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IMMUNE PROFILING: MOLECULAR MONITORING IN RENAL TRANSPLANTATION

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1. ABSTRACT

Molecular techniques have become a mainstay for most biomedical research. In particular, sensitive methods for gene transcript detection and advanced flow cytometry have been crucial in fostering our understanding of the basic mechanisms promoting allosensitization and adaptive immune regulation. These technologies have been validated in vitro, and in pre-clinical settings, and as such their clinical application is now clearly appropriate. It is becoming increasingly clear that these robust techniques hold much promise to better elucidate human transplant biology, and more importantly, guide clinical decision making with mechanistically-based information. This article will discuss our laboratory's use of several novel technologies, including gene polymorphism analysis, real-time polymerase chain reaction transcript quantification, and multi-color flow cytometry in clinical human renal transplantation. Specific technical methodology will be presented outlining keys for effective clinical application. Clinical correlations will be presented as examples of how these techniques may have clinical relevance. Suggestions for the adaptation of these methods for therapeutic intervention will be given. We propose that clinical transplantation should proceed in close step with modern molecular diagnostics.

2. INTRODUCTION

Over the past 20 years advances have been made in essentially every aspect of clinical transplantation from organ preservation to immunosuppression and infectious disease prophylaxis. Accordingly, outcomes for renal transplantation have steadily improved (1). However, these substantial gains have not prevented graft loss so much as they have delayed it, and shifted the problems from acute to

chronic (2). Renal allograft survival steadily declines post transplantation, and both chronic dysfunction and immunosuppressive related morbidity remain significant clinical challenges.

Though chronic problems predominate in transplant management, it remains unclear to what extent they are the result of processes that are mechanistically distinct from acute rejection. Acute cellular rejection (ACR) occurs within the first three months in roughly 20% of renal transplant recipients and can be categorized clinically, based on response to therapy, or histologically, based on the severity of the changes seen on biopsy. The factors that most prominently influence ACR are inadequate immunosuppression and donor-recipient disparity at the human leukocyte antigen (HLA) loci (3). After one year, although the leading cause of graft failure is chronic rejection, the problems of inadequate immunosuppression and ACR remain critical determinants of outcome (2,4,5).

Rejection of a renal graft is initiated by signals within two microenvironments: the allograft itself, which is well defined and clinically accessible, and the draining lymph nodes, which are no doubt important, but poorly defined in humans and essentially not accessible. Between the two, the peripheral blood has been thought to represent the lines of communication, being more of a highway than a battlefield. Nevertheless, diagnostic analysis remains dependent on accessibility, leaving the blood and graft more relevant than the nodes, regardless of their actual mechanistic authority. In this review, we will highlight several emerging methods for molecular gene expression profiling and cellular phenotyping. By allowing for critical analysis of the blood and graft microenvironment, these assays are providing early mechanistic data aiding in the correlation of basic and clinical scientific concepts. With more sensitive analytical methods, it is becoming increasingly clear that efforts to prevent chronic rejection and avoid inappropriate immunosuppression must include an effective means to proximally diagnose ACR, including sub-clinical forms of injury. The role of concomitant viral infection is also being recognized as increasing germane, and these methods may also assist in more precisely identifying the origin and the "intent" of immune cell infiltrates.

Considerable progress has been made in developing molecular-based diagnostics on genetic inheritance immune cell polymorphisms, gene transcriptional profiling and polychromatic flow cytometry (PFC). These techniques have been incorporated into the evaluation of clinical renal transplant trials at the National Institutes of Health (NIH). Practical aspects of their use will also be discussed.

3. CYTOKINE POLYMORPHISMS

3.1. Potential Applications of Polymorphism Analysis

Most cytokines have been demonstrated to be transcriptionally controlled. As such, the inheritance of genetically determined polymorphisms has been implicated in the development of both acute and chronic renal allograft rejection (6-9) and peripheral tolerance (10). Cytokines influence the local activation of cells and play a critical role in the regulation of immune responses. While functional affects have been attributed to cytokine gene polymorphisms (11-13), their role in allograft rejection remains controversial. Based upon the inheritance of specific polymorphisms, each individual represents a mosaic of high, intermediate or low cytokine responses. If cytokine gene polymorphisms influence immunity then they must be independently regulated and result in variations in protein production. The level of production of many of these cytokines may be important in accelerating or slowing the rejection process.

Indeed, some studies have shown an association between the development of ACR and the inheritance, in recipients, of polymorphic alleles for IL-10 and TNF-alpha (11,14). Others have failed to find such a relationship (15-17). Thus, the practical importance of polymorphisms remains debatable. Nevertheless, by imparting subtle changes on cytokine production, allelic variations within cytokine genes might act on the microenvironment of responding T cells and antigen presenting cells (APCs), and thereby regulate allograft survival. Therefore, a more clear definition of cytokine genotypes may indicate how recipients will respond to their transplants and guide both optimal immunotherapy, organ selection, or enable selection of patient subgroups for individualized clinical trials.

Application of this technology remains speculative. However, patients that are high producers of particular genes could be viewed as requiring higher dosed immunosuppressants, while low producers might be seen as benefiting from drug withdrawal. An illustrative example of such benefit would be those individuals that code for high expression of TGF-beta. It has been well established that Cyclosporin A (CsA) causes elevated levels of TGF-beta and may promote early fibrosis in renal allografts ultimately aiding chronic allograft nephropathy (CAN) (18). High producer patients would be selected for those trials without calcineurin inhibitors or given additional anti-fibrotic agents posttransplant. Regardless, the task at hand is to determine the translational effect of the growing number of transcriptional polymorphisms and begin clinical correlations of their potential effects.

3.1.1. Recipient and Donor Polymorphisms

We have analyzed both recipient and donor cytokine gene polymorphisms in six critical immunoregulatory cytokine genes following renal allograft transplantation (Table 1). In each case we can associate gene sequence with cytokine phenotype, so that individuals homozygous for a so-called high producer allele are in fact the highest producers of that cytokine. Likewise homozygotes for the low producer allele are the lowest cytokine producers. Heterozygous genotypes may have variable effects based on the dominance of a particular allele, or simply result in intermediate cytokine producers. All of these cytokines are independently regulated so that each person is a mosaic of high and low cytokine-producing genes.

Table 1. Cytokine Allelic Polymorphisms and Protein Phenotype

Cytokine	Function	Polymorphism	Allele	Phenotype[a]
IL-2	Activating	-330	T	Low
			G	High
IL-6	Activating	-174	G	High
			C	Low
IL-10	Suppression	-1082	G	High
			A	Low
TNF-alpha	Activating	-308	G	Low
			A	High
TGF-beta	Suppression	Codon 10	T (leu)	High
			C (pro)	Low
	Pro-fibrogenic	Codon 25	G (arg)	High
			C (pro)	Low
IFN-gamma	Activating	Intron 1	A	High
			T	Low

[a] Cytokine Production

In our studies, we have compared patient and donor allelic genotypes and established phenotypes to allograft status as defined by Banff criteria in serial protocol biopsies (19). In contrast to many earlier studies, we found no strong associations in the inheritance of cytokine polymorphisms in recipients and renal allograft outcome. When comparisons were made between those patients that had stable allografts and those that showed evidence of subclinical rejection (Banff criteria for ACR with <10% rise in baseline serum creatinine), a slight association was established with high IL-10 production (Table 2). In addition, high production alleles for either IL-10 or IFN-gamma were prevalent in recipients with Banff criteria for chronic changes compared to a control population. Evidence from animal transplant models has suggested a role for IL-10 in promoting graft survival although our studies, and others, have support a role of IL-10 in rejection (9,12). It was also interesting that in the seven rejections that were resistant to initial steroid therapy alone, each of these individuals were of the high IFN-gamma producer phenotype.

In most prior studies, the affects of cytokine polymorphisms have focused on recipient profiles. However, early rejection episodes can be affected by donor related factors, in particular the age and ethnicity of the donor (20-22). Organ procurement itself is a traumatic process potentially altering cytokine production through the effects of brain death and allograft ischemia (23). Given the ever concerning shortage of suitable donor organs, the acceptance of kidneys from marginal donors is rising warranting an assessment of donor specific variables (24,25).

We have characterized the role of donor-specific cytokine polymorphisms from healthy renal allograft donors with their recipient allograft status (Table 2). No relationship was evident between donor polymorphisms within IL-2, IL-6, TNF-alpha or TGF-beta and clinical allograft status (data not shown). However, when compared to controls, high donor-based IFN-gamma production was more common in recipients with either subclinical (p=0.016) or chronic changes (p=0.007). We also noted an impact of high donor IFN-gamma production on recipients presenting with subclinical rejection (p=0.007), ACR (p=0.042) or chronic changes (p=0.004) when compared to those donors with stable recipients and normal allograft histology. In addition, donor-based high producers of IL-10 phenotypes were markedly associated with subsequent recipient subclinical rejection when compared to donors resulting in normal allograft histology (p=0.019) (Table 2).

These results suggest that while triple immunosuppressive therapies mute the influence of IFN-gamma within the recipient (26), donor-derived production may play an essential role in inflammatory processes initiating rejection. Activated APCs within the graft most likely produce donor-derived IFN-gamma. Differential expression of IFN-gamma in the draining lymph nodes, resulting from passenger APCs, could significantly influence subsequent alloimmune activation, perhaps through the up-regulation of HLA-DR. IFN-gamma may also inhibit apoptosis of alloreactive T cells through the induction of nitric oxide (27). As such, those recipients that receive renal grafts from high IFN-gamma producing individuals may be at a greater risk for rejection.

3.1.2. Protein Production

We have also examined the allelic variation in cytokine polymorphisms with their ability to produce cytokines in cultured leukocytes. For the cytokines described, there are considerable differences in the amount of cytokines produced when peripheral blood leukocytes are stimulated *in vitro*. Cytokine production was measured by enzyme-linked immunosorbent assay (ELISA) following stimulation of purified PBL using anti-CD3 and anti-CD28 (anti-CD3/CD28) labeled microbeads for 72 hours in healthy volunteers. We found cytokines secreted at levels 4-10 fold greater than samples stimulated with the mitogen Concavalin A.

Table 2. Distribution of Cytokine Polymorphisms Among Allograft Outcomes

A. Recipient Polymorphisms

Gene	Phenotype[a]	Controls (%)	Stable Allograft (%)	Subclinical Rejection (%)	Acute Rejection (%)	Chronic Rejection (%)	Steroid Resistant Rej (%)
IL-10	High	82 (58.6)	9 (45)	10 (83.3)[c]	13 (61.9)	17 (60.7)[b]	5 (71.4)
	Low	58 (41.4)	11 (55)	2 (16.7)	8 (38.1)	11 (39.3)	2 (28.6)
IFN-gamma	High	89 (63.6)	14 (66.7)	7 (58.3)	17 (81)	19 (67.9)[b]	7 (100)[b]
	Low	51 (36.4)	7 (33.3)	5 (41.7)	4 (19)	9 (32.1)	0 (0)

B. Donor Polymorphisms

Gene	Phenotype[a]	Controls (%)	Stable Allograft (%)	Subclinical Rejection (%)	Acute Rejection (%)	Chronic Rejection (%)	Steroid Resistant Rej (%)
IL-10	High	82 (58.6)	8 (50)	10 (83.3)[c]	12 (80)	14 (77.8)	5 (71.4)
	Low	58 (41.4)	8 (50)	2 (16.7)	3 (20)	4 (22.2)	2 (28.6)
IFN-gamma	High	89 (63.6)	9 (56.3)	10 (100)[b, c]	13 (86.6)[c]	17 (94.5)[b, c]	4 (100)
	Low	51 (36.4)	7 (43.7)	0 (0)	2 (13.4)	1 (5.5)	0 (0)

[a]Cytokine Production; [b]Fisher's exact test, $p<0.05$, compared to Controls, [c]Fisher's exact test, $p<0.05$, compared to Stable Allograft

Table 3. Correlation of Polymorphic Phenotype with Cytokine Production in Control and Posttransplant Patients

Cytokine	Genotype	Phenotype[a]	N=	Controls pg/ml[b]	p Value[c]	N=	Recipients pg/ml[b]	p Value[c]
TNF-alpha	A/A or G/A or G/G	High	8	3774 +/- 666	0.586	4	5972 +/- 1460	0.367
		Low	24	4099 +/- 457		13	4653 +/- 1089	
IFN-gamma	T/T or T/A	High	24	38740 +/- 3514	0.386	12	23635 +/- 6999	0.838
	A/A	Low	6	31461 +/- 6675		4	34079 +/- 9704	

[a]Cytokine Production; [b]Protein production as measured by ELISA in 72 hr anti-CD3/CD28 stimulated PBL supernatants, [c]Mann-Whitney U test used to compared high vs. low cytokine production

Culturing T cells with anti-CD3/CD28 results in optimal and sustained activation and the stabilization of both Th-1 and Th-2-type cytokine transcripts (28-30). While cytokine proteins are generally short lived, our system permits a more accurate analysis of the effects of polymorphisms in various cytokine genes by allowing the greatest accumulation of cytokines in culture. Additionally, stimulation of PBL with anti-CD3/CD28 results in comprehensive polyclonal activation (31) and is more robust than the variable activation seen following stimulation with anti-CD3 alone (32), PMA/Ionomycin (33) or mitogens (34,35). One question raised from our results is whether cytokine protein levels are independently regulated following stimulation of PBL.

We found statistically significant differences between protein production and allelic variants of the IL-2, IL-6, IL-10 and IFN-gamma. However, we noted no correlation between TNF-alpha and TGF-beta polymorphic variants and accumulation of their proteins in supernatants from stimulated cells. Protein levels for TNF-alpha and IFN-gamma from control and transplant recipient assays are presented in Table 3. This result for TNF-alpha is perplexing in light of reports that have strongly associated inheritance of the polymorphic "A" allele with renal allograft rejection (7,10,14) as well as a wide range of conditions including leishmaniasis (36), asthma (37), and infections after renal transplantation (37,38). TNF-alpha production is tightly regulated both at the transcriptional and posttranscriptional levels (30). Recent work by Kroeger *et al* has concluded that the TNF-alpha gene utilizes different sets of transcriptional elements depending on the activation stimuli and the cell type induced (40,41). CD28 ligation regulates transcriptional and post-transcriptional induction of TNF-alpha. Therefore, it is possible that stimulation through CD28 could circumvent influences mediated by the polymorphism on TNF-alpha protein production.

3.1.3. Immunosuppression

Disparate results between previous polymorphism work and our studies could be related to the level of immunosuppression given to the study population. In instances where significant associations between cytokine polymorphisms and outcome were found, patients were often treated with relatively low dose immunosuppression including CsA monotherapy (14) or azathioprine and steroids alone (42). Fewer correlations

were apparent in later studies when patients received greater levels of immunosuppression (43), suggesting that potent immunosuppression could mollify the contribution of high responder genotypes. Asderakis et al, determined that an association between high IFN-gamma production and acute renal graft rejection was highly significant in patients that received CsA monotherapy, but not significant in those patients on triple-drug immunotherapy (26). Clearly immunosuppressive therapies can alter cytokine production. For example, calcineurin inhibitors such as CsA and tacrolimus have been shown to stimulate TGF-beta production, potentially encouraging fibrosis and immunosuppression (18,44). Recipients in the studies presented here were treated using triple immunosuppression including a calcineurin inhibitor (CsA or tacrolimus), mycophenolate mofetil, and prednisone. We have limited data from a subset of patients that demonstrates that triple immunosuppression can mask the influence of cytokine polymorphisms (Table 3). As described above, analysis of cultured lymphocytes demonstrated and confirmed correlations between genotype and protein production for various cytokine polymorphisms. However, as noted in Table 3, IFN-gamma showed dramatically different results in immunosuppressed allograft recipients.

With the use of more aggressive T cell depletional protocols and donor-specific transfusions/ chimerism approaches, a potentially increased influence of donor cell cytokine production could be envisioned. The relationships between cytokine polymorphisms and protein production phenotype may need to be revisited. In the future, extensive work will have to be conducted to elucidate the influence of immunosuppression on in vivo cytokine production. It is likely, however, that a generalized muting of immune cell responsiveness by immunosuppression will minimize the influence of cytokine gene polymorphisms. In trials focused on immunosuppressive minimization, the influence of these factors will likely gain increasing prominence.

3.2. Ethnicity as a Factor Determining Cytokine Gene Polymorphism

Several studies have demonstrated significant health outcome disparities based on patient ethnicity in cancer, and cardiovascular and kidney diseases. Indeed, there is a documented deficit in long-term allograft survival for African-Americans as compared to Caucasian, Hispanic, and Asian populations (45). Prior to the widespread use of CsA, 1 and 3 years post transplant allograft survival was 6% to 10% lower in African-Americans than Caucasians (46-47), and in the modern era, the difference continues to be evident (45). Poor graft survival among African-Americans has been attributed in part to socioeconomic factors and to inferior HLA matching. African American donor kidneys also have been associated with a worse graft survival (21). Studies examining potential effects of polymorphisms on pharmacokinetics and immune responsiveness have also been performed showing that peripheral blood cells from healthy adult African-Americans express significantly more B7 costimulatory molecules (CD80, CD86) than Caucasians and mount more vigorous immune responses to mitogens and antigens *in vitro* (48-50). Factors such as these could represent additional key factors for racial variation in allograft loss (51-53).

We have demonstrated striking differences in the distribution of cytokine polymorphisms among ethnic populations (53,54). Blacks, Hispanics and Asians have marked differences in their inheritance of IL-6 alleles and IL-10 genotypes that result in high expression when compared with Whites. High IL-6 producing individuals have previously been shown to be at heightened risk for ACR (55). Within our renal clinical trial cohort, African-American patients with hypertension, polycystic kidney disease and focal segmental glomerulosclerosis were all genotyped as high producers of IL-6, suggesting a potential association between high IL-6 production and some forms of end-stage renal disease. We suspect that high IL-6 production, in concert with additional pro-inflammatory cytokines, may result in an increase risk for allograft rejection in the African-American population, or at least increase the impact of alterations in immunosuppressive non-compliance. Conversely, Asians exhibit IFN-gamma genotypes that result in low expression as compared to Whites. This may result in diminished cytotoxic effects thereby lessening the chance of acute graft rejection.

While the potential synergistic effects of cytokine gene polymorphisms on disease pathogenesis has yet to be fully established, ethnicity clearly is associated with dramatic differences in cytokine polymorphism distributions. The disparity between graft survival rates among African-American and other populations suggests that the cytokine polymorphisms described by many laboratories, including our own, may play an incremental role in ethnic-based survival rates and subsequent differences in immune reactivity.

3.3. Future Strategies

Multiple studies have indicated that alloresponsiveness after transplantation is influenced by genetic changes within certain cytokine genes. However, the degree to which cytokine gene polymorphisms influence clinical outcome remains undefined. Confounding variables between studies contribute to the uncertainty with the level and type of immunosuppression likely serving as a major variable in determining the significance of any genotype. The work described above has provided evidence within an immunosuppressed patient population that genetic disposition towards high production of inflammatory cytokines can be identified. It remains to be seen if these differences are predictive of those recipients at greater risk of rejection. This observation suggests that a population of recipients more prone to ACR may be predicted through study of cytokine polymorphisms. While a wide range of factors contributes to allograft survivability, routine screening of cytokine gene polymorphisms may have important clinical relevance and therefore should be considered in the design of both pre- and post-treatment regimens.

4. GENE EXPRESSION DURING ALLOGRAFT DYSFUNCTION

4.1. Biopsy Tissue as a Primary Source of Transplant Relevant Material

There are no clearly identified surrogates for chronic rejection in renal transplantation. However, it has been established that seemingly stable allografts have histological and transcriptional evidence consistent with ongoing detrimental inflammation (56,57). Although the significance of these so called "subclinical" rejections remains to be definitively proven, there is little doubt that normal allografts are not necessarily normal kidneys, and given the choice of having inflammatory infiltrates and tubulitis or not, most would opt not to be so encumbered. Sequential monitoring of allografts with surveillance or protocol biopsies may allow for better immunosuppression and earlier rejection diagnosis following renal transplantation. In addition, the allograft remains the single most relevant accessible site in a clinical rejection episode. We have therefore, used the biopsy as a mainstay of our clinical investigation.

Certainly, non-invasive strategies to monitor activated lymphocyte infiltration and gene transcripts in patient peripheral blood and urine have been aided by our knowledge of potential surrogate markers of ACR (58-60). These studies have mainly focused on cytotoxic T effector transcripts such as perforin, granzyme B and FasL (61-63). However, many of these observations are obtained from biopsies or peripheral analyses that have been conducted after clinical diagnosis of ACR. Therefore, it is possible that in many of these trials, allograft damage may have already occurred such that alteration of immunotherapy has not lead to a reduction in chronic rejection.

Early evaluations of renal allograft biopsies relied on the phenotypic and functional characterization of T cells propagated from biopsy tissue (57,64). These studies clearly showed a relationship between the activation state of the cells, their growth capacity, the cytokines produced, and clinical ACR. However, these techniques discounted the importance of other cell types. Our subsequent studies have highlighted the importance of monocytes and the intensity of T cell costimulation-related targets in early infiltrating cell types, and underscore monocytic activation as a potential instigator of alloimmunity. In addition, propagation studies rarely allowed for the evaluation of T cells with potential regulatory function. Controversy exists regarding whether interstitial infiltrates alone are indicative of rejection or may in fact be beneficial, with infiltrates actually representing regulatory or suppressor cell populations (65). However, infiltrates associated with histological damage, such as tubulitis or vasculitis, are unlikely to fall into this category.

Nevertheless, *in situ* analysis of infiltrates seems more likely to yield comprehensive data. In order to better define the transcriptional events following allograft transplantation in humans, and gain, in a comprehensive manner, insight into those factors that may initiate and propagate intragraft alloimmune responses, we have examined infiltrating cells and gene transcripts derived from human renal allograft protocol biopsies. Rush and associates, and several other laboratories, have established the use of protocol biopsies to detect and follow the course of early allograft rejection (56,66-68). The aim of our ongoing studies is to identify key mediators and determinants involved in the molecular pathogenesis of renal allograft dysfunction. Ideally, preemptive treatment of an expanding alloimmune response before clinical recognition of organ pathology (i.e. elevation of serum creatinine), if it could be detected, would be desirable. Such early intervention may be more effective than therapy initiated after organ damage has already occurred and could theoretically limit morbidity associated with the treatment itself.

4.2. Real-Time Quantitative PCR

Simultaneous screening and quantification of a large number of gene transcripts has typically been performed utilizing chip array technology or mRNA differential display. Although these techniques are exemplary in their ability to screen a large number of genes in a given disease state, their quantitative reproducibility certainly has been questioned. More importantly, the use of large sample amounts and their often laborious and expensive nature have limited their use as a robust clinical monitoring tool. We have therefore employed real-time quantitative PCR (RT-PCR) as our method of choice for biopsy transcript analysis. This technique allows for a rapid and precise relative quantification of gene transcripts (69,70). RT-PCR has been shown to exceed the sensitivity of northern blots and RNase protection assays and allows for quantitative study of multiple transcripts with reproducibility equal to or exceeding competitive template PCR (71,72). However, as with many novel methodologies, sample quality control and assurance is vital if we are to incorporate these techniques as standard clinical tools for monitoring allograft dysfunction and guiding individualized immunotherapeutic interventions.

Comprehensive RT-PCR gene expression studies must incorporate aspects of gene mining, profiling and quantitation. In this regard, allograft dysfunction or rejection is a conglomerate of multiple inflammatory, cytotoxic and fibrotic pathways that make isolation of a single surrogate diagnostic marker unlikely. The following sections detail our laboratory's use of both pooled populations and individual biopsy samples to monitor and describe logistical experimental design and data analysis for RT-PCR immune profiling of renal allograft dysfunction.

4.2.1. Primer/Probe Design and Reaction Conditions

The design of primers for use in RT-PCR is clearly of fundamental importance, and several automated design programs have been developed. As one changes their methodology, it is important that primer design methods remain current and relevant to the techniques used. Our gene target primers and probes have been obtained from Applied Biosystems as pre-developed assay reagents. Primer pairs are designed to produce amplicons smaller than 150 base pairs. Furthermore, all primers are

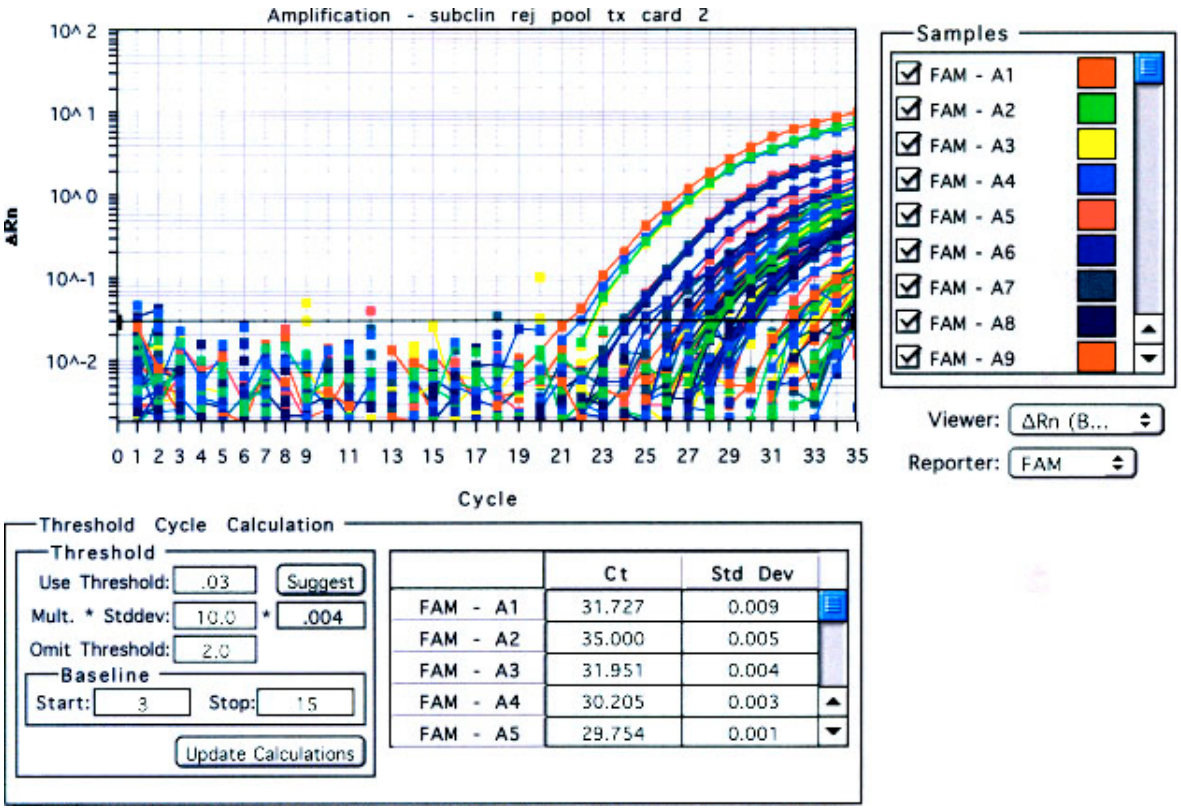


Figure 1. Illustration of sample 96-well Microcard RT-PCR reaction for 24 targets and analyzed by ABI Sequence Detection Software 1.7.1. The amplification plot is the plot of fluorescence signal vs. PCR cycle number. The baseline is defined as those PCR cycles in which signal detection is accumulating, but beneath the level of detection. The threshold is typically defined by the user at 10 standard deviations above baseline detection and crosses the PCR plots during optimal exponential amplification and highest efficiency. The Ct value is that PCR cycle at the intersection of the PCR plot for a specific target gene and the threshold line. The Threshold line must be keep at a constant level for quantitative comparison between samples.

designed over intron-exon junctions to further reduce the risk of genomic DNA amplification as discussed above. Primers and probes are loaded into 96-well Microcards for use in quantitative RT-PCR assays. Each card contains forward and reverse primers and 6-carboxyfluorescein (6-FAM)-labeled probes for 4 replicates of nearly 80 targets chosen based on their potential relevance to the study of allograft biology. In addition, forward and reverse primers for 18s ribosomal RNA; VIC dye (a fluorescein derivative)-labeled probe for 18s (internal control); and 2X TaqMan Universal PCR Master Mix (Applied Biosystems), are combined with the cDNA template and loaded into 96-well Microcards. All probes are also labeled on the 3' end with a minor groove binding protein (MGB). This protein allows for more efficient binding of Taqman probes and increases probe melting temperature and ultimately PCR efficiency. We have found these dual-labeled probes to be superior to other commercially available probes without MGB protein for enhanced sensitivity and detection of low transcript gene amplification.

Reaction mixtures are subjected to the following amplification scheme: one cycle at 50°C for 2 minutes and one cycle at 99°C for 10 minutes, followed by 35 cycles at 99°C for 15 seconds and 60°C for 1 minute. RT-PCR data are analyzed using Sequence Detection System version 1.7.1 software included with the ABI PRISM 7700 Sequence Detector (Applied Biosystems) or downloaded into an excel database for handling and analysis. Accumulation of the PCR products is detected by directly monitoring the increase in fluorescence of the reporter dye. Data points collected in this manner are analyzed at the end of thermal cycling. The mean of the background fluorescence emission for all the tested wells measured between cycles 3 and 15 is recorded and used to set the baseline. A threshold for the amplification of each gene of interest is then set by drawing a line that intersects the exponential phase of the logarithmic amplification curves for all samples being analyzed for expression of target gene. The cycle number at which the threshold line intersects the linear curve for each sample is used to determine the threshold cycle (Ct) value (Figure 1). Ct values decrease linearly with increasing input target quantity. Final quantification is derived using the comparative threshold method as described below and was reported as the n-fold difference of an individual gene expression level from an allograft biopsy relative to the pool of normal kidney biopsies.

4.2.2. The Comparative Ct Method

The fractional cycle number at which the reporter fluorescence generated by the cleavage of the

probe passes a fixed threshold above baseline is defined as the parameter threshold cycle. The detection of multiple target cDNAs in the same well is achieved by labeling probes with separate and distinguishable reporter dyes (FAM & VIC) in multiplex reactions or inclusion of 18s RNA as a separate FAM-labeled target. For quantification, values are expressed relative to a reference (calibrator) sample, and calculated by the comparative Ct method. The sample Ct value for each target amplicon was subtracted from the Ct value of the same gene in the calibrator cDNA (for our studies, 18s RNA) to generate the ΔCt value. The ΔCt for each experimental sample is subtracted from the ΔCt of the calibrator (for our studies, the normal kidney pool). The difference is denoted the ΔΔCt. Since each target and 18s RNA amplicon are designed with comparable PCR efficiency, the fold amount of target is calculated by $2^{-\Delta\Delta Ct}$. Thus, all experimental samples are expressed as n-fold difference relative to the calibrator.

4.3. Biopsy Acquisition and Allograft Status

The precise characterization of biopsy tissue for analysis is important in determining relevant correlations between transcription and disease. The reference point for normal is also critically important. Accordingly, we have used biopsies from normal kidneys acquired from living renal donors during open donor nephrectomy as our standard. As described subsequently (section 4.3.4), using "normal" transplanted kidney as a baseline is not physiologically or immunologically accurate. Furthermore, the use of cDNAs from "uninvolved" poles of kidneys extirpated for malignant disease is equally improper, as we have found the nephrectomy process to induce many genes in response to ischemia.

For our studies, normal renal cortical tissue is obtained prior to renal extirpation or vascular cross clamp and used for quantitative calibrator pool as described below. Post-reperfusion biopsies are obtained 30-60 minutes following renal transplantation and revascularization of live donor or cadaveric renal allografts. All posttransplant allograft biopsies are obtained from recipients treated using either triple immunosuppression, including a calcineurin inhibitor {either CsA (Neoral, Novartis), or tacrolimus (Prograf, Fujisawa)}, mycophenolate mofetil (Cellcept, Roche), and prednisone. Biopsies are taken based on protocol surveillance criteria under real time ultrasound guidance using local anesthesia. All biopsies are obtained from the renal cortex using a 16 gauge needle core biopsy device. This device usually provides ample tissue for histology and RNA extraction from a single pass. Close inspection of the biopsy prior to sectioning is required to ensure that both the histological and transcriptional studies are performed on cortical tissue. All recipients described as having stable allograft function are, at the time of biopsy, free of clinical or subclinical allograft rejection according to the Banff criteria (19). Allograft biopsies defined as subclinical rejection ($\geq$IA rejection (Banff) with a <10% rise in serum creatinine from baseline levels) are obtained from either 1 month or 6 month protocol biopsies according to clinical trial guidelines. Resolved biopsies with stable function and normal histology were taken 3 months after steroid pulse treatment following subclinical rejection diagnosis.

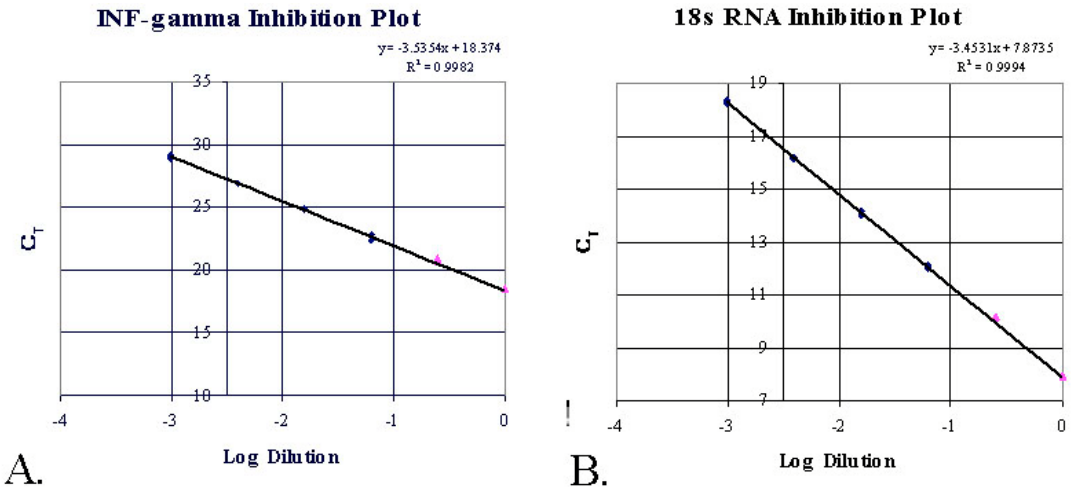
4.3.1. RNA Extraction and Isolation

Biopsy cores are snap frozen in liquid nitrogen at the bedside within 1 minute of procurement. Biopsy samples for RNA extraction are either isolated immediately or kept at –80°C or on dry ice until processing. We have found that significant lengthening of time periods prior to initial liquid nitrogen freezing can cause significant RNA degradation, leading to limited RNA recovery and poor quality RNA for RT-PCR. For total cellular RNA extraction, samples are homogenized in 1 ml TRIzol reagent (Life Technologies, Gaithersburg, MD) and extracted according to manufacturer's instruction. Total RNA concentration is quantified by UV spectrophotometry at 260nm converted to cDNA with random hexamers and AMV reverse transcriptase (Roche) from commercially available kits.

Since we have utilized 18s RNA as a calibrator and normalization gene transcript, oligo dT primers should not be used for cDNA conversion. In addition, all subsequent RT-PCR reactions are performed from a single cDNA conversion sample. Inefficient or incomplete conversion of RNA to cDNA may cause variable amplification and decreased reproducibility of data if separate conversions are utilized for multiple assays. In addition, DNAse is not used during RNA isolation since we have found that residual contamination with DNAse may strongly inhibit both cDNA conversion and the PCR reaction. Since probes are designed over intron-exon junctions, DNAse treatment is typically not necessary. Residual or contaminating genomic DNA amplification has been shown to represent <1% of amplified products compared to highly expressed housekeeping genes or not be amplified due to probe specificity amplicon size, thus having not effect on transcript quantification.

4.3.2. 18s RNA Normalization

With the increased interest in surrogate markers of rejection, the choice of a housekeeping gene for an internal control for various assays becomes an important consideration that could affect the sensitivity and reproducibility of the RT-PCR. In order to perform appropriate gene expression normalization and quantification, we evaluated the variation in RT-PCR amplification (ΔCt values) from several housekeeping genes, namely, 18s RNA, beta-actin, cyclophilin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-glucuronidase (GUS). 18s RNA was shown to be an appropriate candidate housekeeping control for transcript normalization since its expression levels were not found to vary among different tissues, cell types, with or without stimulation, or from experimental treatments. In contrast GAPDH and beta-actin expression varied widely, exhibiting ΔCt fluctuations and nearly 10-fold differences in some activated cell populations. Our studies further complement previous analyses that shown variation in both GAPDH (74) and beta-actin (75) expression under several experimental regimens. In addition, cyclophilin is not a practical calibrator for transplantation-related studies due to its direct role in the calcineurin pathway effected by standard immunosuppressive therapies. GUS was found to be a fairly constant and reliable expression target.



Dilution	Conc	dCt IFN-g*	dCt 18s RNA*	ddCt
neat	100 ng	18.47	7.93	10.54
1:4	25 ng	20.78	10.16	10.62
1:16	6.25 ng	22.59	12.07	10.53
1:64	1.5 ng	24.83	14.08	10.75
1:256	0.4 ng	26.88	16.15	10.73
1:1024	0.1 ng	29.00	18.31	10.70

C. * Mean threshold value for triplicate runs

Figure 2. RNA inhibition plots of Ct value vs. log of sample cDNA (RNA) concentration are displayed for IFN-g (A) and the target calibrator, 18s RNA (B). Triplicate runs of serial dilutions of each cDNA validate samples over 6 orders of magnitude. Comparative Ct calculations are presented in (C). Lack of inhibition and optimal sample integrity without inhibition indicates no change in ΔΔCt with variant starting sample concentrations.

However, the level of GUS transcript expression may compete with several immune targets and therefore, does not allow for multiplex assays and could invalidate quantitative precision. GUS may be used in singleplex assays when all targets are individually FAM-labeled targets. Based on these findings and our predominant use of 96-well multiplex RT-PCR, we have chosen 18s RNA as our internal housekeeping control for all patient analyses.

4.3.3. Patient Sample Validation

For each converted cDNA sample, we generate RNA inhibition plots over 6 orders of magnitude. Serial dilutions of all samples are made (Neat, 1:4, 1:16, 1:64, 1:256, 1:1024) and critical threshold values established for standard PCR assays detailed above for 18s RNA and/or select target genes. The neat concentration is 100ng cDNA. With optimal sample integrity and PCR efficiency, 2-fold differences in sample concentration should equal a change in one Ct. An optimal plot of Ct value against the log of sample concentration should yield a highly efficient PCR amplification that is >90% for all amplicons (Figure 2). Log plots are made using the dilutions between 1:16 and 1:1024, to predict values for higher concentrations according to the equation of the line. Under these conditions ΔCt values should remain constant over a range of sample input concentrations and thus, optimally validates the integrity of each of our biopsy cDNA samples.

For each Microcard assay, we typically load 100ng sample cDNA and retrieve expression data from quadruplicates of 24 genes per reaction. Limiting dilution assays as described above indicate that 100ng is sufficient to reproducibly and reliably give expression levels above baseline detection for low copy number transcripts.

4.3.4. Normal Kidney Biopsy Pool for Calibration

Previous studies in humans have been limited by their reliance on biopsies obtained from patients for clinical cause, and thus are likely not indicative of the earliest post transplant events, nor are they representative of the conditions seen in a clinically stable patient posttransplant. In addition, most studies have been descriptive rather than comparative in nature. That is to say that they have not been compared to the transcription seen in normal kidney. This is, in part, due to the absence of an accepted transcriptional standard for normal kidney. Those transcriptional analyses that have been compared to control cDNA have used as their standard, gene bank cDNAs, typically from post-excision, and hence strongly ischemic, kidney. Others have been compared to stable functioning allografts or to cadaveric donors. However, neither of these are acceptable references for truly "normal" kidney. Therefore, our initial efforts were to develop a strong pool of pre-reperfusion kidney biopsies that were obtained from live donors at the time of transplant. Equal concentrations

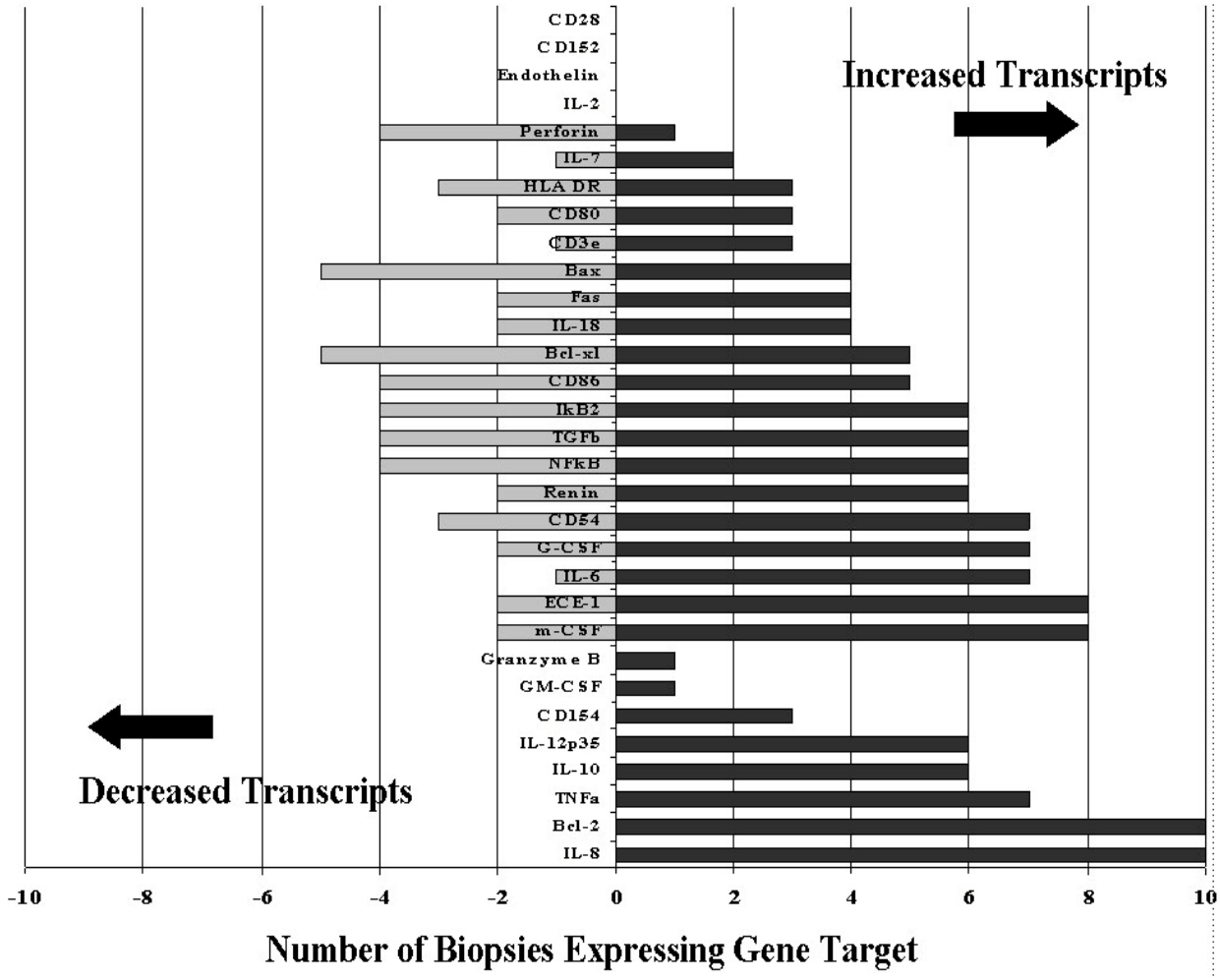


Figure 3. The consistency of changes in RNA prevalence within biopsies from the post-reperfusion biopsies. Bars represent the number of 10 individual post-reperfusion biopsies that express the analyzed gene and have increased (black) or decreased (gray) RNA transcripts compared with the RNA levels from the normal kidney pool. Biopsies with undetectable levels of RNA transcripts for a particular gene do not appear on the figure.

of cDNA obtained from these individual donor biopsies were pooled to create a reference calibrator for all subsequent allograft biopsies.

4.4. Posttransplant RT-PCR Expression Analysis

We have applied the RT-PCR methods outlined above to analyze transcriptional profiles characterizing multiple post-transplant conditions. It is important to point out that analyses such as these requires strict attention to establishing proper diagnoses. As such, all diagnoses used for these pilot characterizations have been reached by thorough retrospective analysis taking into account histopathological analysis, response to therapy and clinical outcome. In addition, cases for analysis were chosen based on unequivocal single diagnoses. That is to say that to establish a characteristic phenotype of a particular condition, one must insure that the condition is present in its isolated form, i.e. only acute rejection, not acute rejection superimposed on calcineurin toxicity and chronic changes.

4.4.1. Ischemia-Reperfusion Injury

Ischemic injury to the donor organ during procurement and subsequent transplantation is a common cause of impaired post-transplant kidney function. It is well appreciated that injury resulting from the initial allograft reperfusion augments the immunogenicity of the graft leading to increased rates of both acute and chronic rejection (75). Reperfusion-induced infiltration of APCs into a graft's pro-inflammatory milieu is one method by which this is suspected to occur. We compared post-reperfusion biopsies, obtained 30-60 minutes following renal transplantation (n=10) to a normal kidney pool calibrator sample. Screening of gene targets compared to a pooled calibrator sample allows for an initial determination of those gene involved in the process of interest. The type of analysis shown in Figure 3 highlights the presence or absence of a particular target gene and whether the sample expressed contains a greater or lesser number of RNA transcripts than that observed in normal kidney. This method of analysis is the simplest iteration of RT-PCR

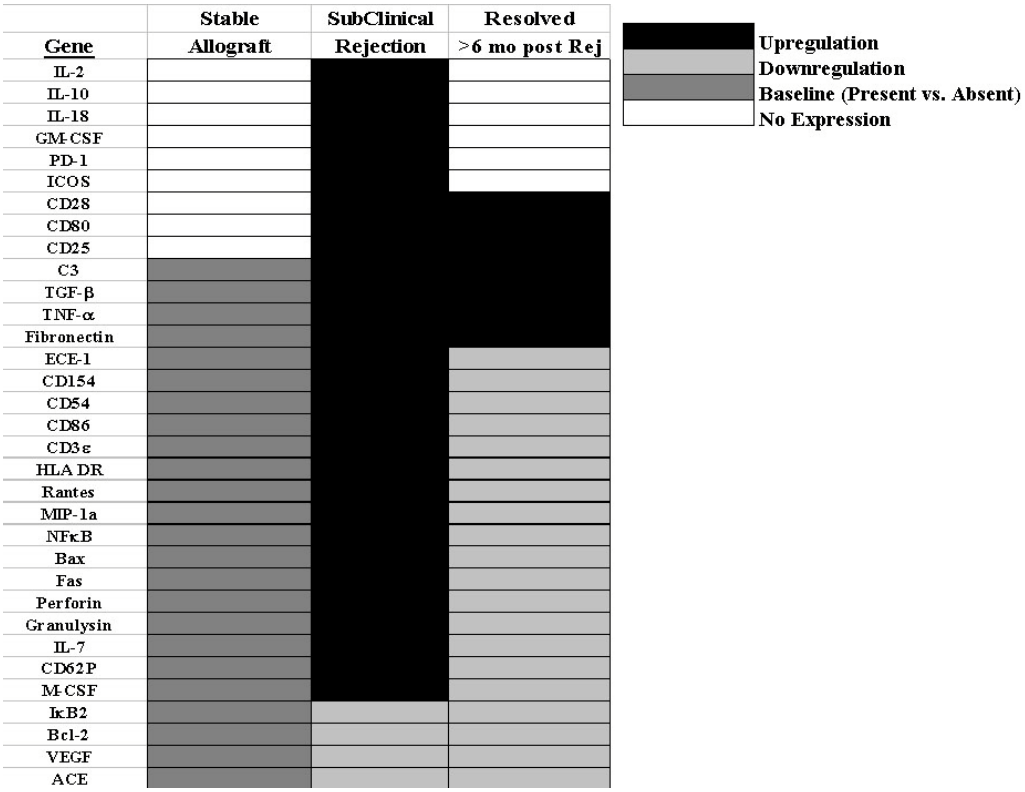


Figure 4. Relative gene expression of human renal allograft biopsy pools. Level of RNA transcript prevalence in pooled samples from subclinical rejection biopsies (n=10) and resolved (post-treatment for subclinical rejection; n=5) compared to stable allograft biopsies (n=10). All fold-expression were determined compared to the normal kidney pool (n=12). Black bars represent increased RNA transcripts, light gray decreased transcripts, as compared to a stable allograft baseline (dark gray). Empty cell indicate undetectable levels or no expression.

data, and although it does not give robust quantitative data, it is useful for initial analysis of large numbers of transcripts in a screening fashion. It is also a simple method for demonstrating changing transcriptional motifs.

Immediate post-reperfusion biopsies are histologically characterized by mild to moderate degrees of proximal tubular necrosis and vacuolation as well as interstitial edema. This injury was associated with a mild infiltration of monocyte/macrophage lineage cells into the interstitium. All 10 post-reperfusion biopsies demonstrated increased relative expression of IL-8 and Bcl-2. Eight of ten biopsies had elevated levels of m-CSF and ECE-1. Seven of ten had enhanced presence of G-CSF, IL-6, CD54 and TNF-alpha. Thus, transcription of genes related to adhesion, monocyte recruitment, and activation appear to be upregulated in post-reperfusion biopsies. However, we found a paucity of T cell-associated RNA species such as CD3, IFN-gamma, perforin, IL-2 or costimulatory molecules CD80, CD86 or CD154 (76). These data are consistent with a prevailing view that reperfusion injury initiates APC infiltration and activation prior to T cell infiltration.

4.4.2. Subclincial Rejection

The single most important risk factor for development of chronic rejection is prior ACR, and reduction of ACR episodes is associated with a decreased incidence of chronic rejection. It has now been recognized that histological ACR can be found in normally functioning kidneys, and it is a growing concern that these subclinical rejections are a subtle and insidious cause of late graft fibrosis (56,66,77-80). We have taken the stance that these mild rejections are representative of the earliest phases of acute rejection and are thus excellent opportunities to examine ACR in its most primordial form. The sensitivity of RT-PCR is well suited to analyze low-grade lesions such as these.

Clinical trials at the NIH are generally designed to obtain protocol biopsies giving us the opportunity to discover and analyzed subclinical rejection. As described previously, we generated homogenous cDNA pools from biopsies obtained from patients greater than 1 month post transplantation with stable creatinine and Banff grade 1A or greater histology on protocol biopsies. These were compared to biopsies from stable patients without histological rejection, and patients who had rejection but showed functional and histological resolution on 3 month follow-up biopsies. Expression results of these pooled populations were compared to normal kidney. In Figure 4, profiles indicate the presence or absence of target genes in each of the pooled populations and also whether transcripts are increased or decreased compared to the level of expression in stable allografts with normal histology.

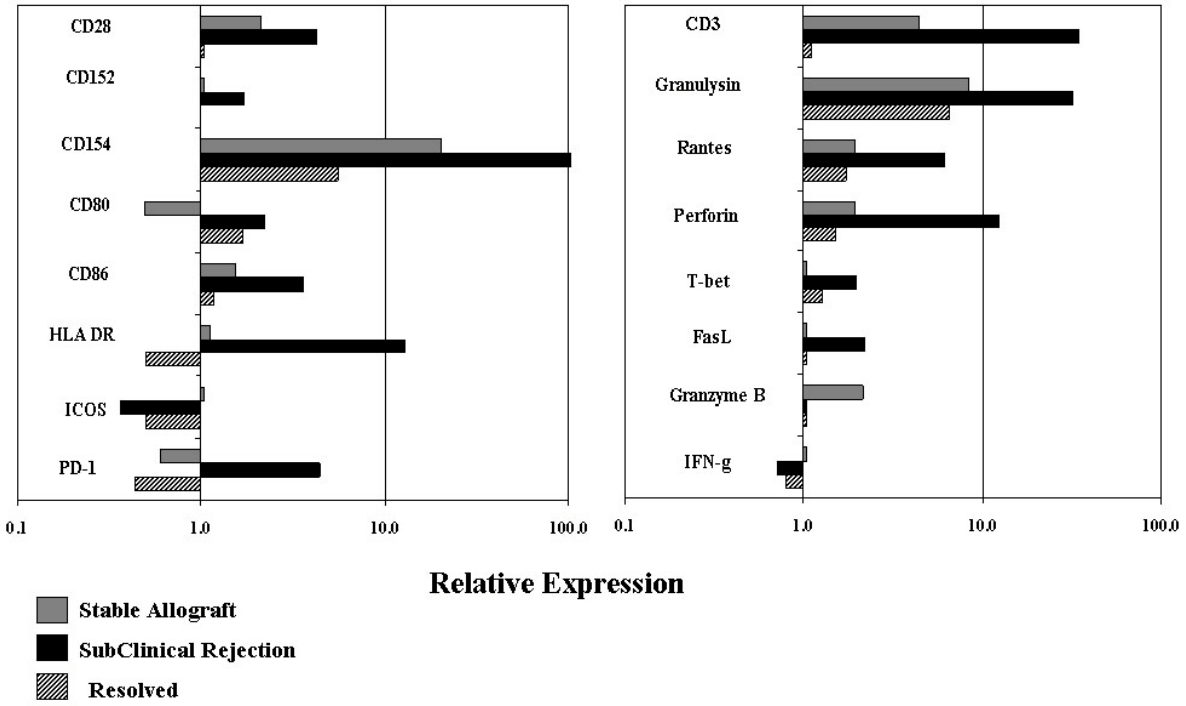


Figure 5. Quantitative gene expression monitoring of allograft dysfunction in sample pools. Level of RNA transcript prevalence in pooled samples from stable allograft biopsies (dark gray; n=10), subclinical rejection biopsies (black; n=10) and resolved (slanted bards; n=5) were determined and are displayed as fold-level above baseline (the normal kidney pool; n=12).

Results show that nearly all the targets analyzed in this study were elevated during subclinical rejection. In addition, several genes associated with T cell activation, which were not expressed in stable allografts, (IL-2, PD-1, CD28, CD25) were present in the subclinical samples. Patients undergoing subclinical rejection were treated with bolus methylprednisolone and re-biopsied after 3 months. Interestingly, most inflammatory, cytotoxic and monocyte activation transcripts were reduced to levels even lower than in stable allografts. The predominant T cell activation markers were also not expressed. However, the continued elevated presence of TNF-alpha, TGF-beta, C3 and several costimulation markers, suggested continual tissue inflammation and detrimental cellular activation despite scoring by standard clinical histology assessments as stable function. As in the reperfusion studies, initial pooled population analysis may be very descriptive of trends and pathways that are involved in allograft function.

We have taken these largely qualitative assessments of the subclinical milieu and evaluated them using the quantitative power of RT-PCR (Figure 5). These data demonstrate a more vivid snapshot of the transcripts, and therefore the critical cell populations and pathways, that may be the target of immunosuppression modification. Using these quantitative analytical methods, we have found that stable functioning allografts with normal histology, though clearly more muted than subclinical rejection biopsies, have surprisingly elevated levels of CD154, CD3, Granulysin and cytotoxic transcripts, perforin and granzyme B. These transcripts are more prominently elevated in the subclinical rejection pool. In addition, class II and other costimulatory molecules are significantly upregulated suggesting the presence of activated cellular infiltrates that may predispose the allograft to dysfunction. These findings are in agreement with other studies showing that a significant percentage of renal biopsies from stable allografts have early borderline and subclinical markers of acute and chronic rejection (56,57,65-67). However, our findings suggest that this concept should be extended further in showing that T cell activity is augmented even in the absence of findings that meet the criteria for rejection. This finding is certainly a product of a more sensitive assay at the transcriptional level, but also may be highlighted by our use of a truly normal comparative state, i.e. the normal kidney. These data may help elucidate the mechanisms involved in allograft rejection under standard immunosuppression. Indeed, these studies also underscore the necessity for a sensitive and precise quantitative measure of the allograft microenvironment to be able to correctly diagnose renal dysfunction to ultimately individualize patient immunotherapy.

5. POLYCHROMATIC FLOW CYTOMETRY

Flow cytometry was developed in the late 1960s and came into widespread clinical use in the 1990s. The earliest technology used one laser and two light detectors that were able to detect forward scatter and a single fluorescent dye (color). It became apparent that detection of multiple colors simultaneously was necessary to accurately identify lymphocyte subsets. Even as 4- and 5-color flow

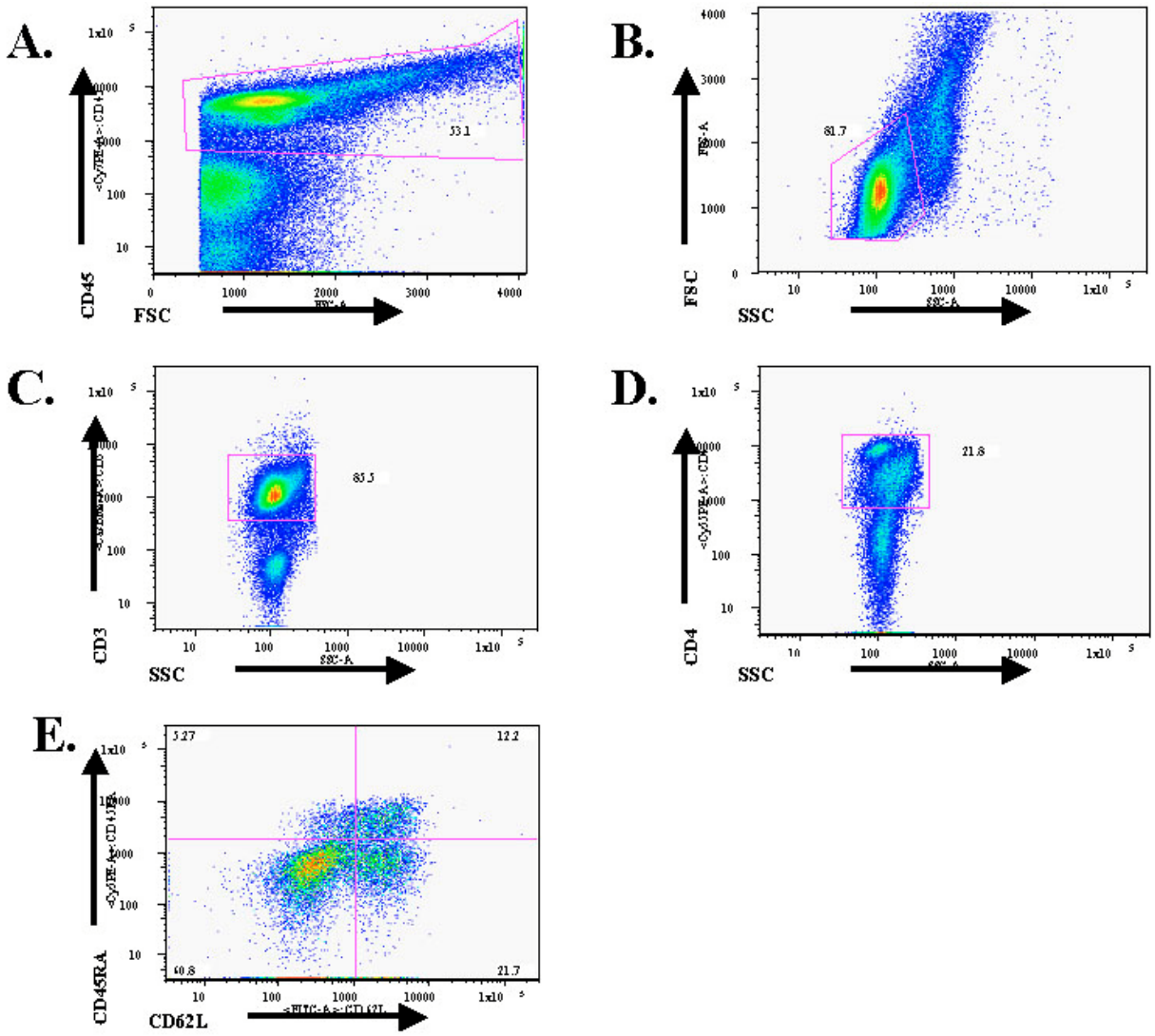


Figure 6. Identification of naïve and memory cells in a normal patient. (a) Forward scatter vs. CD45 identifies the common leukocyte antigen positive cells; (b) side scatter vs. forward scatter identifies lymphocytes by cell size and complexity; (c) and (d) side scatter vs. CD3 and CD4 identifies CD4+ T cells; (e) CD62L vs. CD45RA identifies naïve and memory cells. Naïve cells are double positive for CD62L and CD45RA. The remainder of the cells function as memory cells.

cytometry came into clinical use, its limitations were apparent. The complexity of the immune system is such that some lymphocyte subsets require measurement of at least 6 cell surface antigens for accurate immunophenotyping (81). For example, 95% accuracy in identifying naive T cells mandates three antigens to delineate T cell families (CD3, CD4, CD8). Detection of three additional functional antigens (e.g., CD45RA, CD62L, CD11a) is required to yield a clean subset of naïve cells. This type of exhaustive identification of lymphocytes is imperative so that subsequent functional studies with these populations are not confounded by misidentification of functionally distinct cells. We currently use 12-color, 14-parameter flow cytometry, deemed polychromatic flow cytometry (PFC) (82), in the evaluation of renal transplant patients at the NIH. This technology was developed at Stanford University in the Herzenberg laboratory (83) and brought to the NIH by Dr. Mario Roederer.

5.1. Technique

The current scheme of PFC uses three lasers to excite the 12 colors. A krypton laser excites Cascade Blue and Alexa 430; the argon laser excites fluorescein isothiocyanate (FITC), phycoerythrin (PE), Texas Red PE, Cy5PE, Cy5.5PE, and Cy7PE; a dye laser excites Alexa 594, allophycocyanin (APC), Cy5.5APC, and Cy7APC. Peripheral blood mononuclear cells are obtained from renal transplant recipients through density centrifugation prior to administration of induction agents, on the day of surgery, and on post-operative days 7, 14, 21, and 28. Peripheral blood is obtained monthly thereafter. Between 10^6 and 10^7 cells are stained with each of 2 separate 10 to 12-reagent panels as shown in Figure 6. The

Table 4. The 2 antibody panels used for PFC to interrogate the peripheral blood in human renal transplant recipients

TUBE	FITC	PE	Cy5PE	APC	Alexa 594	Cy5.5APC	Cy7APC	Cy7PE	CasB	Cy5.5PE	TRPE
A	CD56	CD19	CD163	HLA DR		CD16	CD3	CD14	CD45	CD20	CD8
B	CD62L	CD25	CD45RA	CD56	CD16	CD45RO	CD11a	CD45	CD3	CD4	CD8

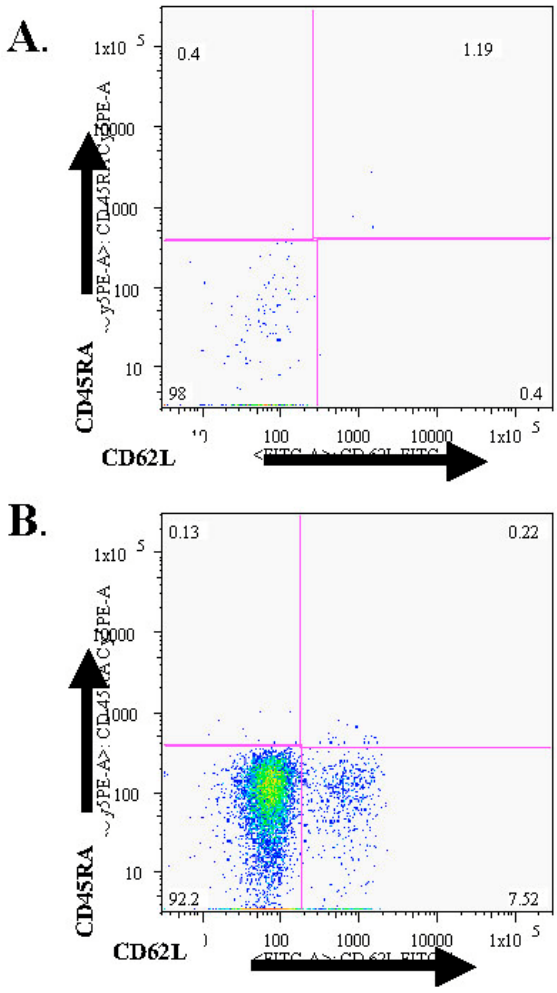


Figure 7. (a) Naïve and memory cells on post-operative day 7 in a patient who received depleting doses of thymoglobulin; (b) Naïve and memory cells in the same patient one day before confirmation of acute clinical rejection. Note the marked increase in memory T cells.

cells are stained for 30 minutes at room temperature then washed 3 times. Data are collected on a modified FACS DiVa (Becton Dickinson, San Jose, CA) connected to CellQuest software. The data are compensated and analyzed using FlowJo (Tree Star, San Carlos, CA).

Analysis of PFC data can be complicated and relies on proper compensation of the samples (84). In compensation, the spectral overlap between the fluorochromes is mathematically eliminated. Compensation must be set conservatively, meaning undercompensating where necessary. Failure to do so may cause overcompensation with certain fluorochromes, obfuscating the separation of bright and dim and leading to errors in data interpretation. Thus, the recognition of inaccurate compensation is a necessity.

The most rigorous method of discerning background staining from positive controls is to formulate the proposed mixture of reagents but eliminate the single reagent of interest. This should be done for all reagents by eliminating each separate reagent from an individual cocktail. For example, in a 6-color stain, multiple 5-color combinations could be constructed by eliminating each of the six reagents from the complete stain. Figure 6 depicts analysis of peripheral blood mononuclear cells for memory and naïve cells. Six colors and 8 parameters (Table 4) are used to immunophenotype these distinct cell subsets. Interrogating surface markers such as CD62L enriches these data by providing functional information.

5.2. Investigating Rejection

We are beginning to use PFC to evaluate small subsets of cells in renal transplant recipients and find that it is an ideal tool for investigating factors associated with allograft rejection following T cell depletion with rabbit anti-thymocyte globulin (Thymoglobulin, Sangstat) and Alemtuzumab (Campath-1H, Millennium). With 5 ml of peripheral blood, the populations of naïve and memory T cells, regulatory T cells, NK cells, monocytes, and B cells can be discerned with a high degree of fidelity. Using flow cytometry, we have seen a profound peripheral depletion of all lymphocyte subsets at one week post-transplant. In some patients, episodes of clinical rejection correlate with a rise in the absolute number of CD4+ memory T cells independent of other populations (Figure 7). The absolute lymphocyte count remains very low (100-200 cells/microliter) and essentially unchanged from the preceding week, confirming specific elevation of memory T cells rather than pan-lymphocyte proliferation.

Regulatory T cells may also play a role in protection of allografts from rejection. The cell surface markers that identify regulatory T cells include CD4 and CD25. We have observed a positive correlation between allograft integrity and percentage of CD4+ T cells also expressing CD25 (Figure 8). The timing of the peak in percentage of regulatory T cells corresponds with the elevation in the absolute CD4+ memory T cell count. At the same time as the rise in CD4+CD25+ in healthy allograft recipients, those patients who reject their allografts have very low percentages of CD4+CD25+ T cells. This indicates a possible role for regulatory T cells in protection of a kidney allograft.

5.3. Advantages

The advantages of PFC are legion. The information obtained rises geometrically with the increase in colors and parameters. Also, the data obtained from PFC could not be generated from any other source. Small

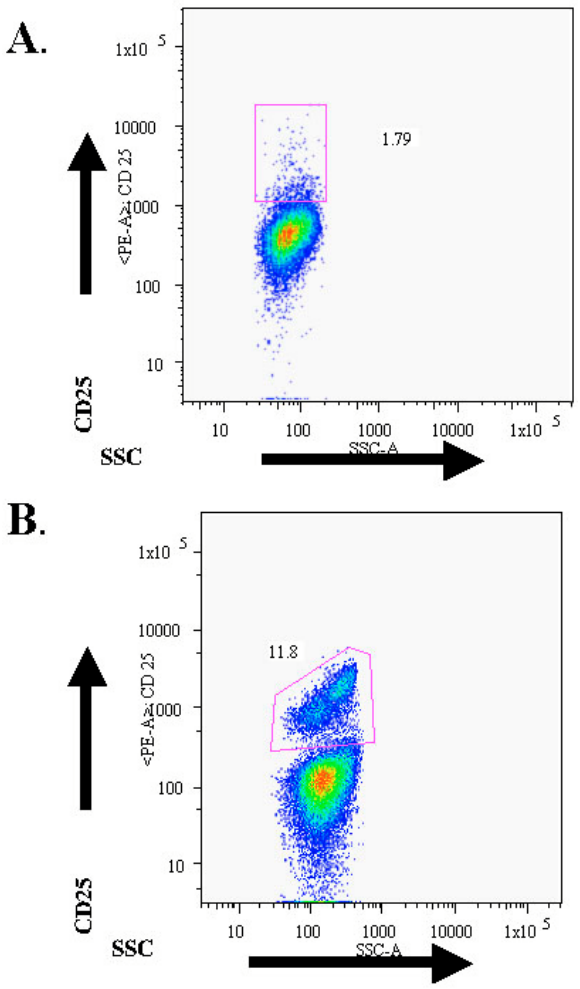


Figure 8. (a) Preoperative levels of CD25+ T cells in a preoperative patient. Previous gating included CD45+, forward scatter and side scatter for lymphocytes, CD3+ and CD4+. Over 90% of these cells also express CD45RO without expression of CD45RA. (b) CD4+CD25+ T cells in the same patient on post-operative day 20. This patient did not demonstrate acute allograft rejection. Note the variation in background fluorescence between the samples, which commonly occurs in PFC.

subsets of rare lymphocytes can be unambiguously identified. For example, immunophenotyping and sorting memory and naïve T cells with a high degree of fidelity could not be done with any combination of one-color stains or even with the use of a more sophisticated 4-color flow cytometer.

From a practical standpoint, PFC conserves antibody by not duplicating reagents for multiple samples. The labor required for sample preparation is diminished, although data analysis is much more complicated and time consuming. Many current transplant protocols use depleting agents for induction. Polychromatic flow cytometry can identify small numbers of rare lymphocyte populations, such as early NK cells, even after depletion, and can also be used to interrogate additional cell markers on previously described lymphocyte subsets. For example, we have observed a transition of CD62L on regulatory T cells from CD62L+ preoperatively, to CD62L- at approximately 3 weeks after the renal transplant. These cells have also been shown to consistently express the CD45RA+, CD45RO- phenotype, as would be expected.

Very precise cell sorting can be accomplished with a high degree of purity using PFC. This allows subsequent functional studies in vitro and in experimental animals that yield reliable data. Also, combinations of antibodies against cell surface markers and intracellular cytokines can demonstrate cell function from very specific lymphocyte subsets.

5.4. Disadvantages

The difficulty with compensation has already been discussed. Although understanding of the compensation process allows acquisition of reliable data and recognition of improperly compensated data. Additionally, finding a salutary combination of fluorochromes can be labor intensive. A combination of fluorochromes should be tested by stepwise addition of reagents to a panel. Without such testing, signals from different fluorochromes can overlap and obscure data from precious samples. As the number of colors available for PFC increases, the availability of commercial reagents diminishes. Many reagents require conjugation and titration before clinical use. Some combinations will not be bright enough for good separation of high and low expression of a marker, thereby requiring additional conjugations and one-color FACS testing.

5.5. Future Application of Polychromatic Flow Cytometry

The unambiguous identification of rare lymphocyte subsets afforded by PFC will certainly be instrumental in future transplant immunology discoveries. This is particularly the case in depletional protocols where analysis of rare sub-populations appears critical. The technology has provided insight into the mechanisms of allograft rejection and integrity and has prompted in vitro studies to test our hypotheses. The capability for sorting and functional studies makes this technique widely applicable in the field of immunology. Addition of colors beyond the current limit of 12 will promote even closer scrutiny of the immune system. Polychromatic flow cytometry is still a few years from widespread clinical use. Cooperation between industry and academia is accelerating the development of new technology, making this valuable technique more widely available.

6. PERSPECTIVE

Our understanding of transplantation has become increasingly fueled by basic immunobiological discoveries. As we carry this knowledge to the clinic, tools must be used to relate clinical syndromes to the basic concepts thought to be germane to their development. In this review, we have outlined three emerging technologies that appear robust enough to evaluate transplant patients and derive

mechanistic insight into the origins of allograft rejection and acceptance. These techniques, and others like them, will aid clinical investigators in understanding patient disease, and will also likely highlight situations whereby seemingly logical basic observations will be found to be overly simplistic when placed in the context of human illness. In both situations, a seamless interface between the laboratory and clinic will benefit all involved.

7. ACKNOWLEDGEMENTS

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