung-transplant-recipient reported a treated tuberculosis infection during childhood. Four lung- and 6 renal transplant-patients were born in a country with 25-299 tuberculosis cases/100.000 population; 3 lung- and 2 renal transplant-recipients had an occupational risk, i.e. health care workers. Moreover, 13 renal transplant-recipients were studied prospectively before as well as three and twelve months after transplantation. Initial immunosuppression comprised tacrolimus (n=9) or cyclosporine A (n=4), methylprednisolone, azathioprine, and daclizumab (1 mg per kilogram of body weight on day 0 and after 2, 4, 6, and 8 weeks). Patients did not receive any isoniazide-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 70.9 years of age, range 53.7-85.6). To analyze the effect of trough and peak levels of immunosuppressive drugs on T-cell function ex vivo, blood from 8 renal transplant-recipients on a cyclosporine A based drug regimen (median 7.8 years after transplantation, range 1.4-15.7) was analyzed before intake of immunosuppressive drugs and 2 hours thereafter. The study was approved by the local ethics committee (reference number 142/02) and all individuals gave oral informed consent.

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

Specific stimulation of CD4 T-cells was performed directly from heparinized whole blood for a total of 6h as previously described [12, 16, 17]. Cells were stimulated with 222 IU/ml PPD (Tuberkulin-GT-1000; Chiron-Behring, Marburg, Germany) and, where indicated, with a mixture of recombinant ESAT-6 and CFP-10 (10 µg/ml each, Lionex, Braunschweig, Germany). As negative controls, cells were stimulated with diluent (Chiron-Behring), stimulation with 2.5 µg/ml *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 anti-CD28 and anti-CD49d (BD, Heidelberg, Germany), respectively. Staining was done as described before [12, 16, 17] using anti-CD4, anti-IFN-γ, and anti-CD69 (all from BD). At least 15.000 CD4 T-cells were analyzed on a FACS Calibur (BD) 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 as compared to 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.

Quantitation of the suppressive effect of calcineurin inhibitors in vitro

To analyze 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 hours 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-IL-2 (clone MQ1-17H12).

Statistical analysis

Statistical analysis was performed using Prism-V4.01 software (Graphpad, USA). The paired Friedman-test was used to compare differences in PPD-reactive T-cell frequencies before, 3 and 12 months after transplantation. The non-parametric paired Wilcoxon-test was used to compare drug levels in patients analyzed 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 analyze differences in calcineurin inhibitor levels between lung- and renal transplant-recipients. The Fisher's exact test was used to analyze 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 analyze differences in PPD-reactive T-cell frequencies between long-term renal and lung-transplant-recipients. Unless stated differently, the mean±standard deviation is given to describe the distribution of a given parameter within the study population. If normality can not be assumed, median and range is given.

Results

PPD-specific CD4 T-cell frequencies significantly decrease within the first three months after renal transplantation

To address the effect of different dosages of immunosuppressive drugs after transplantation, PPD-specific T-cells were longitudinally quantified in 13 patients before as well as three and twelve months after renal transplantation. As expected, both the number of drugs as well as actual levels of calcineurin inhibitors were significantly different at three and 12 months after transplantation (table 1). The frequency of PPD-specific CD4 T-cells was determined after a 6h-stimulation and is given as the percentage of CD69 and IFN-γ positive CD4 T-cells (figure 1A). In general, the diluent failed to induce any specific cytokines (data not shown). Dotplots from a representative patient is shown in figure 1A. Interestingly, median PPD-specific T-cell frequencies showed a significant decrease from an initial 0.55% (range from 0.07 to 3.27%) to 0.15% (range from 0.04 to 2.63%) within the first three months after transplantation ($p<0.0001$; figure 1B). Thereafter, specific T-cell frequencies re-reached pre-transplant levels when immunosuppressive drugs were tapered to maintenance dosages (median 0.46%, range from 0.06 to 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 characterize the effect of high dosages of immunosuppressive drugs on PPD-specific immunity, PPD-specific T-cell frequencies were cross-sectionally analyzed 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 gender, but differ in the number and dosage of immunosuppressive drugs. Specific risk factors did not differ between lung- and renal transplant-recipients (see material and methods). Differences in immunosuppressive drug regimens and dosages 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 as compared to 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 as compared to patients after lung transplantation (figure 2; $p<0.0001$, median frequencies kidney: 0.06%, range from 0 to 3.25% versus lung: 0.004%, range from 0 to 0.28%). Moreover, a significantly higher percentage of renal transplant-recipients had PPD-specific CD4 T-cells above detection limit (53.1%, 26/49) as compared to patients after lung transplantation (16.3%, 8/49; $p=0.0002$, Fisher´s exact test). An overall lack of T-cell reactivity as a cause of this low percentage of PPD positive individuals in lung transplant-recipients was excluded by the fact that all tested patients readily reacted towards the polyclonal stimulus SEB (median frequencies 3.44%, range from 0.08-26.7%, n=46, data not shown). Among patients with PPD-specific CD4 T-cells above detection limit, the median frequencies of PPD-specific CD4 T-cells were 0.27% (range 0.06-3.25%) in renal and 0.15% (range 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 analyzed according to age groups, as BCG vaccinations had been recommended in Western Germany between 1945 and 1975 [19]. PPD-reactive CD4 T-cell frequencies were below detection limit in 9 patients born after 1975 (5 lung- and 4 renal transplant-recipients). Within 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- as compared to renal transplant-recipients ($lung_{<1945}$ vs. $kidney_{<1945}$, p=0.0001; $lung_{1945\text{-}75}$ vs. $kidney_{1945\text{-}75}$, p=0.0001).

Calcineurin inhibitors dose-dependently affect 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 that is used as 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 non-immunosuppressed 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 did not only affect the percentage of PPD-specific CD4 T-cells (figure 3A and B) but also the cytokine production on the single-cell basis (mean fluorescence intensity of cytokines, figure 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 (figure 3E-H). As with PPD-reactive T cells, both ESAT-6/CFP-10 specific T-cell frequencies (figure 3E and F) and cytokine-production on the single cell level (figure 3G and H) were reduced with increasing dosages of drugs, although the suppressive effect appeared to be less pronounced as compared to 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. 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 two hours thereafter. Stimulation was carried out directly from whole blood using SEB. As shown in figure 4A, median trough levels were 106 ng/ml (range 74-122) and peak levels increased to 459 ng/ml (range 313-712, p=0.008). As expected, peak levels were associated with a significantly lower frequency of antigen-reactive CD4 T-cells (figure 4B, median 6.25% (range 1.18-18.84) versus 7.78% (range 2.63-20.67) SEB-reactive CD4 T-cells at peak and trough levels, respectively, p=0.008). This corresponds to a decrease by up to 55.1% (24.7±15.8%).

Discussion

In recent years, IGRA have been developed that have the potential to eventually replace skin-testing in routine clinical practice [11, 20]. Among the main advantages, IGRA have been shown to be of higher specificity due to the use of *M. tuberculosis*-specific antigens and of potentially increased sensitivity due to optimized in vitro culture conditions [11]. Up to now, however, limited data exist on the sensitivity of IGRA 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 ~~IFN-γ~~ assay that is based on intracellular IFN-γ staining after specific stimulation with PPD or with *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 hemodialysis 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 analyzed. To at best exclude other potential confounding effects of early transplant surgery on antigen-specific T-cell reactivity (i.e. transient cytokine release, rejection, blood transfusion, anaesthesia), patients were analysed at least three months after transplantation in a clinically stable state. Together, these findings may have important general implications for the interpretation of IGRA 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 simultaneously assess 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 hours 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 emphasize the particular importance to perform in vitro-testing from blood drawn prior to intake of immunosuppressive drugs, as T-cell frequencies may be reduced by more than 50% when analyzed at peak levels. Moreover, as the amount of drugs *in vivo* continuously cycles from trough to peak levels within twelve hours [21, 22], the dose-response curve shown in this study gives a considerably good estimate of the overall suppressive strength that daily acts 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 ng/ml cyclosporine A to peak levels of approximately 600 ng/ml, [22]), whereas the overall suppressive effect is much stronger in lung-transplant-recipients, where

drug levels range between 150 and >1500 ng/ml [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 (i) PPD-reactive T-cell frequencies are lower after three months as compared to the first days post-transplant (data not shown), although immunosuppressive drugs have already been tapered, and (ii) the prevalence of a positive PPD-reactive CD4 T-cell response is significantly lower in long-term lung transplant-recipients as compared to patients after renal transplantation. Together these data emphasize 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 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 what we have previously found 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 and 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 for evidence of *M. tuberculosis* contact already prior to transplantation. In the presence of clinical signs of infection post 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 ~~other~~ IFN-γ-based assays are commercially available, the ELISPOT based T.SPOT.*TB* and the ELISA based QuantiFERON 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 only inadequate evidence on the value of the two commercial IGRA for the use in immunocompromized individuals [20, 25]. In line with results from flow-cytometry [12, 16, 17], there are case reports indicating that ELISPOT or ELISA-based IGRA are of higher sensitivity as compared to skin-testing upon moderate immunosuppression such as monotherapy with steroids [26] or azathioprine [27] or upon hematological diseases [28].

However, there are also reports that show a higher rate of indeterminate results in patients on immunosuppressive drug therapy as compared to 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 as compared to 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 IGRA 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 to yield specific T-cell frequencies that are approximately 10fold lower than respective frequencies obtained after stimulation with a complex mixture of approximately 200 antigens such as PPD [12, 16, 17]. Therefore it seems conceivable, that such a low T-cell response may more easily drop below 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 due to their higher cytokine production on the single cell level (legend to figure 3 and data not shown). As a consequence, this may result in a less pronounced effect of immunosuppressive drugs on ESAT-6 and CFP-10 specific T-cell reactivity as compared to 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 IGRA in patients with high levels of immunosuppressive drugs will have to be assessed in future. Based on the present study and recent meta-analyses [20, 25], we have now launched a large European clinical multicenter study among patients with various levels of immunosuppression within the “Tuberculosis Network European Trials group” (TBNET) to comparatively evaluate the use and limitations of the commercially available IGRA and skin-testing in a head-to-head manner.

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Figure legends

Figure 1. Lowest frequencies of PPD-specific CD4 T-cells three months after transplantation. (**A**) Representative dotplots of an individual before transplantation, as well as three and twelve months after transplantation. Numbers indicate percentages of PPD-specific CD4 T-cell frequencies. The depicted patient received an immunosuppressive therapy consisting of tacrolimus, methylprednisolone, azathioprine and induction therapy with daclizumab. (**B**) Time course of PPD-specific T-cell frequencies in 13 renal transplant-recipients before and after renal transplantation. The bold line indicates median frequencies. Mean through levels of tacrolimus at the time of analysis three and 12 months after transplantation were 11.4±2.6 and 7.6±2.6 ng/ml (n=9), respectively. Mean through levels of cyclosporine A at the time of analysis three and 12 months after transplantation were 167.9±20.7 and 100.6±78.5 ng/ml (n=4), respectively.

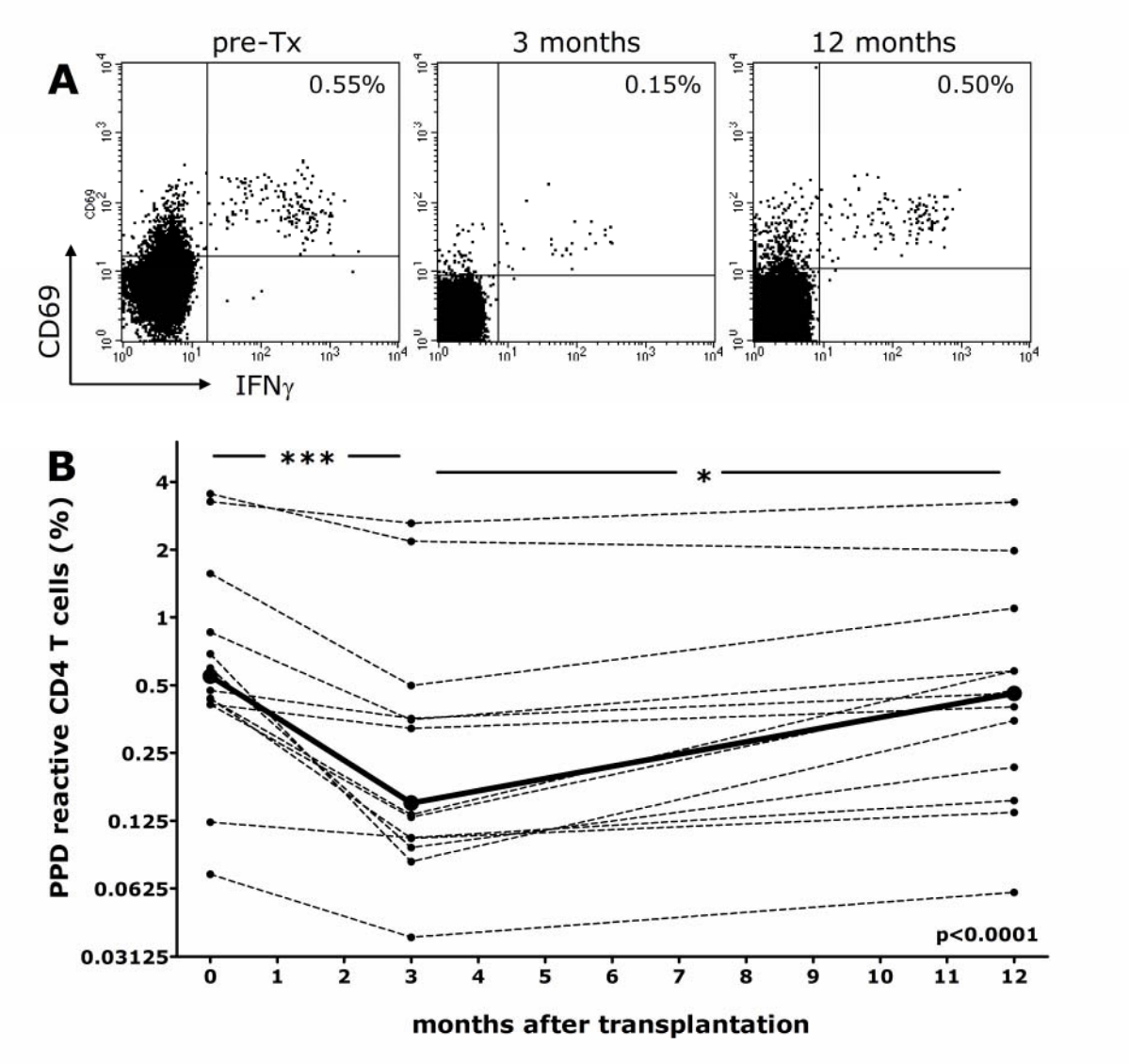

Figure 2. Lower frequencies of PPD-specific CD4 T-cells in long-term lung-transplant-recipients. PPD-specific CD4 T-cells were quantified in age-matched long-term renal and

lung transplant-recipients using flow-cytometry (48 patients in each group). One lung-transplant-recipient with a history of treated childhood tuberculosis had 0.12% PPD-reactive CD4 T-cells.

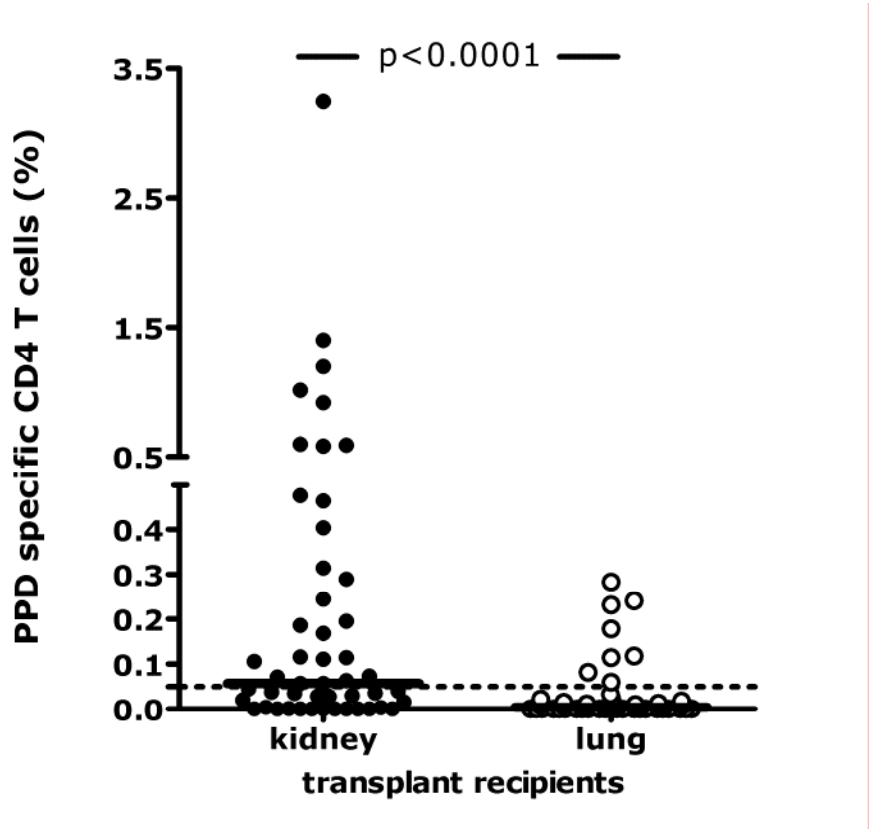

Figure 3. Dose-dependent decrease in PPD- and ESAT-6/CFP-10 specific CD4 T-cell reactivity upon incubation with increasing doses of calcineurin inhibitors. Whole blood from four non-immunosuppressed individuals was preincubated with various concentrations of cyclosporine A (**A**, **C, E, G**) or tacrolimus (**B**, **D, F. H**) and the percentage of IFN-γ and IL-2 producing cells was quantified after six hours of stimulation with PPD (**A**, **B**) or ESAT-6/CFP-10 (**E, F**). Moreover, the production of IFN-γ and IL-2 on the single cell level was determined after stimulation with PPD (**C**, **D**) and ESAT-6/CFP-10 (**G, H**). Respective values are normalized 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 20842±5937) as compared to PPD as a stimulus (MFI 13909±5072, and data not shown).

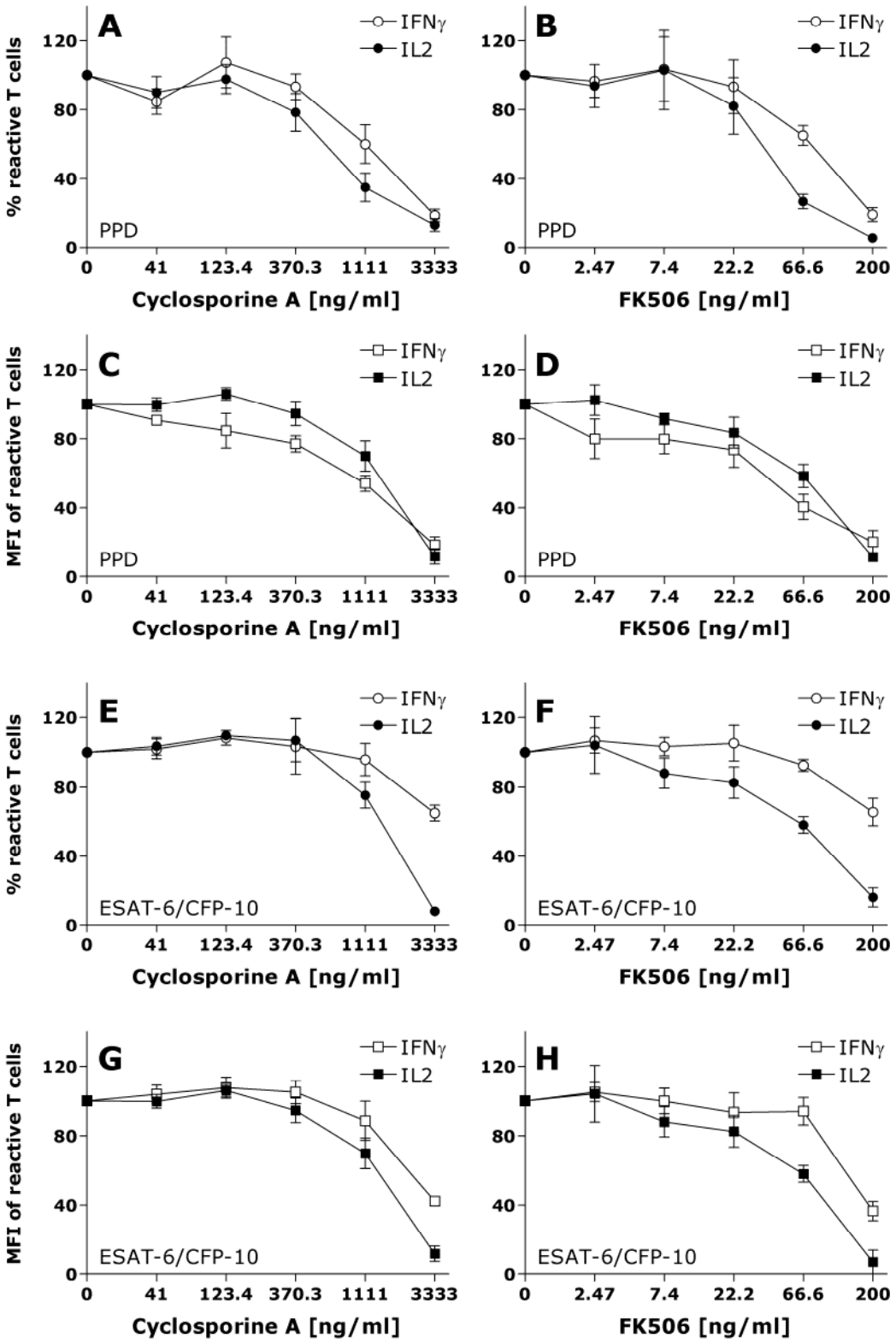
A
B
C
D
E
F
G
H
IFNγ
IL2
% reactive T cells
MFI of reactive T cells
PPD
ESAT-6/CFP-10
0
40
80
120
0
41
123.4
370.3
1111
3333
Cyclosporine A [ng/ml]
0
2.47
7.4
22.2
66.6
200
FK506 [ng/ml]

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 two hours thereafter, and stimulated with SEB for 6h. (**A**) Cyclosporine A levels significantly increased and (**B**) SEB-reactive CD4 T-cells concomitantly decreased by up to 55.1% (24.7±15.8%). Each symbol in panel A and B refers to data from the same patient.

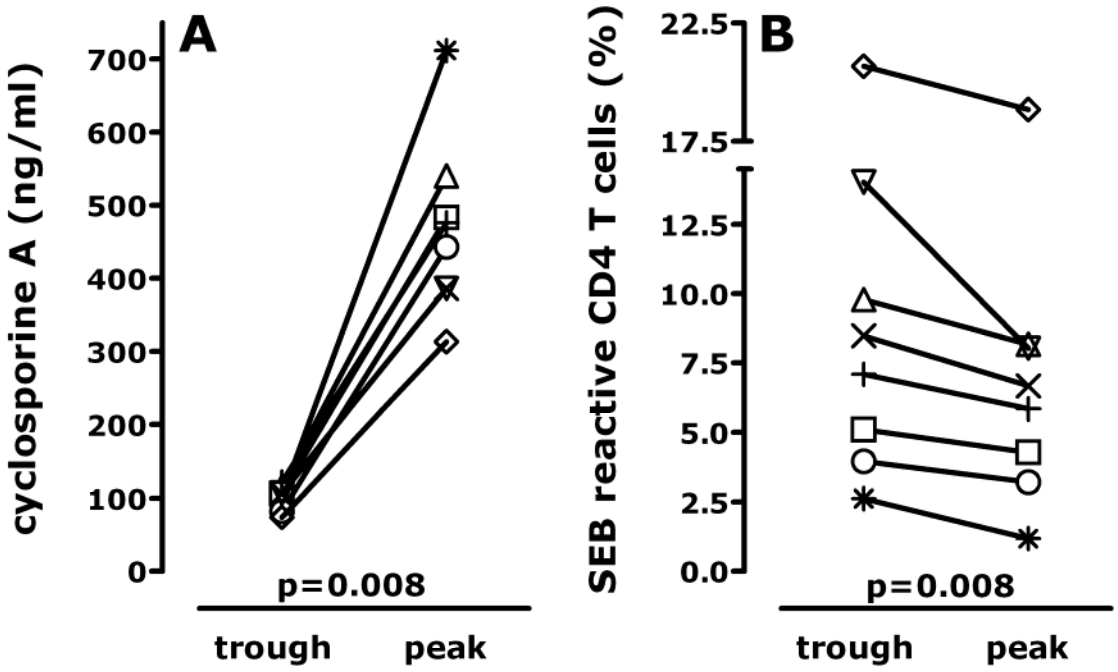

Table 1

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

	Renal (n=13) longitudinal			Renal (n=49) cross-sectional	Lung (n=49) cross-sectional	
time after transplantation years (mean±SD)	3 months	12 months		>12 months 5.7±3.7	>12 months 4.2±2.5	
age (years, mean±SD)	55.1±8.3			48.4±14.1	48.4±14.3	
male/female	8/5			26/23	26/23	
triple/double drug regimen	13/0 patients	2/11 patients	p<0.0001	5/44 patients	49/0 patients	p<0.0001
calcineurin inhibitor						
cyclosporine A (ng/ml, actual mean±SD)	167.9±20.7 (n=4)	104.1±6.7	p=0.004	109.1±26.0 (n=28)	176.4±90.5 (n=35)	p<0.0001
tacrolimus (ng/ml, actual mean±SD)	11.4±2.6 (n=9)	7.6±2.6	p=0.04	7.8±1.9 (n=21)	8.9±3.6 (n=14)	p=n.s.
azathioprine (mg/d)	50-150	50-150		50-150mg/d	50-150mg/d	
methylprednisolone*/prednisolone* (mg/d)	8-20	up to 4		up to 4	7.5	
mycophenolate mofetil (g/d)	n. a.	up to 2		up to 2	up to 2	

*renal transplant-recipients, **lung transplant-recipients, SD: standard deviation