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Brief Communication

Renal Allograft Recipients Fail to Increase Interferon-γ During Invasive Fungal Diseases

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Invasive fungal diseases are a major cause of death in renal allograft recipients. We previously reported that adjunctive recombinant human interferon-γ therapy has clinical utility for invasive fungal diseases after renal transplantation. We have now developed a rapid peripheral blood-based quantitative real-time PCR assay that enables accurate profiling of cytokine imbalances. Our preliminary studies in renal transplant patients with invasive fungal diseases suggest that they fail to mount an adequate interferon-γ response to the fungal infection. In addition, they have reduced IL-10 and increased TNF-α when compared to stable renal transplant patients. These preliminary cytokine profiling-based observations provide a possible explanation for the therapeutic benefit of adjunctive human interferon-γ therapy in renal allograft recipients with invasive fungal diseases.

Key words: Cytokines, diagnosis, interferon-γ, invasive fungal disease, renal allograft

Abbreviations: EORTC/MSG, European Organisation for Research and Treatment of Cancer/Mycoses Study Group; IFN-γ, Interferon-γ; IL-10, Interleukin-10; Th-1, T-helper 1; TNF-α, Tumor Necrosis Factor-α.

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[Correction made after online publication September 13, 2012: Abbreviations have been updated.]

Introduction

Invasive fungal diseases are a major cause of morbidity and mortality in renal allograft recipients (1). This clinical problem is a consequence of an increasing requirement for organ transplantation, and the aggressive immunosuppression treatment protocols required to sustain allogeneic transplant programs (2). Transplant immunosuppressive drug regimens primarily target T cell mediated graft rejection, and it is notable that solid organ transplant recipients develop invasive fungal diseases without neutropenia being a major risk factor (3). This clinical observation suggests that T cell-based immune responses are important for protective immunity against invasive fungal diseases in this patient population, and it draws parallels with the emergence of invasive fungal diseases in patients with late-stage AIDS.

There is also increasing evidence that susceptibility to invasive fungal diseases is associated with an impaired interferon-γ response in the lung, and that the inflammatory pathology is driven by a Th17 response when Th1 responses are impaired (4). These observations have led to the evaluation of recombinant human interferon-γ therapy in the treatment of invasive fungal diseases in renal allograft recipients who are receiving T cell-directed immunosuppressive drug regimens (5). Our clinical studies indicated that such an approach can be useful because it led to the complete clearance of refractory and disseminated invasive fungal diseases (5). Although there still remain concerns that recombinant human interferon-γ therapy could provoke allograft rejection in transplant patients, we have not yet seen any evidence of this. In addition, we are reassured by the established literature in chronic allograft rejection and in graft-versus-host disease, that recombinant human interferon-γ therapy can be therapeutically useful (5,6).

Given these observations, we investigated whether renal allograft recipients with invasive fungal diseases had impaired IFN-γ responses. A rapid and robust peripheral blood based cytokine mRNA based assay was developed to try to identify those renal allograft recipients who had an impaired Th-1 immune response, and who could potentially benefit from adjunctive recombinant human interferon-γ therapy.

Methods

Patients were recruited from the West London Renal Transplant Centre at Imperial College Healthcare NHS Trust, UK. All clinical studies were approved by the UK National Research Ethics Committee. Patients with invasive fungal diseases had either proven or probable invasive fungal disease according to the European Organisation for Research and Treatment of Cancer/Mycoses Study Group's (EORTC/MSG) diagnostic criteria (7). They

were compared with stable renal transplant recipients who were matched for age, time from transplantation and cytomegalovirus status.

For patients with invasive fungal diseases, blood was sampled at the time of diagnosis of their fungal infection and before adjunctive recombinant human interferon-γ therapy. For stable renal allograft recipients, blood was sampled during routine clinic follow-up visits. Twenty milliliters of blood was collected and peripheral blood mononuclear cells separated by Ficoll–Paque. They were washed twice with phosphate-buffered saline and resuspended in RPMI 1640 supplemented with 200 IU/mL penicillin–streptomycin and 10% v/v autologous plasma. Cells were then adhered to 48 well plastic tissue culture plates and incubated at 37°C in 5% CO_2. After 3 h, they were recovered, lysed in TRIzol® (Invitrogen, Paisley, Scotland) and the RNA isolated. RNA was reverse transcribed to cDNA after removal of genomic DNA with the Quantitect Reverse Transcription Kit (Qiagen, Crawley, England) according to the manufacturer's instructions.

Gene expression was quantified by quantitative real-time PCR assay and normalized against hypoxanthine-guanine phosphoribosyltransferase (HPRT) using a gene plasmid standard as previously described (8,9). The following intron-spanning primer pair sequences were used.

IFN-γ	(f)	AAACGAGATGACTTCGAAAAGCTGA
IFN-γ	(r)	CTTCGACCTCGAAACAGCATCTGACT
IL-10	(f)	CTGAGAACCAAGACCCAGACATC
IL-10	(r)	CACGGCCTTGCTCTTGTTT
TNF-α	(f)	AGGCGGTGCTTGTTCCTCA
TNF-α	(r)	GTTCGAGAAGATGATCTGACTGCC
HPRT	(f)	GCTCGAGATGTGATGAAGGAG
HPRT	(r)	TCCCCTGTTGACTGGTCATT

All data was analyzed using GraphPad Prism software and a Mann–Whitney test. Results shown as mean ± SEM.

Results

Ten renal allograft recipients with proven or probable invasive fungal diseases were studied over a 5-year period. They were from a cohort of 850 renal allograft recipients. The invasive fungal disease group included three patients with invasive aspergillosis, three patients with disseminated phaeohyphomycosis, two patients with proven candidemia (by culture) and radiological evidence of hepatosplenic candidiasis, one patient with disseminated histoplasmosis and one patient with pulmonary mucormycosis.

Their time from transplantation was 1178 ± 464 days. Four patients were receiving MMF in addition to tacrolimus and prednisolone. No patient had been neutropenic in the 3 months before their diagnosis with an invasive fungal disease. Their median absolute lymphocyte count in the month before the invasive fungal disease was 0.75 ± 0.32 × 10^9 /L. Eight of the 10 patients were CMV IgG positive, one patient had a coexisiting BK nephropathy and one patient had a concurrent *Citrobacter freundi* isolated from the lung: this was the patient with pulmonary mucormycosis. There were no other concomitant bacterial of viral infections. They were compared with an age-matched group of 15 stable renal allograft recipients. Their mean time from transplantation was 1227 ± 373 days. Thirteen patients were on prednisolone and five patients were also receiving MMF in addition to their tacrolimus. None had been neutropenic in the previous 3 months. The median absolute lymphocyte count in the month before analysis was 1.1 ± 0.17 × 10^9 /L. Eleven of the 15 stable transplants were CMV IgG positive.

We first established baseline cytokine levels in 10 healthy volunteers which were as follows: IFN-γ = 30 500 ± 12 500 copies/10^5 HPRT; IL-10 = 27 500 ± 7800 copies/10^5 HPRT and TNF-α = 780 000 ± 245 000 copies/10^5 HPRT. We then established cytokine levels in the 15 stable transplants (Figure 1). As expected, IFN-γ was much lower in stable transplants than in healthy controls ($p < 0.001$). IL-10 was much higher in stable transplants than in healthy controls ($p < 0.001$). TNF-α was not significantly different between stable transplants and healthy controls.

We then compared stable renal allograft recipients with renal allograft recipients who had developed an invasive fungal disease. Surprisingly, patients with invasive fungal diseases were unable to mount an incremental IFN-γ response (Figure 1A). There was no relationship between IFN-γ levels and lymphocyte counts or tacrolimus trough levels at the time of sampling. IL-10 was reduced fivefold in patients with invasive fungal diseases as compared to stable renal allograft recipients (Figure 1B). There was no effect of time from transplantation or tacrolimus level on IL-10 levels. TNF-α was increased 10-fold in patients with invasive fungal diseases as compared to stable renal allograft recipients (Figure 1C).

Discussion

We report preliminary results of the use of a rapid and reproducible bioassay for the accurate measurement of three important cytokines in the peripheral blood of renal allograft recipients. Surprisingly, renal allograft recipients (whose IFN-γ is usually low) were unable to mount an IFN-γ response when they developed an invasive fungal disease. As IFN-γ has been clearly shown to be a major cytokine for protective immunity against fungi (10), our preliminary observations suggest that this bioassay could be used prospectively to both define and support the use of adjunctive human interferon-γ therapy in invasive fungal diseases (5). The additional observation of reduced IL-10 and increased TNF-α requires further study in future studies.

The failure to mount an adequate endogenous IFN-γ response could be the key functional host immune defect that predisposes renal allograft recipients to invasive fungal diseases. This appears to be independent of the

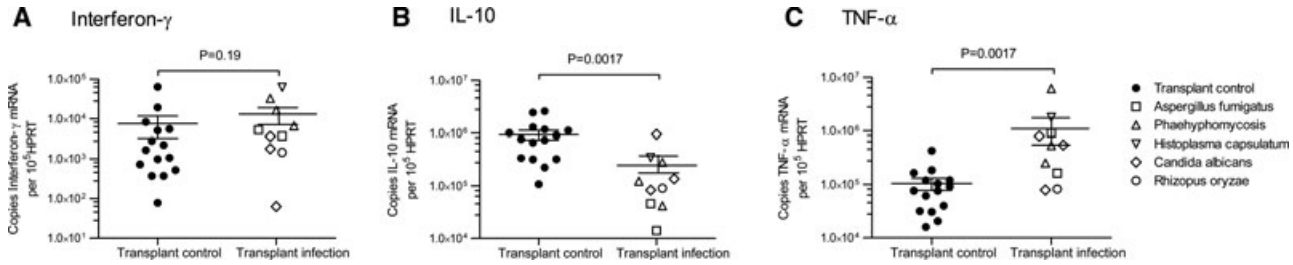

Figure 1: Cytokine profiling in renal allograft recipients with invasive fungal diseases. Peripheral blood mononuclear cell cytokine mRNA expression is shown for: (A) IFN-γ, (B) IL-10, (C) TNF-α in stable renal allograft recipients (n = 15) compared to renal allograft recipients with invasive fungal diseases (n = 10). Data shown as mean ± SEM. K- thousand; M- million. Note that p values determined using a two-tailed Mann–Whitney test.

fungal infection as both stable renal allograft recipients and those with invasive fungal diseases have low levels of IFN-γ expression. Our ongoing studies in a new mouse model of invasive aspergillosis with tacrolimus-induced immunosuppression leads us to speculate that the transplant immunosuppressive drugs could be responsible for this IFN-γ anergy (paper submitted). Calcineurin inhibitors are known to be inhibitors of T cell-dependent IFN-γ production (11). However, the clinical observation that only a minority of stable renal allograft recipients develop these serious fungal diseases suggests that additional, and as yet undefined, factors also play a part.

Our patient based observations also raise the intriguing possibility that the reduction in IL-10 in invasive fungal diseases was the consequence of sustained calcineurin mediated inhibition of innate immune responses to fungal pathogens. IL-10 expression in response to activation of the fungal pattern recognition receptor Dectin-1 is under the control of the calcineurin pathway (12,13). Furthermore, the reduction in IL-10 expression and the increase in TNF-α expression seen in renal allograft recipients with invasive fungal diseases is consistent with TLR-dependent macrophage activation (14). The secretion of IL-10 by alternatively activated macrophages has also been shown to be an important early host defense mechanism against invasive fungal diseases (13). There is also an association between transplant tolerance and high circulating levels of IL-10, albeit with high levels of IFN-γ in the early post-transplantation period (15,16). IL-10 polymorphisms are a major determinant of IL-10 expression in man: (a) they influence diseases from graft-versus-host-disease to systemic lupus erythematosus (17); (b) lead to an association between IL-10 promoter polymorphisms and susceptibility to aspergillosis in stem cell transplantation (18); and (c) promoter polymorphisms impair IL-10 production in chronic pulmonary aspergillosis (19). It is therefore possible that our observations reflect IL-10 polymorphism driven differences in IL-10 expression, and that this increases susceptibility to invasive fungal diseases in renal allograft recipients. Further sequencing based studies will be required to determine this.

Although there is recent interest in defining the *Aspergillus*-specific T cell response in patients at risk of invasive aspergillosis, our intention was to develop a simple, rapid, robust and quantitative assay for peripheral blood mononuclear cell cytokine expression that might be clinically useful (20). Importantly, the approach described does not require pathogen-specific reagents. Our preliminary findings suggest that renal allograft recipients with invasive fungal diseases could have an impaired Th-1 immune response, and that this can be corrected by a short course of adjunctive human interferon-γ therapy.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation.*

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