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Incidence, Outcomes, and Long-Term Immune Response to Tuberculosis in Organ Transplant Recipients

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AUTHOR CONTRIBUTIONS

Y.N., A.H. and D.K participated in research design, the writing of the paper, performance of the research and data analysis. V.H.F and S.N. participated in the data analysis and interpretation. S.H., C.R. participated in writing of the paper.

DISCLOSURE

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ABBREVIATIONS

CFP, culture filtrate protein

CMI, cell-mediated immunity

DMSO, dimethyl sulfoxide

ESAT, early secretory antigenic target

FBS, fetal bovine serum

IFN, interferon

IGRA, interferon gamma release assay

LTBI, latent tuberculosis infection

PBMC, peripheral blood mononuclear cells

SOTR, solid organ transplant recipient

TB, tuberculosis

T_{CM}, central memory T cell

T_{EM}, effector memory T cell

T_{EMRA}, terminally differentiated T cell

ULN, upper limit of normal

ABSTRACT

Background: Tuberculosis (TB) is a significant opportunistic infection in solid organ transplant recipients (SOTR). There are limited data on TB incidence in transplantation from low prevalence countries as well as on long-term TB specific immune responses. **Methods:** We performed a single-center retrospective review of SOTR diagnosed with active TB between 2000 and 2015 and further contacted the available patients for a study of long-term T cell responses using an interferon-gamma (IFN-γ) release assay and a flow cytometry-based assay.

Results: We identified 31 SOTR with active TB for an incidence of 62 cases/100,000 patient-years. 19/31 (61.3%) patients were diagnosed within the first year after transplant. Nineteen (61.3%) were born in countries with high TB prevalence and disseminated disease occurred in 22.6%. No patient had been screened for latent TB infection pretransplant. The majority of patients received isoniazid and a rifamycin as part of multidrug regimen. In addition, 13/29 (44.8%) patients received quinolones. One-year mortality in this population was 19.4%. Eight patients were available for long-term immune responses. Of these, all had detectable IFN-γ response by interferon-gamma release assay testing and 7/8 had detectable TB-specific T cells, primarily central and effector T cell responses in the $CD4^+$ compartment and terminally differentiated T cells in the $CD8^+$ compartment.

Conclusions: TB has high incidence in SOTR even in low-prevalence regions but especially targets patients who originated from TB-endemic countries. Long-term TB-specific T cell responses were found in the majority of patients.

INTRODUCTION

Active *Mycobacterium tuberculosis* (TB) infection can cause pulmonary or extrapulmonary disease in organ transplant recipients.[1] Development of active TB posttransplant in low incidence countries occurs mainly as a consequence of reactivation of latent tuberculosis infection (LTBI) and rarely due to new community-exposure posttransplant or donor-derived infection. Solid organ transplant recipients (SOTR) have a higher risk of developing active TB, estimated at 20-74 times that of the general population.[2,3] The majority of active TB occurs within the first year posttransplant.[2,3] In previous studies, transplant recipients who develop active TB disease have a mortality rate of up to 30%.[4]

T cell immunity, especially via the cytokine interferon-gamma (IFN-γ), plays an important role in protection from *Mycobacterium tuberculosis* reactivation in the general population. A previous study has shown that TB-specific memory T-helper cells secrete interferon gamma.[5] Cell-mediated immunity (CMI) can be determined by stimulating whole blood or peripheral blood mononuclear cells (PBMC) with TB-specific antigen and measuring the quantity or expression of IFN-γ. A commercially available interferon-gamma release assay (IGRA) is QuantiferonTB (Qiagen Inc, USA) whereas flow cytometry can be used to determine IFN-γ

expression on specific T cell subsets. There are no studies that look at persistence of long-term immune responses to TB in transplant patients that have previously been treated. Immune responses would provide important information about the immunologic profile required for the long-term control of TB and could be used to determine risk for active TB and recurrence while on immunosuppression. Therefore, the objectives of this study were to define the incidence and clinical characteristics of active TB in SOTRs in a low TB incidence area and to determine whether previously treated SOT patients have long-term immune responses to TB.

METHODS

Study Design

We first performed a retrospective cohort study of SOTRs with active tuberculosis. The study was conducted at the Multi-Organ Transplant Program, University Health Network, Toronto, Canada. Approval for the study was obtained from the institutional research ethics board according to the Tri-Council Policy Statement on Ethical Conduct for Research Involving Humans. Patients were included if they had received an organ transplant as an adult (age ≥18 years) and had a positive microbiology result for *M. tuberculosis* after transplant by TB-specific polymerase chain reaction

(PCR) or culture between January 1, 2000 and December 31, 2015. Patients with failed kidney grafts at the time of TB development were excluded since they were not routinely followed by the transplant program. The transplant program has a long-standing recommendation that in the pretransplant period, patients be screened for TB using the TB skin test or interferon-gamma release assay if available. However, screening of the patients was done by primary care physicians and was not a prerequisite for transplant listing.

Data regarding demographics, transplant-specific variables (date of transplant, organ type, immunosuppression), and TB-specific variables (history of TB screening, clinical presentation, therapy, outcomes) were collected. Hepatotoxicity was defined as liver enzymes more than 3 times greater than upper limit or normal (ULN) with symptoms or 5 times greater than ULN without symptoms. In order to determine the persistence of a TB-specific CMI response, we contacted transplant recipients who were identified in the retrospective review, and were still alive and on immunosuppression with a functional graft. Informed consent was obtained to conduct TB-specific CMI testing using an interferon-gamma (IFN-γ) release assay (IGRA; QuantiferonTB , Qiagen Inc, USA) and a flow cytometry-based assay.

Laboratory Methods

The QuantiferonTB assay was collected and performed as per manufacturer's instructions. Briefly, 1mL whole blood was collected in each of 3 tubes containing mitogen (positive control), nil (negative control) or TB antigens: ESAT-6, CFP-10, and TB7.7. After overnight incubation, tubes were centrifuged and plasma was obtained. An ELISA was performed for IFN-γ and the final result was expressed as the IFN-γ concentration in the TB antigen tube minus the nil tube. For flow cytometry, peripheral blood mononuclear cells (PBMCs) were isolated from 10mL of heparinized blood by Ficoll-gradient separation. Cells were resuspended in freezing media (FBS + 10% DMSO) and stored in liquid nitrogen. Thawed cells were resuspended in complete RPMI 1640 culture media containing L-glutamine and sodium bicarbonate (Sigma) supplemented with 10% FBS, 1x penicillin/streptomycin and 25mM HEPES. A total of $2x10^5$ PBMCs in 500 µL complete media were incubated with no antigen (media only control) or with PMA and ionomycin (positive control; BD Biosciences). PBMCs were stimulated with recombinant *M. tuberculosis* antigens ESAT-6 and CFP-10 (each at 0.1 µg/mL) (BEI Resources, NIAID, NIH Reference standard NR-14868 for ESAT-6 and NR-49425 for CFP-10) for 16 hours at 37°C in 5% CO_2. After the initial hour, a protein transport inhibitor (BD Biosciences) was added to the

cells to prevent cytokine release from stimulated cells. After incubation, cells were pelleted and stained with Zombie Aqua Fixable Viability Stain (BioLegend), Human BD Fc Block (BD Biosciences) and a cell-surface antibody cocktail consisting of mouse antihuman CD3-PE/Cy7 (clone 17A2, BioLegend), CD4-Pacific Blue (clone OKT4, BioLegend), CD8-APC (clone RPAT8, BD Biosciences), CD45-FITC (Clone HI30, BD Biosciences), CD45RA-PE-Cy5 (clone HI100, BioLegend) and CCR7-PE (clone 4B12, BioLegend). For intracellular cytokine measurement, cells were fixed and permeabilized using BioLegend's Fixation and Intracellular Staining Perm Wash Buffers and stained using mouse antihuman IFN-γ-APC-eFluor 780 (clone 4S.B3, eBioscience). Frequencies of IFN-γ^+ $CD4^+$ and IFN-γ^+ $CD8^+$ T cells were acquired on a BD LSRII cytometer using FACSDiva software and analysis was performed using FlowJo version 10. Frequencies of central memory (T_{CM}), effector memory (T_{EM}) and terminally differentiated effector memory cells reexpressing CD45RA (T_{EMRA}) were identified using the CCR7/CD45RA staining convention described elsewhere in detail.[6]

Statistical Methods

Demographics and outcomes were described using descriptive statistics. Overall incidence was calculated using total patients active in the transplant program from 2000-2015. Follow-up time

was calculated until graft failure or death. Frequencies of TB-specific T cell subsets expressing IFN-γ were calculated by subtracting the frequency of IFN-γ-positive cells in unstimulated negative controls from the frequency of IFN-γ-positive cells in the antigen exposed cells. Frequencies were expressed as a percentage of total $CD4^+$ or $CD8^+$ T cells. Correlation between the IFN-γ values obtained by IGRA and TB-specific IFN-γ^+CD4^+ and IFN-γ^+CD8^+ T cells was performed using Spearman test. Mann-Whitney U test was used to determine association between T cell responses and clinical variables. All statistical analysis was performed using IBM SPSS v. 22 (Chicago, IL) and Prism GraphPad v. 6.0 (La Jolla, CA).

RESULTS

Patient Population

During the time period of 2000-2015, a total of 33 organ transplant recipients were treated for active TB. Of these, 2 patients (both lung transplants) were excluded due to presumed donor-derived TB and empiric TB treatment. Patient characteristics for the remaining 31 patients are shown in Table 1. Median age was 51 (range 25-75) years and transplant types were liver (14/31;45.2%), kidney (8/31; 25.8%), lung (6/31;19.4%), and heart (3/31;9.7%). The

majority of patients were receiving triple immunosuppressive maintenance therapy at the time of TB diagnosis including corticosteroids, calcineurin-inhibitor and mycophenolate. No patient had documentation of TB skin testing or TB-specific IGRA testing prior to transplant. 30/31 patients had pretransplant chest imaging available for review. Of these only 5/30 (16.7%) had imaging that suggested latent TB infection (apical fibrosis, upper lobe granuloma, pleural thickening, mediastinal lymph node calcification). Most patients (19/31, 61.3%) were born in countries with high TB prevalence while 4 (12.9%) were born in Canada and 8 (25.8%) were unknown. During this time, there were 8,376 patients in active follow-up and 6,135 new transplants in the program. Therefore, the incidence of active TB in SOTRs was 62 cases/100,000 transplant-years. TB incidence in each transplanted organ were 38, 89, 87, 117 cases/100,000 transplant-years for kidney, liver, heart and lung transplant, respectively (Figure 1).

Active TB diagnosis and treatment

The median time to TB diagnosis after transplantation was 8 months (range 1-327 months) and 21/31 (67.7%) were diagnosed within the first year after transplant. Twenty-six patients (83.9%) had pulmonary TB and 12 (38.7%) had extrapulmonary involvement. All of the *M. tuberculosis*

isolates cultured were sensitive to isoniazid, rifampin, pyrazinamide and ethambutol.

In this cohort, 22/31 (70.9%) patients were hospitalized for the diagnosis of TB. Two patients died prior to starting treatment. Of the remaining 29, all but 1 patient received isoniazid and a rifamycin (rifampin or rifabutin) as initial therapy as part of a multidrug regimen (Table 2). Median duration of therapy was 9 months (range 6-18). A total of 13/29 (44.8%) patients were treated with moxifloxacin. In 9 patients, quinolones (8 moxifloxacin and 1 gatifloxacin) were used as part of the initial treatment regimen generally as a 4^{th} or 5^{th} drug (in addition to isoniazid, rifamycin, pyrazinamide, ethambutol) due to the potential for resistance; 4 patients were switched to moxifloxacin due to adverse effects. Adverse events are described in Table 2. Liver enzyme abnormalities developed in 11/29 (37.9%) patients although a discontinuation or change in treatment regimen was instituted in only 4 patients. Biopsy-proven graft rejection occurred in 5/29 (17.2%) and of those, 2 patients developed graft loss within 1 year after diagnosis of active TB. Of these 5 patients, 3 were receiving rifabutin at the time of rejection and none were on rifampin. One-year all-cause mortality in the overall population was 6/31 patients (19.4%).

Mycobacterium tuberculosis-specific immunity

Of the 31 patients included in the retrospective review, 10 patients continued to be actively followed in our program. Of these, 8 provided consent for CMI testing. IFN-γ levels by IGRA ranged from 0.16 IU/mL to >100 IU/mL. The lowest results were seen in lung transplant recipients. Demographics of these patients are shown in Table 3. By flow cytometry, 7 out of 8 transplant recipients tested had a T cell response to *M. tuberculosis* antigens (Table 3). Median frequencies of TB-specific IFN-γ^+CD4$^+$ and IFN-γ^+CD8$^+$ T cells were 0.57% (range 0-6.97%) and 0.79% (range 0-16.5%), respectively. These were further subclassified into T_{EM}, T_{CM} and T_{EMRA} subsets. For IFN-γ^+CD4$^+$ T cells, the greatest proportion of TB-specific T cells, in a majority of subjects (5/8), were found in the T_{EM} (effector memory) compartment, followed by lower frequencies of IFN-γ^+CD4$^+$ T_{CM} (central memory) cells (Figure 2A). The T_{EM} compartment is important in response to pathogen rechallenge.[7] In 2/8 patients, the highest frequency of TB-specific IFN-γ^+CD4$^+$ T cells were T_{CM} cells followed by lower frequencies of T_{EM} cells. IFN-γ^+CD4$^+$ T_{EMRA} (terminally-differentiated) cells were at the lowest frequency. In comparison, the frequency of antigen-specific IFN-γ producing T_{EMRA} cells were consistently much higher within the CD8$^+$ T cell compartment. In fact, in 3 subjects (#1, 3 and 8), the highest frequency of

antigen-specific IFN-γ^+CD8^+ cells were T_{EMRA} cells, followed by T_{EM} and T_{CM} (Figure 2B). In 4 additional subjects, antigen-specific IFN-γ^+CD8^+ T_{EM} cells made up the most prominent population, followed by antigen-specific IFN-γ producing T_{EMRA} cells. In the 1 lung transplant recipient (subject #7), TB-specific IFN-γ producing $CD4^+$ or $CD8^+$ T cells were not detected, and we found overall decreased frequencies of total $CD4^+$ and $CD8^+$ T_{CM} and T_{EM} cells, suggesting perhaps a global deficit in these memory compartments in addition to abrogated TB-specific responses (Figure S1, SDC, http://links.lww.com/TP/B595). This patient also had a low IFN-γ response by IGRA. There was moderate correlation between IGRA results and IFN-γ producing $CD4^+$ and $CD8^+$ T cells (Spearman rho 0.63, p=0.094). There was no association of T cell response with extent of disease, time from diagnosis or current immunosuppression (data not shown).

DISCUSSION

We examined the incidence of active TB at a large-volume organ transplant center and found that the incidence was greatest in lung followed by liver, heart and kidney transplants. We also conducted a cross-sectional study of TB-specific CMI and in a subgroup of patients with a

history of active TB and found that the majority of patients retain T cell immunity to TB many years after resolution of infection.

The incidence of TB after organ transplantation is reported to be up to 500-638 per 100,000 patient-years.[8-10] However, these incidence rates are mostly derived from countries that are intermediate- or high-prevalence for TB (TB incidence >10 per 100,000 population per year) such as Spain, South Korea, China and Taiwan.[8,11-14] Incidence rates of TB after transplantation from low incidence countries such as Canada and the United States are limited. Older literature from 1990s shows the incidence of active tuberculosis in solid organ transplant recipients in the United States varied from 0% to 6.4%. [1,15-18] However, since then there has been a rapid increase in the number of transplants as well as in immigration rates which may change the demographics of the patient population undergoing transplant. We found the incidence of active TB in our cohort was 62 per 100,000 transplant-years. In comparison, this is at least 14-fold greater than the published incidence of active TB in the general population of Ontario (4.3 per 100,000).[19] Lung transplants had the greatest incidence of TB and this is similar to a report from Spain, where lung transplant recipients had a TB incidence of 2,072/100,000 transplant-years.[12] Although screening for latent TB infection is recommended by American

Society of Transplantation and international guidelines,[20] screening was not consistently done at our center until 2016. Since our program performs transplantation for patients from a large catchment area and 2 clinic visits are required for TB skin testing, patients were asked to have TB testing done with their local primary care physician. However, it was likely that pretransplant testing was not done in many cases. There was no documentation of pretransplant TB skin test or IGRA for any of the patients that developed active TB in our series and none received therapy for latent TB infection previously. Chest imaging showed evidence of latent TB in only 16.7% of patients although it may be difficult to differentiate changes of prior TB exposure from underlying lung disease in lung transplant recipients. A previous study has shown that transplant centers in Canada with centralized TB screening programs appear to have a lower risk of TB.[21] As a result of these findings, our program now ensures the performance and documentation of TB screening test results. The majority of patients with TB in our study (61%) were born in TB-endemic countries which is similar to overall Canadian statistics where 71% of TB occurred in the foreign-born population.[22] Therefore, it may be important to prioritize screening in those from TB-endemic countries with TB skin test or IGRA combined with chest imaging.

Hepatotoxicity in our study appeared to be common and occurred in 42% of patients although only 12.9% required a change in therapy. This is in keeping with other studies that have shown hepatotoxicity ranged from 10-25%. [11,23] Quinolones are generally reserved as second line TB therapy although appear to be increasingly used in transplantation for treatment of latent and active TB. [24-26] In our cohort, approximately 40% of patients received moxifloxacin primarily due to disseminated disease and the risk of drug-resistant TB in patients from some endemic countries; moxifloxacin was generally well tolerated with no significant adverse effects. The high rate of rejection observed in our study may have been due to a combination of reduction of immunosuppression and rifamycin use despite careful monitoring of levels. Although reduction of immunosuppression is beneficial for many infections, many patients in our cohort recovered from TB without significant changes to their immunosuppression.

A novel aspect to our study was the determination of TB-specific immunity in transplant patients who were treated for active TB. We first used an IGRA (QuantiferonTB) and found that all patients had some antigen-specific IFN-γ response. Although this is a screening test for latent TB, it was interesting to note that 2 patients, both lung transplants, would have been negative using the screening cut-off for IFN-γ levels (<0.35 IU/mL) despite having had active TB in the past. It

is not clear whether IGRA testing would have been positive prior to development of TB.

Except for 1 lung transplant recipient, all patients had detection of TB-specific T cell response by flow cytometry. Overall, long term TB-specific T cell responses were primarily comprised of the T_{EM} (effector memory) subset. Specifically, $CD4^+$ T cell responses were primarily in the T_{EM} and T_{CM} compartments, while $CD8^+$ T cells were predominantly of a T_{EM} and T_{EMRA}. In general, T_{EM} and T_{CM} cells may proliferate vigorously in response to pathogen challenge whereas terminally differentiated cells (T_{EMRA}) may be limited in their ability to proliferate in rechallenge to the pathogen but remain potent effector cells.[7,27] Therefore, presence of CMI especially in the $CD4^+$ T_{EM} and T_{CM} compartments and $CD8^+$ T_{EMRA} compartment may be a marker of resolved TB infection. Further studies would need to determine whether the presence of CMI offers long-term protection from relapsing TB or disease due to reexposure. The low levels of $CD8^+T_{CM}$ in our study is consistent with a TB-CMI study in the nontransplant population that showed a decline in CD8+ T_{CM} over time.[28]

Our study has some limitations. It is limited in its retrospective nature and potentially missed cases although we have a comprehensive follow-up for all transplant patients in our program, making missed TB cases unlikely. The absolute number of clinical cases is small although to

our knowledge represents the largest original case series from a low prevalence country in the published literature. Although some cases occurred early posttransplant, we do not have donor information to determine whether these are donor-derived. Strengths of our study include data on TB therapy in the modern era including the use of quinolones and the novel assessment of long-term TB-specific CMI.

In conclusion, we show that active TB has a significant incidence and impact in transplant recipients. This supports the need for pretransplant screening even in low-prevalence countries especially in patients with epidemiologic risk factors. Long-term CMI responses appear preserved in most patients.

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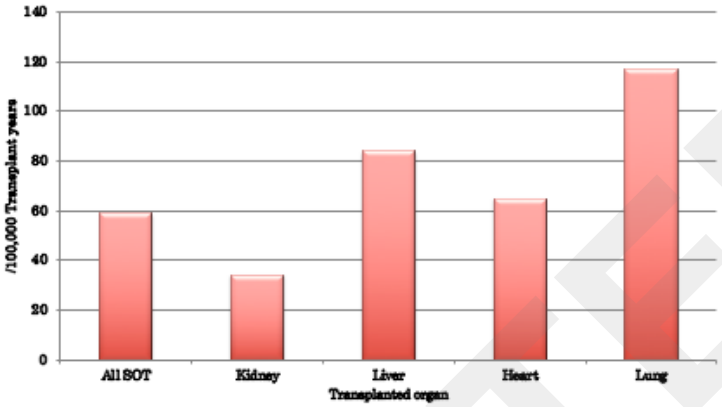
FIGURE LEGENDS:

Figure 1. Incidence of active tuberculosis in Organ Transplant Recipients by transplant type.

Figure 2: Frequencies of $CD4^+$ (Panel A) and $CD8^+$ (Panel B) T cell subsets expressing IFN-γ in response to TB antigens in long-term survivors of posttransplant TB.

FIGURE 1



*SOT, Solid Organ Transplant.

Figure 2

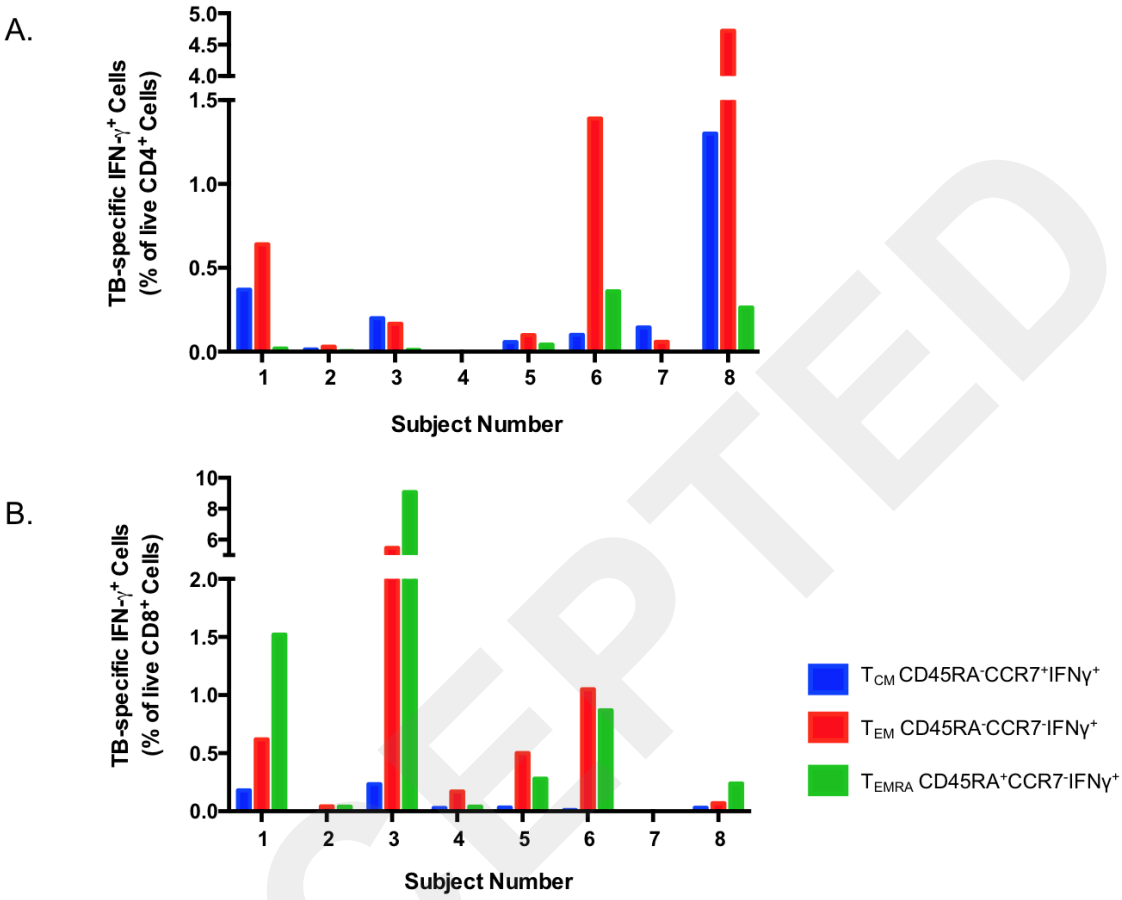


Table 1: Characteristics of patients with Posttransplant Tuberculosis (TB).

Characteristic	**N=31**
Age (years) (median; range)	50 (25-75)
Sex (Male/Female)	13/18
Type of Transplant • Liver • Kidney • Lung • Heart	 14 (45.2%) 8 (25.8%) 6 (19.4%) 3 (9.7%)
Country of Birth • Canada/United States • Outside of Canada/United States* • Unknown	 8 (25.8%) 19 (61.3%) 4 (12.9%)
Time Posttransplant to TB diagnosis (Months)(median;range)	8 (1-327)
Diagnosis within 1 year of transplant	19 (61.3%)
Biopsy-proven acute rejection in the 90 days prior to TB diagnosis	3 (9.7%)
Use of antithymocyte globulin in 6 months prior to diagnosis	14 (45.2%)
Immunosuppression at Diagnosis	

• Prednisone	29 (93.5%)
• Cyclosporine	15 (48.4%)
• Tacrolimus	14 (45.2%)
• Mycophenolate	14 (45.2%)
Pre transplant TB screening test	0 (0%)
Positive specimen	
• Sputum	16 (51.6%)
• Broncho-alveolar lavage	8 (25.8%)
• Pleural fluid	6 (19.4%)
• Lymph node	5 (16.1%)
• Liver	2 (6.5%)
• Other sites**	6 (19.4%)
Diagnosis	
• TB-specific PCR	11 (35.5%)
• Culture	20 (64.5%)
Site of Infection	
• Pulmonary	19 (61.3%)
• Extrapulmonary only	5 (16.1%)
• Disseminated§	7 (22.6%)

*Country of origin includes: India (n=6), Pakistan (n=4), China (n=3), Vietnam (n=3), Philippines (n=2), Ethiopia (n=1), Iraq (n=1), Somalia (n=1)

** one each of urine, blood, cerebrospinal fluid, parathyroid gland, bone marrow, small bowel

§≥ 2 organ involvement

Table 2: Therapy and Outcomes for Active tuberculosis (TB).

	N=29*
TB Treatment Duration (months) (median;range)	9 (6-30)
Reduction of Immunosuppression	15 (51.7%)
TB Therapy	
• Isoniazid	28 (96.6%)
• Rifampin/Rifabutin	29 (100%)
• Pyrazinamide	22 (75.9%)
• Ethambutol	27 (93.1%)
• Amikacin	2 (6.9%)
• Quinolones	13 (40.9%)
Treatment-associated Adverse Effects	
• Hepatotoxicity	11(37.9%)
• Rash	3 (10.3%)
• Peripheral Neuropathy	3 (10.3%)
1 Year Transplant Outcomes	
• Graft rejection	5 (17.2%)
• Graft loss	2 (6.9%)

*excludes 2 patients who died prior to starting treatment

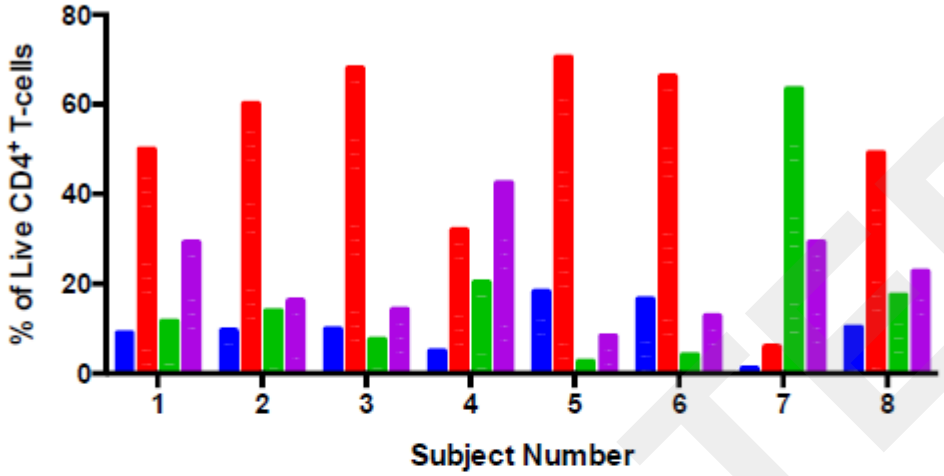
Table 3. Summary of TB-specific Cell-mediated Immune Responses in patients previously treated for active TB (n=8).

Subject number	Age at transplant /sex	Organ Transplanted	Time from transplant to TB diagnosis (months)	Time from diagnosis to QFT (months)	IFN-γ by ELISA (QFT-TB result IU/mL)*	TB-specific IFN-γ^+CD4$^+$ T cells (%)	TB specific IFN-γ^+CD8$^+$ T cells (%)
1	51/F	Kidney	2	26	0.44	2.7	4.0
2	36/M	Kidney	6	128	0.53	0.054	0.09
3	48/F	Kidney	86	109	>100	6.97	16.47
4	24/F	Liver	2	106	83.5	0.7	0.75
5	64/F	Liver	8	57	1.24	0.44	0.83
6	38/M	Liver	53	36	1.5	1.24	2.34
7	50/F	Lung	4	59	0.16	0.0	0.0
8	37/F	Lung	192	15	0.16	0.21	0.48

*IFN-γ value after subtraction of nil tube

Figure S1: Total CD4+ (Panel A) and CD8+ (Panel B) T cell frequencies (unstimulated) in long-term survivors of post-transplant TB.

A.



B.