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Original Article

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Improved efficiency in detecting cellular immunity towards *M. tuberculosis* in patients receiving immunosuppressive drug therapy

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Abstract

Background. Reactivation of a latent *Mycobacterium tuberculosis* infection in immunocompromised individuals is associated with significant morbidity and mortality. The limited sensitivity of the established tuberculin skin-test in identifying latently infected patients on immunosuppressive drug therapy represents a major obstacle to better tuberculosis control after transplantation.

Methods. In this study, a quantitative flow-cytometric whole-blood assay and the skin-test were comparatively evaluated towards both diagnostic power and practicability in 117 long-term renal transplant recipients (age 53.1 ± 14.8 years; 7.0 ± 5.0 years after transplantation) in a low-prevalence region.

Results. Among the aforementioned renal transplant recipients, a high proportion (52.14%) had purified protein-derivative (PPD)-specific T-cell-immunity *in vitro*. Despite immunosuppression, prevalence as well as median frequencies of PPD-specific T-cells (0.22%; >0.05–4.71%) were as high as previously reported for immunocompetent individuals and haemodialysis patients. In contrast to *in vitro* testing, skin testing was less practicable in an ambulatory setting. Moreover, skin-test reactivity was significantly reduced as only 50.0% of patients with PPD-reactivity *in vitro* were skin-test positive. T-cell reactivity towards early secretory antigenic target-6 (ESAT-6), a protein specific for *M. tuberculosis* but absent from the bacillus Calmette–Guerin BCG-vaccine strain, was found in 52.9% of all individuals with PPD-reactivity *in vitro*.

Conclusions. In conclusion, the whole-blood assay reveals a high prevalence of latent tuberculosis infection in renal transplant recipients. It may represent a valuable alternative to skin testing as the test result is not adversely affected by immunosuppression. Moreover, reactivity towards ESAT-6 allows the distinction of a latent infection from BCG-induced reactivity. The assay is well-suited for use in screening programmes and may facilitate the management of tuberculosis infection in immunocompromised individuals.

Keywords: bacterial infection; human; immunosuppression; *Mycobacterium tuberculosis*; T-cells; transplantation

Introduction

Tuberculosis is among the most frequent causes of death from infection in humans, accounting for around 1.6 million deaths annually [1]. The disease is caused by infection with *Mycobacterium tuberculosis* complex and represents an important cause of morbidity and mortality, especially among immunocompromised individuals such as transplant recipients [2]. Even in low-prevalence countries, the incidence of tuberculosis in transplant recipients is higher compared with the general population and attendant mortality was recently reported to be 23% [2,3].

M. tuberculosis is largely controlled by the cellular arm of immunity. A latent infection is characterized by a strong cellular immune response in the absence of detectable bacterial load. In immunocompetent individuals, this persisting immunity is thought to keep bacterial replication to below detectable levels. As iatrogenic immunosuppression in transplant recipients accounts for a considerable decrease in cellular immune function [4], progressive impairment in cellular immunity may contribute to an increased incidence of reactivation from latent infection. In addition, a higher rate of primary infections due to regular visits of dialysis units and/or graft transmission may further increase the risk for infectious complications in immunocompromised patients [5].

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Despite the low overall incidence of symptomatic tuberculosis infection in low-prevalence countries, the potential lethality and morbidity mandate constant vigilance to identify patients at risk for reactivation. Therefore, current guidelines, aimed at preventing tuberculosis infection in immunocompromised individuals, recommend a generalized screening for evidence of latent infection prior to and after transplantation to target appropriate preventative prophylaxis [6–9]. At present, tuberculosis control programmes exclusively rely on the tuberculin skin-test to identify a latent infection in asymptomatic individuals [10–12]. Among others, a major drawback of the tuberculin skin-test is its impaired sensitivity in immunocompromised patients. As a consequence, the threshold defining a positive skin-test reaction has been reduced to an induration of $\geq$5 mm in this patient group. Nevertheless, depending on the degree of immunosuppression, the actual number of latently infected individuals may be considerably underestimated due to false-negative diagnoses [6,10]. Moreover, the skin-test is logistically demanding as it requires at least two visits by the patient for placing and reading of the test. This may not only affect overall compliance, but may significantly decrease the adherence to recommended routine screening programmes in out-patient settings. In addition, the widespread application in transplant recipients may further be precluded by test-related exclusion criteria, as several of those criteria such as skin disorders or intercurrent pyrexiel illness are most frequently observed in transplant patients. Finally, cross-reactivity of purified protein derivative (PPD)-specific T-cell immunity towards the *Mycobacterium bovis* bacillus Calmette–Guerin (BCG) vaccine strain may contribute to a decreased specificity in BCG-vaccinated individuals. Thus, based on the low specificity, sensitivity and practicability, the skin-test is of limited use for widespread screening programmes and identification of latently infected individuals among transplant recipients.

At present, there is no standard test to diagnose a latent tuberculosis infection in patients receiving immunosuppressive combination therapy. This would be of considerable clinical interest, as this could help targeting preventative chemotherapy in patients prior to or after transplantation. We and others previously reported that flow cytometry or ELISPOT-based assays are suitable to detect and quantify cellular immune responses towards a number of clinically relevant pathogens such as cytomegalovirus (CMV) or HIV [13–16]. As both *in vitro* assays and skin testing rely on the induction of cytokines after stimulation [17], *in vitro* analysis of PPD-specific immunity may represent a promising alternative. Moreover, stimulation using the recombinant early secretory antigenic target-6 (ESAT-6) protein that is absent from all strains of the BCG-vaccine has been established to distinguish true *M. tuberculosis*-specific immunity from vaccination-induced responses [18,19]. Promising studies comparing *in vitro* assays and skin testing have already been performed in immunocompetent individuals [18–21] and patients with uraemic [15] or HIV-associated immunodeficiency [22]. Interestingly, although a reliable gold standard for defining a latent tuberculosis infection is lacking, *in vitro* tests seem equal or superior in both sensitivity and specificity [18–21]. Up to now, similar evaluations in patients receiving immunosuppressive drug therapy were lacking. This cohort study was carried out to comparatively evaluate a quantitative rapid whole-blood assay and the skin-test towards both diagnostic power and practicability in immunosuppressed patients.

Subjects and methods

Subjects

The study was conducted among a total of 117 long-term renal transplant recipients in our out-patient clinic (53.1 ± 14.8 years of age; 7.0 ± 5.0 years after transplantation; range from 0.52 to 19.37 years). The geographical area served by this institution covers a distance of up to 200 km. There was no history of skin testing prior to transplantation in this cohort. A subgroup of the cohort ($n = 83$; 53.7 ± 15.2 years of age; 7.0 ± 5.1 years after transplantation; range from 0.52 to 19.37 years) was evaluated for both skin testing and flow-cytometric analysis during March 2002 and February 2003. After that period, only flow-cytometric characterization was performed. All transplant patients were of Caucasian origin, were transplanted for at least 6 months, had stable graft function, and had no signs or symptoms of active tuberculosis during the study period. BCG-vaccination status was not consistently available for all patients, but vaccination was recommended in Germany up to 1998. All patients received an immunosuppressive maintenance double or triple drug regimen consisting of either ciclosporin A (120–150 ng/ml) or tacrolimus (5–10 ng/ml) and methylprednisolone alone (4 mg/day) or combined with azathioprine (50–150 mg/day) or mycophenolate mofetile (up to 2 g/day). There was no influence of these immunosuppressive drug regimens on PPD-specific T-cell reactivity. Blood was drawn in the morning before the intake of drugs. Data on skin-test induration and PPD-specific T-cell reactivity *in vitro* from two age-matched cohorts of immunocompetent individuals and haemodialysis patients have been previously described [15] and served as control groups in the present study. There was no routine pre-transplant screening programme, but 17 transplant recipients (six PPD negative, 11 PPD positive; 51.7 ± 9.8 years of age at the time of transplantation) were available for analysis before and 1 year after transplantation. Dotplots shown in Figure 1 were from immunocompetent individuals without end-stage renal disease (age range from 27 to 51 years). A group of 21 individuals (age 66.3 ± 21.5 years) was comparatively tested using both flow-cytometric assay and ELISPOT assay. The CMV serostatus was determined by a commercial CMV IgG test (IMX, MEIA; Abbott Diagnostics, Wiesbaden, Germany). The study was approved by the local ethics committee and all individuals gave informed consent.

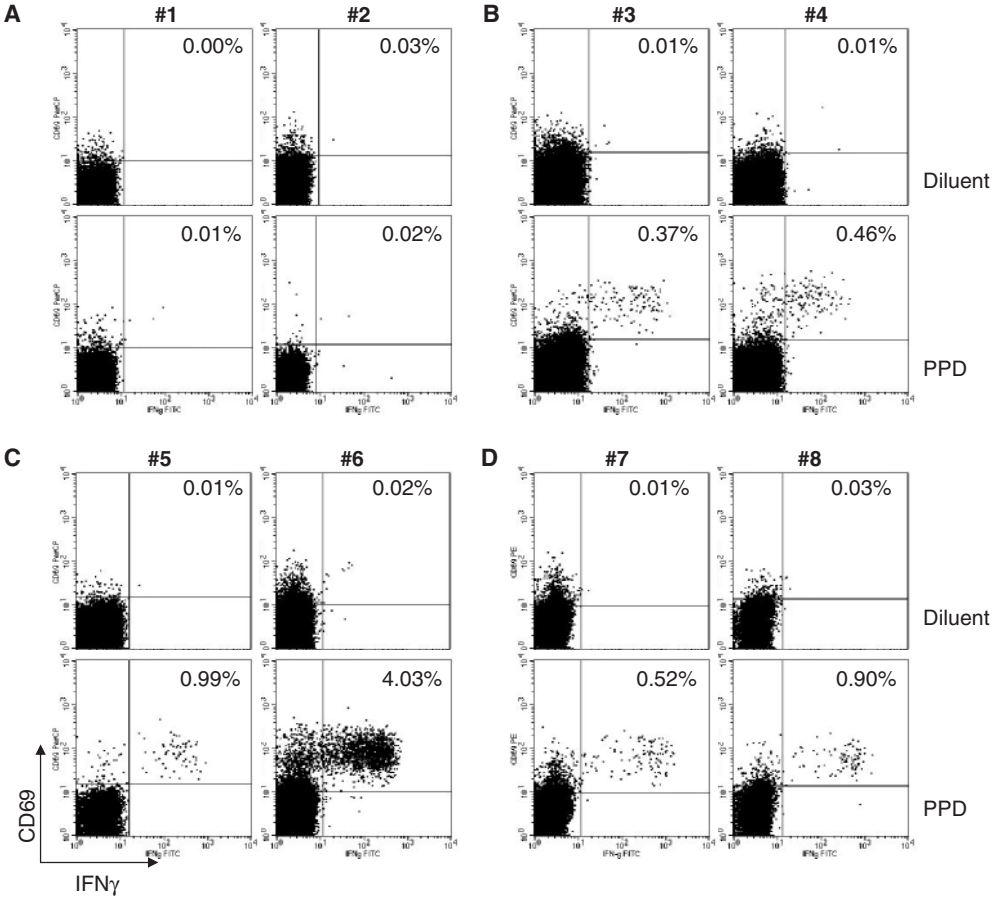

Fig. 1. Quantitation of PPD-specific CD4 T-cells in immunocompetent individuals using flow cytometry. Dotplots of two individuals, respectively, of the following groups are shown: (**A**) non-BCG-vaccinated individuals without known history of tuberculosis contact (skin-test negative), (**B**) latently infected individuals with recent contact to a patient with active tuberculosis (skin-test 20 and 30 mm, respectively), (**C**) patients with known history of treated tuberculosis and (**D**) patients with active tuberculosis (acid fast bacilli-positive from sputum). Patients in panels C and D had no skin-test performed due to contraindication for skin testing. Patients in panels B–D were also ESAT-6 positive (data not shown).

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

Specific stimulation of CD4 T-cells was performed from heparinized whole blood. Titred amounts of PPD (222 IU/ml, Tuberkulin-GT-1000; Chiron Behring, Marburg, Germany), recombinant ESAT-6 (10 μg/ml, kindly provided by Lionex, Braunschweig, Germany), CMV antigen (32 μl/ml; BioWhittaker, Verviers, Belgium or Virion, Würzburg, Germany), or *Staphylococcus aureus* enterotoxin B (SEB; 2.5 μg/ml; Sigma, Deisenhofen, Germany) were used as stimulus in the presence of 1 μg/ml anti-CD28 and anti-CD49d (clones L293 and 9F10; BD, Heidelberg, Germany), respectively, as previously described [13,15,23]. As negative controls, cells were stimulated with diluent (Chiron Behring) or control antigen (BioWhittaker or Virion). Stimulation and staining was done as described before [13,15,23] using anti-CD4 (clone SK3), anti-interferon (IFN)-γ (clone 4S.B3), anti-tumour necrosis factor (TNF)-α (clone Mab11), anti-CD69 (clone L78; all antibodies from BD). At least 15 000 CD4 T-cells were analysed 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 [13].

Quantitation of ESAT-6 reactive T-cells using ELISPOT assay

ESAT-6 reactive T-cells were quantified from 200 000 peripheral blood mononuclear cells (PBMC)/well using the ELISPOT assay (AID GmbH, Straßberg, Germany) according to the manufacturer's instruction. Mock-stimulation and stimulation using pokeweed mitogen served as negative and positive controls, respectively.

Determination of PPD-reactivity in vivo using the Mendel–Mantoux skin-test

Mendel–Mantoux skin testing was carried out by the intracutaneous inoculation of 10 U of PPD (Tuberkulin-GT-10; Chiron Behring) into the volar surface of the forearm in accordance with standard national German guidelines as described before [15]. The resulting induration was determined by two qualified persons (U.S. and H.J.) 48–72 h

thereafter and compared with the diluent. Clinical exclusion criteria for skin testing were a known history of tuberculosis infection, skin disorders such as malignancies or suffusion or intercurrent pyrexiel illness. Practical exclusion criteria were a distance of more than 50 km between our study centre and the place of residence, or the lack of informed consent for the skin-test.

Statistical analysis

Statistical analysis was performed using Prism V4.01 software (Graphpad, San Diego, USA). The Mann–Whitney test was applied to analyse differences in CMV- or *S. aureus* enterotoxin B (SEB)-reactive T-cell frequencies between PPD-positive and negative patients. The Wilcoxon matched pairs test was used to analyse differences in PPD reactive T-cell frequencies before and 1 year after transplantation. The Fisher's exact test was used to analyse differences between two groups of individuals with respect to the prevalence of PPD reactivity or skin-test responses. Correlations were calculated according to Spearman. The χ^2 test and the Kruskall–Wallis test were used to calculate differences in prevalence and PPD-specific T-cell frequencies, respectively, among renal transplant recipients as compared with previously characterized control groups of haemodialysis patients and immunocompetent individuals (Tables 1 and 2).

Results

Detection and quantitation of PPD-specific CD4 T-cells in immunocompetent individuals directly from whole blood

IFN-γ based *in vitro* tests such as ELISPOT or QuantiFERON-assays have previously been established for the detection of T-cell reactivity against *M. tuberculosis* in immunocompetent individuals [18–21]. Similarly, flow-cytometric tests rely on cytokine induction after specific stimulation and have already been used for the quantitation of antigen-specific T-cells against various viral antigens such as CMV, adenoviruses or HIV [13,14,23]. As illustrated in Figure 1 and [15], PPD-specific CD4 T-cells may specifically be quantified using flow cytometry. Heparinized whole blood was incubated with PPD-antigen or diluent for 6 h *in vitro* and antigen-specific cytokine induction was analysed. The frequency of PPD-specific CD4 T-cells is given as the percentage of CD69 and IFN-γ positive CD4 T-cells. PPD reactivity was below detection limit in BCG non-immunized individuals without any known tuberculosis contact (Figure 1A). Conversely, PPD-specific CD4 T-cells may be detected from immunocompetent individuals with latent infection (after recent contact to a patient with tuberculosis, Figure 1B), in individuals with a known history of treated tuberculosis (Figure 1C), and in patients with active tuberculosis prior to therapy (Figure 1D). In general, the diluent failed to induce any specific cytokine production. Thus, as with other established cytokine-based *in vitro* tests, these

Table 1. PPD-prevalence and antigen-specific CD4 T-cell frequencies in transplant recipients as compared with haemodialysis patients and immunocompetent health care workers[a]

Prevalence	Transplant recipients $n=117$		Health care workers[a] $n=107$	Haemodialysis patients[a] $n=127$	
	PPD-negative	PPD-positive	PPD-positive	PPD-positive	
	47.86% $n=56$	52.14% $n=61$	48.6% $n=52$	53.5% $n=68$	$\chi^2=0.60$ $P=0.74$[b]
PPD-specific CD4 T-cells (median and range)	<0.05% $n=56$	0.22% (>0.054–0.71%) $n=61$	0.17% (>0.053–0.75%) $n=52$	0.26% (>0.054–0.12%) $n=68$	$P=0.38$[b]
CMV-specific CD4 T-cells (median and range)	2.43% (0.123–8.58%) $n=38$	1.75% (0.112–2.53%) $n=52$	ND	ND	$P=0.51$[c]
SEB-reactive CD4 T-cells (median and range)	8.07% (0.483–0.69%) $n=12$	5.61% (1.182–2.30%) $n=20$	ND	ND	$P=0.98$[c]

PPD-prevalence is defined as the percentage of individuals with PPD-specific CD4 T-cell frequencies >0.05%; CMV-specific CD4 T-cell frequencies are shown for CMV seropositive individuals; respective frequencies in CMV seronegative individuals were all <0.05%.
[a]Data from haemodialysis patients and immunocompetent health care workers are derived from our previously published report [15] using the same methodology.
[b]PPD-prevalence and PPD-specific CD4 T-cell frequencies in the three groups of PPD-positive individuals are not significantly different (χ^2-test and Kruskall–Wallis test, respectively).
[c]CMV or SEB-reactive T-cell frequencies do not differ between PPD-negative and positive transplant recipients (Mann–Whitney test).

Table 2. Prevalence of ESAT-6-reactivity among PPD-positive individuals

Prevalence	Transplant recipients $n=60$		Controls[a] $n=37$	Haemodialysis patients[a] $n=36$	
	PPD-negative ($n=26$)	PPD-positive ($n=34$)	PPD-positive ($n=37$)	PPD-positive ($n=36$)	
ESAT-6-specific CD4 T-cells > 0.05% (%)	0% ($n=0$)	52.9% ($n=18$)	40.5% ($n=17$)	50.0% ($n=18$)	$\chi^2=0.49$ ($P=0.78$[b])

[a]Data from haemodialysis patients and immunocompetent controls are derived from our previously published report [15] using the same methodology.
[b]ESAT-6-prevalence in the three groups of PPD-positive individuals is not significantly different (χ^2-test).

results indicate that the flow-cytometric whole-blood assay is suitable for the detection of PPD specific CD4 T-cells in immunocompetent individuals.

Detection and quantitation of PPD-specific CD4 T-cells in immunosuppressed transplant recipients

PPD-specific CD4 T-cells can also be detected among transplanted patients on immunosuppressive drug therapy (Figure 2A). Dotplots of flow-cytometric analyses of four representative renal transplant recipients with and without PPD-specific T-cell reactivity, respectively, are shown in Figure 2A. Interestingly, despite the presence of immunosuppressive drugs, 0.55 and 0.34% of PPD-specific CD4 T-cells were detectable in the two PPD-positive patients. Within the same individuals, the diluent failed to activate any T-cells. To exclude that a lack of specific PPD-reactivity in the PPD-negative transplant recipients was due to a drug-related suppression of overall T-cell reactivity, patients were analysed for their reactivity towards recall antigens such as SEB or, in case of CMV seropositive individuals, CMV. This analysis showed that T-cells reactive towards CMV or SEB were readily detectable and respective median frequencies did not differ in PPD-negative and PPD-positive individuals (Figure 2A and B; Table 1). This indicates that the failure to detect PPD-specific T-cells does not result from a drug-mediated suppression of T-cell reactivity *in vitro*. In order to directly address the effect of immunosuppressive drugs in individual patients with and without immunosuppression, PPD-specific T-cells were quantified in 11 PPD-positive patients before and 1 year after transplantation. Interestingly, despite immunosuppression, there was no significant difference in PPD-specific CD4 T-cell frequencies before and after transplantation and individual PPD reactivity remained detectable in a similar frequency range (Figure 2C). One patient with PPD-specific T-cell frequencies below the detection limit after 1 year had already very low-level reactivity before transplantation. Taken together, the assay allows the distinction of individuals with or without specific T-cell immunity towards PPD directly from whole blood. Moreover, it is suitable for the detection of PPD-reactive CD4 T-cells in patients even while under immunosuppressive therapy.

The prevalence and frequency distribution of PPD-specific CD4 T-cells in immunosuppressed transplant recipients does not differ from haemodialysis patients and immunocompetent health care workers

Similar frequencies of T-cells towards recall antigens indicated that immunosuppressive drugs did not affect T-cell reactivity *in vitro*. In order to get a general estimate on the prevalence and frequency distribution of PPD-specific T-cells in transplant patients on long-term immunosuppression, PPD-specific CD4 T-cells were quantified from whole blood of 117 long-term renal transplant recipients and compared with two previously characterized control groups of haemodialysis patients and immunocompetent health care workers (Figure 3; Table 1 [15]). No individual had signs or symptoms of active tuberculosis at or around the time of analysis. In the transplant cohort, 61 patients (52.14%) showed PPD-specific CD4 T-cells that exceeded detection limit of 0.05%. Among those, the frequency distribution ranged from >0.05% up to 4.71% with a median of 0.22% (Figure 3A). Interestingly, despite immunosuppressive double or triple drug therapy, neither the prevalence nor median frequency of PPD-specific T-cell reactivity was different from haemodialysis patients and immunocompetent health care workers (Table 1). In line with previous observations in healthy individuals and haemodialysis patients [15], the majority of PPD-specific CD4 T-cells in transplant recipients had a memory phenotype characterized by the expression of CD45RO (data not shown) and there was a highly significant correlation between the expression of the Th1 cytokines IFN-γ and TNF-α (Figure 3B, $R=0.95$, $P<0.0001$). Thus, together with this similarity in phenotype, this comparative cohort analysis further demonstrates that the *in vitro* assay is not adversely affected by immunosuppression and indicates its suitability for detection and quantitation of PPD-specific T-cell immunity in immunocompromised individuals.

Standard skin testing is of inferior negative predictive value and is less practicable in a routine out-patient setting

At present, the quantitative Mendel–Mantoux skin-test is clinically applied for the detection of

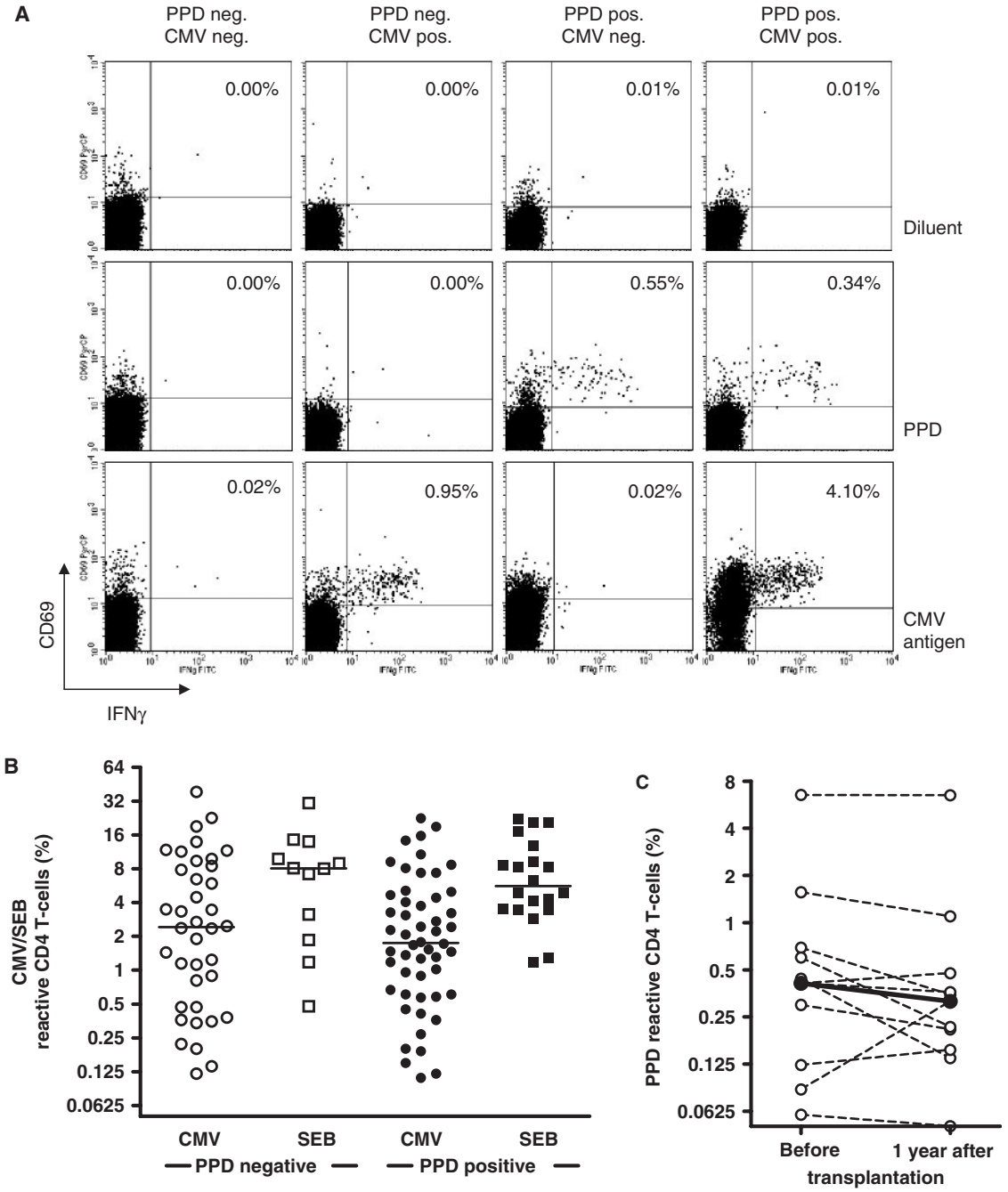

Fig. 2. Quantitation of PPD-specific CD4 T-cells in immunosuppressed individuals using flow cytometry. (**A**) Representative dotplots of renal transplant recipients with or without detectable specific T-cell reactivity towards PPD or CMV, respectively. Whole blood was stimulated with diluent, PPD, CMV antigen, or control antigen (for CMV, data not shown) and specifically induced IFN-γ induction in CD4 T-cells was analysed using flow cytometry. Numbers indicate the percentage of specifically stimulated CD4 T-cells. (**B**) PPD-negative and positive individuals do not differ in recall responses towards CMV antigen or SEB. (**C**) PPD-specific T-cell frequencies do not differ in patients before and 1 year after transplantation ($n = 11$). Black circles indicate median frequencies. Specific T-cells from six PPD-negative individuals remained below detection limit (data not shown).

PPD-specific immunity. Despite its well-known low sensitivity in immunocompromised individuals, it is considered a gold standard and is recommended for routine screening of patients at risk for bacterial reactivation. We comparatively evaluated both the skin-test and the novel flow-cytometric approach for (i) the diagnosis of PPD-specific immunity and (ii) with respect to the applicability for routine screening of transplant recipients in an out-patient setting (Figure 4). Between 03/2002 and 02/2003, a total of 82 patients were included in this analysis. Among them, the skin-test could only be applied in 31.7% of all cases ($n = 26$), whereas flow-cytometric testing was possible in all patients. In 18.3% of all cases,

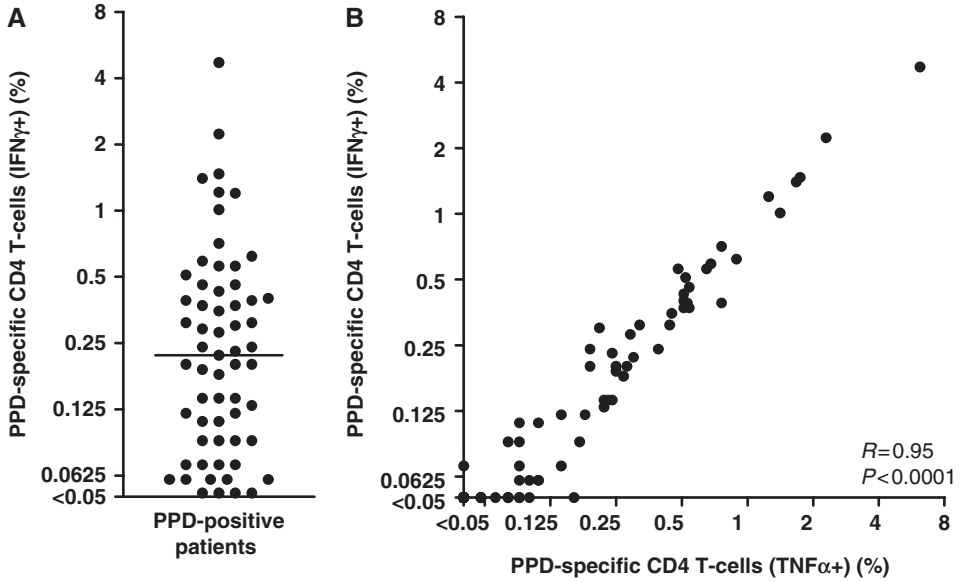

Fig. 3. Heterogeneous frequencies of PPD-specific CD4 T-cells in renal transplant recipients with PPD-specific T-cell reactivity. **(A)** Median T-cell frequencies and frequency distribution ($n = 61$, median 0.22%, from >0.05 to 4.71%) is shown for PPD-positive transplant recipients. (**B**) A significant correlation exists between the percentage of PPD-specific CD4 T-cells producing IFN-γ or TNF-α ($R = 0.95$, $P < 0.0001$; $n = 93$).

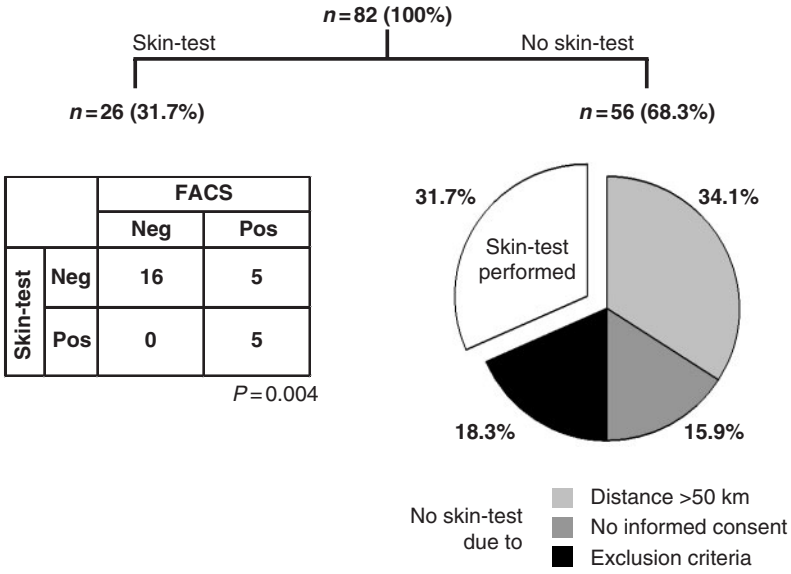

Fig. 4. Skin testing is of inferior negative predictive value and is less practicable in a routine out-patient setting. In 56 patients, no skin-test was performed due to clinical exclusion criteria ($n = 15$) such as a known history of tuberculosis infection ($n = 2$), intercurrent pyrexiel illness ($n = 3$) or skin disorders such as suffusion ($n = 9$) or malignancies ($n = 1$). Practical exclusion criteria were a >50 km distance between our study centre and the place of residence ($n = 28$) or the lack of informed consent for the skin-test ($n = 13$). An induration of ≥5 mm and PPD-specific T-cell frequencies ≥0.05% were deemed positive. There was a significant correlation between skin-test induration and PPD-specific T-cell frequencies ($R = 0.49$, $P = 0.01$; data not shown).

the skin-test could not be performed due to test-specific clinical exclusion criteria such as a known history of tuberculosis infection ($n = 2$), intercurrent pyrexiel illness ($n = 3$) or skin-disorders (suffusion, $n = 9$; malignancies, $n = 1$). Practical exclusion criteria including a distance of more than 50 km between clinic and place of residence applied to 34.1% of all patients. Moreover, the compliance for both tests differed considerably, as a total of 15.9% refused to have the skin-test performed whereas all individuals readily gave informed consent for flow-cytometric testing. Taken together, although practical reasons such as long distance and lack of informed consent would not form an obstacle for vitally important clinical

conditions, these figures emphasize that the skin-test is of poor use for routine screening of large cohorts in an out-patient setting.

The similarity in both prevalence and frequency of PPD-specific T-cells in healthy individuals and immunocompromised patients (Table 1) prompted us to comparatively evaluate the flow-cytometric approach and the skin-test as a measure to detect PPD-specific immunity (Figure 4, left panel). Both tests yielded concordant results in 21 out of 26 cases, as all patients who were FACS-negative were skin-test negative ($n = 16$) and all skin-test positive patients were identified as FACS-positive ($n = 5$, $P = 0.004$). Moreover, there was a significant correlation between skin-test induration and PPD-specific T-cell frequencies ($R = 0.49$, $P = 0.01$; data not shown). As no patient was tested skin-test positive and FACS-negative, the flow-cytometric approach seems of equal or even higher negative predictive value as compared with the skin-test. Therefore, it is at least equally effective in excluding a latent tuberculosis infection. Based on the low sensitivity of skin testing in immunocompromised individuals one might expect a relevant proportion of PPD reactivity among skin-test negative individuals. This was indeed the case, as five out of 21 patients with a negative skin-test had PPD-specific T-cell reactivity *in vitro*. Together this suggests that the flow-cytometric assay may be of particular value for *ex vivo* detection of PPD reactivity in immunocompromised individuals.

ESAT-6 specific T-cell frequencies obtained by flow cytometry and ELISPOT assay show a significant correlation

A detectable reactivity towards PPD in skin-test or flow cytometry may result from an actual *M. tuberculosis* infection and/or from previous vaccination with the BCG strain. To distinguish between these possibilities, T-cells may be stimulated using the recombinant ESAT-6 protein that is absent from all strains of the BCG vaccine. ESAT-6 specific T-cell frequencies were analysed in a subgroup of 21 individuals and compared with T-cell frequencies obtained with the ELISPOT assay, another well-established test to quantify antigen-specific T-cell immunity. Representative examples of four individuals with different levels of ESAT-6 specific T-cells are shown in Figure 5. As expected, ESAT-6-specific T-cell frequencies obtained by both assays show a significant correlation ($n = 21$, $R = 0.53$, $P = 0.01$).

Differential reactivity towards ESAT-6 among PPD-positive transplant recipients

To distinguish whether the detectable reactivity towards PPD in transplant recipients may result from an actual *M. tuberculosis* infection and/or previous vaccination with the BCG strain, T-cells from 60 patients were stimulated using ESAT-6 (Figure 6). This analysis revealed a strong correlation between frequencies of T-cells reactive towards PPD and ESAT-6 ($R = 0.64$, $P < 0.0001$). All PPD-negative patients showed no response towards ESAT-6 ($n = 26$). Conversely, among 34 PPD-positive patients, 18 (52.9%) had ESAT-6-specific T-cells above detection limit. Interestingly, as with PPD, this prevalence of ESAT-6-reactivity did not differ from controls and haemodialysis patients (Table 2). Although the actual status of latent infection or BCG vaccination was not consistently known, the potential to identify patients with detectable and absent immunity towards ESAT-6 may be useful to distinguish a latent infection from BCG vaccination.

Discussion

In this study, the prevalence and frequency of PPD-specific T-cell immunity was characterized in long-term renal transplant recipients using a rapid flow-cytometric whole-blood assay. Unlike skin testing, the *in vitro* assay does not seem to be adversely affected by maintenance immunosuppression. This was evident (i) from positive control stimulations using recall antigens (ii) from paired analysis of patients before and after transplantation and (iii) from the fact that the prevalence of PPD-reactivity *in vitro* was similar to two previously characterized matched control groups with comparable risk of exposure, namely immunocompetent health care workers and haemodialysis patients [15]. Due to the lack of reliable gold standards to precisely define an individual as latently infected, sensitivity and specificity of the flow-cytometric approach cannot be directly quantified and superiority towards the skin-test cannot formally be proven. However, the analysis using PPD as a stimulus allowed a direct comparison of both tests. Based on the well-known impaired responsiveness of transplant recipients in skin-tests [6,12], our findings suggest that the whole-blood assay may have improved sensitivity for the detection of PPD-specific T-cell immunity in patients on immunosuppressive drug therapy. Moreover, stimulation using ESAT-6 may lead to a concomitant increase in specificity as it enables distinction of latent infections from BCG-vaccination responses [15,18,19,24]. Nevertheless, exact definition of sensitivity and specificity in our cohort remains impossible without knowing the true number of patients with latent tuberculosis.

Apart from a case report on a patient receiving azathioprine monotherapy [25] and a recent subgroup analysis of immunosuppressed patients among a hospital-wide screening programme [26], this report is among the first to quantify PPD-specific immunity in patients with iatrogenic immunosuppression. In immunocompetent individuals, the ELISA-based QuantiFERON-approach [20] or the ELISPOT assay [18,24] have already been evaluated and seem similarly effective in detecting reactivity towards *M. tuberculosis*. However, in immunosuppressed patients, the QuantiFERON-approach was recently

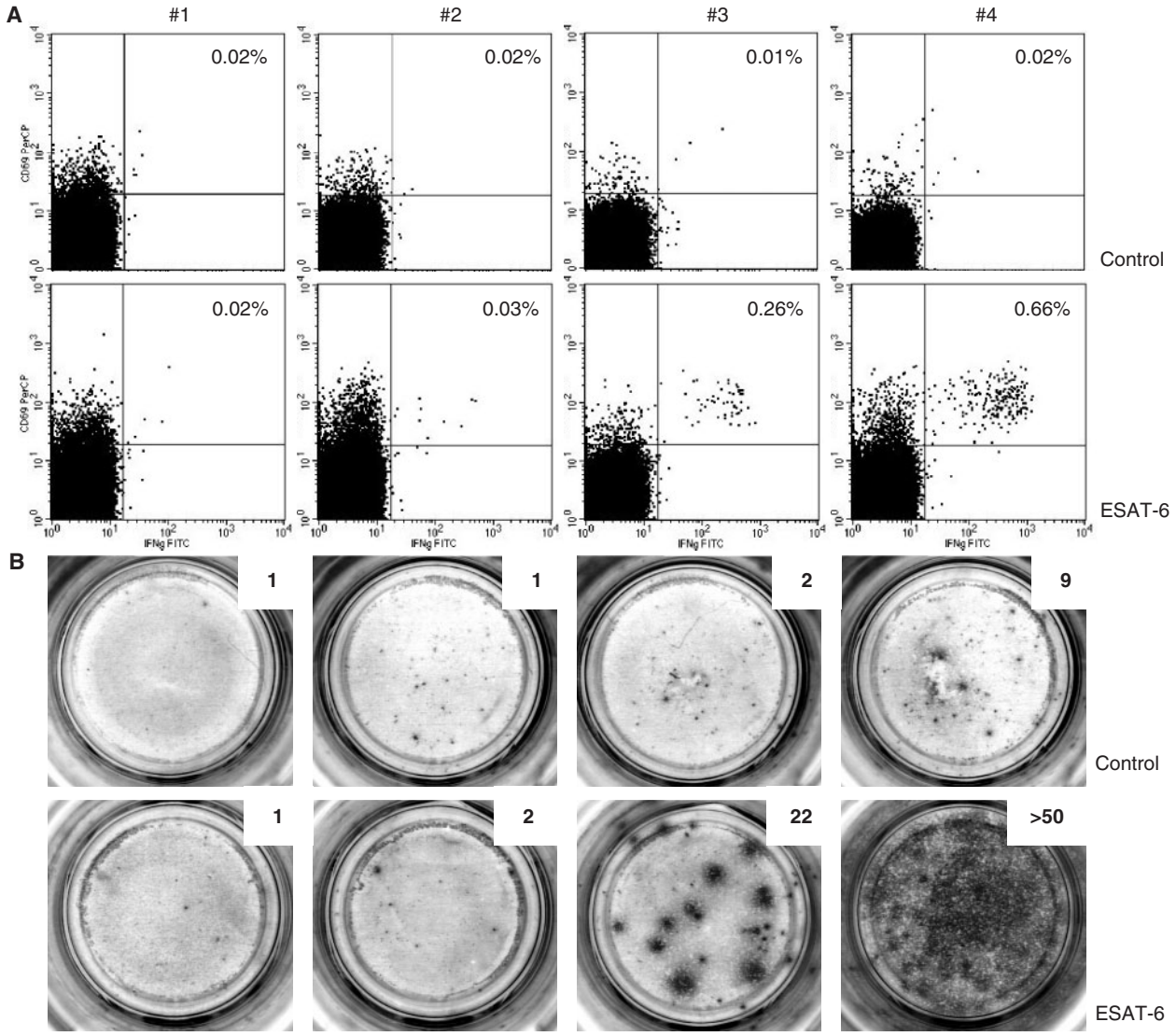

Fig. 5. Correlation between ESAT-6-specific T-cell frequencies obtained by flow cytometry and ELISPOT assay. ESAT-6-specific T-cell frequencies were analysed in 21 individuals using both methods. Four representative examples using flow cytometry (**A**) and ELISPOT assay (**B**) are shown. Numbers represent (**A**) the frequency of ESAT-6-specific CD4 T-cells (%) or (**B**) the number of ESAT-6-specific T-cells from 200.000 PBMC.

reported to yield a significant rate of indeterminate results due to either background reactivity in the negative control or low reactivity in the mitogen control [26]. In our cohort, there was no evidence for indeterminate results, as diluent controls were always low and reactivity towards recall antigens such as CMV or SEB was always detectable (Figure 2B and data not shown). In addition, as reactivity towards recall antigens in long-term transplant recipients is as high as in immunocompetent controls [13,23], the flow-cytometric assay seems less affected by anergy. The same holds true for the ELISPOT assay, as both tests have the potential to control for the number of cells in each assay (Figure 5). There is indeed evidence that the ELISPOT assay may have utility in HIV infected patients [22]. As compared with other *in vitro* tests, the flow-cytometric approach is of similar cost, but has several advantages, as it may be performed directly from whole blood within a single day from small sample volumes (<1 ml of whole blood) and it allows for simultaneous immunophenotyping of reactive T-cells with respect to T-cell subpopulations, differentiation status and cytokine profiles [15].

The use of the whole-blood assay may have various applications that improve the management of tuberculosis in immunocompromised individuals. First, as both compliance and practicability for *in vitro* testing were significantly higher as compared with skin testing, it has the potential for a widespread application in screening programmes in out-patient settings. Second, as the skin-test seems of inferior negative predictive value, the whole-blood assay may more safely rule out the presence of a latent infection in asymptomatic patients. This would avoid unnecessary

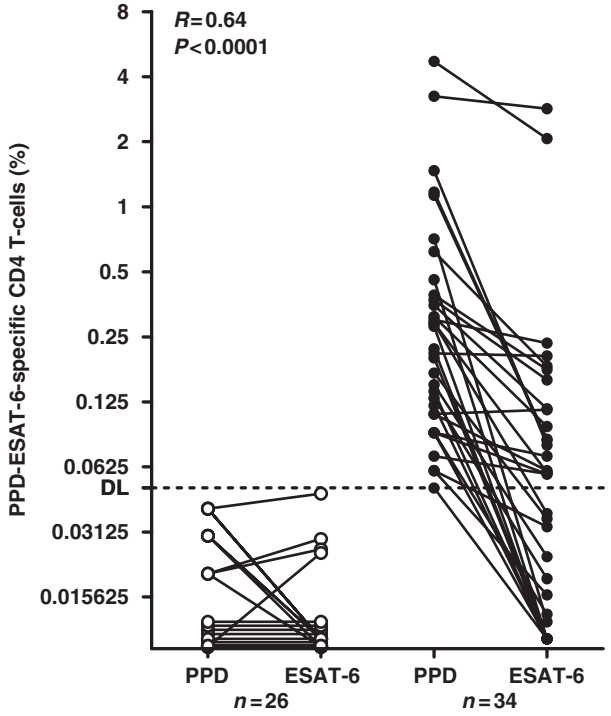

Fig. 6. Identification of latently infected individuals using the ESAT-6-antigen. ESAT-6-specific T-cell frequencies were analysed in 26 PPD-negative and 34 PPD-positive patients. Among PPD-positive individuals, 52.9% had ESAT-6-specific T-cells above 0.05%. T-cell frequencies towards PPD and ESAT-6 showed a significant correlation ($R = 0.64$, $P < 0.0001$). Moreover, a significant correlation exists between the frequency of ESAT-6-specific CD4 T-cells and the induration in skin-test ($R = 0.91$, $P < 0.001$; data not shown). DL denotes detection limit of 0.05%.

chemoprophylaxis and its attendant toxicity. Third, unlike skin testing, the whole-blood assay allows for serial analyses of PPD-specific T-cell immunity without being confounded by the booster phenomenon that carries the risk of inducing false positive indurations [10,12]. Consequently, *in vitro* testing may easily be implemented in monitoring programmes prior to and after transplantation to facilitate the identification of recent converters. Fourth, as patients starting therapy with anti-TNF-α agents are at increased risk for tuberculosis reactivation, screening for latent infection is mandatory prior to treatment [27]. As most of these patients already receive immunosuppressive drugs [28], the flow-cytometric test may have the advantage of being less susceptible to false-negative results. Finally, although not formally proven, *in vitro* testing may well be extended for the use in graft donors to estimate the risk of bacterial transmission from graft to recipients.

A negative test result obtained by *in vitro* testing may certainly help to more clearly exclude a latent infection without anxiety about a false negative decision. What are, however, the individual consequences that should be drawn from a positive test result? Should all these patients be treated prophylactically? In general, a diagnostic test should be performed only in persons who are at high risk for infection and who would benefit from treatment [10,29,30]. Obviously, this risk will critically depend on the overall prevalence of tuberculosis infection in the study population. Current guidelines refrain from recommending a widespread screening of immunocompetent individuals in low-prevalence regions. Instead, screening should be restricted to patients with suspicious clinical findings or to individuals with recent exposure to infectious tuberculosis [10,29,30]. In immunocompromised individuals such as transplant recipients, however, recommendations for screening are rather extended to the patient population as a whole [6,10]. This mainly results from the low sensitivity of the established skin-test as well as from the documented increased risk for reactivation and attendant mortality among immunocompromised individuals [3]. The benefit to provide preventative prophylaxis to patients at risk for reactivation is clear. As, however anti-bacterial drugs are associated with a variety of side effects such as hepatotoxicity [31] or rejections [7], its benefit is less obvious for the transplant population at large. Thus, diagnostic tests that allow a precise decision on anti-bacterial treatment are of utmost clinical importance for the management of tuberculosis infections among transplant recipients. Unlike the skin-test, the whole-blood assay is not adversely affected by maintenance immunosuppression. Thus, it may serve as a more reliable parameter to provide evidence for latent infection in transplant recipients. Future studies will have to establish thresholds in PPD- and/or ESAT-6-reactivity that allow precise targeting of preventative therapy. These thresholds will certainly depend on the individual anamnestic risk as well as on the respective prevalence of tuberculosis in the study population.

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