Trends in Transplant. 2010;4:68-77

Biomarkers of Tolerance in Transplantation: Hope or hype?

Rachel Hilton[1] and María P. Hernández-Fuentes[2]

[1]Renal, Transplant and Urology Services, Guy's and St Thomas' NHS Foundation Trust, London, UK; [2]King's College London, Department of Nephrology and Transplantation and MRC Centre for Transplantation, Guy's Hospital, London, UK

Abstract

Although transplantation has long been regarded as the treatment of choice for solid organ failure, unwanted side effects of immunosuppressive drugs and poor long-term allograft survival remain important limitations. Achieving tolerance to transplanted organs should solve both problems, but this remains elusive for the majority of transplant recipients. Recent advances in immunological techniques have led to a resurgence of interest in studying those rare transplant recipients who have achieved tolerance to their allografts. The development of biomarkers indicative of transplantation tolerance would constitute a major advance in the care of organ transplant recipients in that it would allow individualized minimization of immunosuppressive therapy in the clinic, so reducing side effects and costs and optimizing long-term graft outcomes. The objective of this review is to discuss current and evolving techniques for immunological monitoring in clinical transplantation, to explain how these techniques are being adapted to meet the challenge of characterizing tolerant liver and kidney transplant recipients, and to explore the opportunities and risks of translating these techniques into routine clinical practice in order to guide individualized therapy.

(Trends in Transplant. 2010;4:68-77)

Corresponding author: María Hernández-Fuentes, maria.hernandez@kcl.ac.uk

Key words

Tolerance. Transplantation. Biomarkers. Immune monitoring.

Introduction

Since 1954, when the first successful kidney transplant was performed, the art of transplanting organs, tissues, and cells from one individual into another has expanded many fold and has been employed for diverse purposes, including treating end-stage organ failure, repairing or replacing defective or diseased tissues, treating diseases of the blood, bone marrow, and certain types of cancer, and resetting the immune system in severe autoimmune diseases. Most allograft recipients require life-long immunosuppressive medication, with consequent increased susceptibility to infection, increased risk of malignancy, and accelerated cardiovascular disease. This contributes substantially to morbidity and mortality among transplant recipients and limits the efficacy and durability of transplantation as a therapy. Other key

Correspondence to:
María Hernández-Fuentes
Department Nephrology and Transplantation
MRC Centre for Transplantation
Guy's Hospital
London SE1 9RT, UK
E-mail: maria.hernandez@kcl.ac.uk

Table 1. Illustrative clinical cases

Case 1
A patient living in a rural area received a kidney transplant from his brother six years previously. The patient has difficulty attending the hospital and obtaining regular supplies of immunosuppressive medication. He wants to withdraw from immunosuppressive medication with the support of his transplant physician. The patient is counseled against this on the basis that this would increase his risk of acute allograft rejection and compromise the survival of his transplant. The patient nevertheless decides to embark upon an unsupervised weaning process over the course of the year. The weaning is successful, and when the patient is seen again he is already immunosuppression-free with stable kidney function. The question for the transplant physician is how long can this immunosuppression-free period be sustained, and how can this most effectively be supported by the clinical team.
Case 2
A patient received a cadaveric kidney transplant 15 years ago. Her graft function is excellent, but she has multiple skin lesions, including squamous cell carcinomas and viral warts. She would like to know if it would be safe to reduce or discontinue her immunosuppressive medication in order to reduce these skin problems. She is very anxious above all to preserve her kidney function. Her transplant physician tells her that there is no way of knowing whether tolerance to the allograft has been established and she agrees to remain on her current level of immunosuppressive medication rather than risk any loss of kidney function.

causes of allograft loss are immunologically mediated allograft damage and direct toxicity from immunosuppressive drugs, which have additional undesirable side effects including hypertension, weight gain, and increased risk of diabetes, osteoporosis, hyperlipidemia, hirsutism, or alopecia. For each patient, a constant balance has to be struck between an acceptable level of side effects and the risk of damaging the allograft through rejection. This is particularly challenging for adolescent patients. To date there is no clinical test that reliably determines how much immunosuppressive medication an individual needs at a particular time-point to avoid allograft rejection. Dose ranges and target drug levels are based on data from large-scale clinical trials rather than on individual needs. More widespread availability of information and a concomitant increase in patient engagement in healthcare will lead to ever increasing demand for individualized therapy. Some illustrative clinical cases are shown in table 1.

Given the worldwide shortage of donated organs for transplantation, there is an ever more pressing need to maximize the survival of both patients and allografts. The ability to induce, identify, and monitor transplant tolerance would obviate the need for immunosuppressive medication, so addressing the problem of attrition, maximizing allograft survival, and ensuring optimal use of a scarce resource.

Definitions of transplant tolerance

The definition of "true" transplant tolerance has been proposed as "a well-functioning graft lacking histological signs of rejection, in the absence of any immunosuppressive drugs, in an immunocompetent host capable of accepting a second graft of the same donor origin, while being able to reject a third-party graft"[1]. Although achieved in several animal models[2], this has rarely been reported in clinical practice and only after administration of aggressive conditioning regimens[3,4]. In the clinic, therefore, a less stringent definition of "operational tolerance" is used to describe a state of stable graft function, years after stopping all immunosuppression in recipients who are otherwise immunocompetent[5]. Although approximately 20% of liver transplant recipients become operationally tolerant after immunosuppression weaning[6], the frequency of operational tolerance in the kidney transplant population is unknown as to date there are no controlled trials and no reliable indicators to

identify suitable patients for this approach. The identification and validation of such indicators or biomarkers would aid the development of weaning protocols and open up the possibility of offering individualized therapy and safe immunosuppression minimization or withdrawal to a wider group of transplant recipients.

Definition of a biomarker

The National Institutes of Health Biomarkers Definitions Working Group has defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention"[7]. Biomarkers are commonly used as tools for diagnosis, for staging disease progression, as indicators of disease prognosis, and as predictors of clinical response. Characteristics of ideal biomarkers are shown in table 2. Within immunological systems, candidate biomarkers might include particular cell types, genes, proteins, or peptides. They may also be the results of specific tests that indicate the function of cells present in peripheral blood or in tissues, or of genes expressed by particular cell subsets.

Table 2. Characteristics of ideal biomarkers

- Quick, reliable, and inexpensive to measure
- Readily quantifiable in accessible clinical samples such as blood or urine
- Differ specifically in the relevant biological process or disease
- No overlap in level between patients and healthy controls
- Not subject to wide variation in the general population
- Correlate closely with the relevant biological process or disease
- Unaffected by unrelated conditions and associated comorbid factors
- Correlate closely with established clinicopathologic parameters of disease
- Vary rapidly in response to specific therapies
- Have predictive power for disease severity and prognosis

Conventional biomarkers of kidney and liver function

Established biomarkers of kidney and liver function are widely used but carry major limitations. Glomerular filtration rate (GFR), the sum of the filtration rates in all functioning nephrons, is a convenient and time-honored way of quantifying kidney function. However, GFR varies under normal physiological conditions as well as with disease[8]. Gold standard methods of assessing GFR, such as inulin clearance, are technically demanding, expensive, and time-consuming and therefore unsuitable for everyday use. Measuring serum creatinine, a breakdown product of muscle creatine phosphate produced by the body at a constant rate and filtered out of the blood by the kidneys, is simple to do and is a universally accepted biomarker for kidney function. However, although cheap and easy to measure, serum creatinine has significant limitations when used as an isolated biomarker in this way. The rate of production of creatinine is dependent on muscle mass, which is subject to the major modifying effects of age, gender, and ethnicity. Furthermore, changes in serum creatinine may lag behind changes in GFR by many days. Measurement of 24-hour urinary creatinine clearance gives a more accurate assessment of GFR, but remains subject to the same nonrenal influences on creatinine generation, compounded by the potential for inaccuracy and the inconvenience of 24-hour urine collection.

The liver, metabolically the most complex organ in the body, performs many vital functions such as metabolism of ingested nutrients, synthesis of key immune, inflammatory, and blood coagulation proteins, and processing of endogenous and exogenous toxins. It is perhaps therefore unsurprising that the reliability of any single laboratory test of liver

function is relatively limited. The most commonly used biomarker of hepatocellular injury is the measurement of serum transaminases, of which alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are considered the most important. These are present in high concentration in hepatocytes, where they catalyze the transfer of amino groups from alanine and aspartate to the α-keto group of ketoglutaric acid to produce pyruvic acid and oxaloacetic acid, respectively. These enzymes leak into the blood when hepatocyte cell membranes are damaged. Alanine aminotransferase is the more specific marker of hepatocellular injury because it occurs exclusively in the liver, whereas AST occurs to some extent also in heart, skeletal muscle, kidney, and pancreas. Although elevation in serum transaminase levels may be regarded as a sensitive indicator of hepatocyte damage, the finding is nonspecific[9]. Furthermore, measurements of liver enzymes correlate poorly with liver histopathology and generally provide no prognostic measure of how liver function may further change over time.

In summary, although both serum transaminase levels and serum creatinine levels are widely used in clinical practice as biomarkers of liver or kidney injury, respectively, they correlate poorly with actual liver or kidney function and offer little useful prognostic information regarding the likelihood of organ failure or recovery. In the context of solid organ transplantation, a further major limitation is that these biomarkers signify tissue injury, but provide no information about the immune status of the organ recipient. Of far greater diagnostic and prognostic value would be biomarkers of immune reactivity, which would assist clinical monitoring by indicating the risk of allograft rejection versus over immunosuppression, would aid individualized tailoring of immunosuppressive therapy, would facilitate development of new therapeutic strategies, and would help identify potentially tolerant individuals.

Emerging biomarkers in posttransplant monitoring

Blood and urine samples are readily accessible in clinical practice and are therefore ideal starting points in the search for useful biomarkers. Urine is the least invasive clinical sample to collect, and is most likely to be of value in the context of kidney transplantation as the effluent of the organ of interest. Unfortunately, high variability in concentration and volume makes the quantification of biomarkers often difficult and occasionally unreliable. Nevertheless, useful biomarkers have been identified in urine and studies are underway to validate these. In 2001 it was first demonstrated that messenger RNA (mRNA) analysis of urine from kidney transplant recipients could be used as a noninvasive marker of acute allograft rejection[10]. In this study, levels of mRNA of perforin and granzyme B genes were measured. These genes encode proteins that are found in cytoplasmic granules in cytotoxic T-cells and natural killer cells, and cooperate to induce target cell death. Levels of perforin mRNA in urine above a predetermined threshold predicted biopsy-confirmed acute rejection with high sensitivity and specificity, whereas levels of granzyme B mRNA were less specific. Subsequently, it has been shown that expression levels of CD3, perforin, and CD25 in urine sediment cells are higher in kidney transplant patients with acute rejection, and expression of forkhead box protein 3 (FOXP3) at the time of rejection correlates with response to treatment[11]. Since then, numerous biomarkers of cytotoxic T-cell activity have been identified in urine, including mRNA for serine protease inhibitor-9 (PI-9), CD103, granulysin, inducible protein 10 (IP-10), chemokine (CXC motif) receptor 3 (CXCR3), Fas ligand (FasL), NKG2D, T-cell immunoglobulin domain (Tim-3) and Interferon gamma (IFN-γ). These biomarkers, alone or in combination, have been shown by a number of different laboratories to be able to predict acute rejection in kidney allografts with a high degree of accuracy[12].

Other studies have assessed peripheral blood gene expression using real-time polymerase chain reaction (PCR) in correlation with clinical outcomes in transplant recipients. Several studies have shown increased expression of cytotoxic genes in the peripheral blood of patients undergoing acute rejection[13-15]. In another study, expression of selected genes in peripheral blood, biopsy tissue, and urine was simultaneously evaluated in kidney transplant recipients with delayed allograft function. Expression of cytotoxic and apoptosis-associated genes, such as perforin, granzyme B, and FasL, was significantly higher in patients with delayed graft function due to acute rejection than in patients with acute tubular injury[16].

Similarly, a number of immune monitoring assays have been developed to assess recipient T-cell activity using peripheral blood cells. These assays may be donor antigen-specific or antigen-nonspecific and include mixed lymphocyte reaction, limiting dilution analysis, enzyme-linked immunosorbent spot (ELISpot) assay, *trans-vivo* delayed-type hypersensitivity (DTH) assay, and flow cytometric techniques using carboxyfluorescein succinimidyl ester (CFSE) to detect cell division, cytokine detection, or HLA-tetramer staining. While it could be argued that responses in peripheral blood may not mirror intra-graft events, there are some experimental data to suggest that circulating lymphocytes do indeed share phenotypic and functional characteristics with those infiltrating the graft[16,17]. Applying some of these methods in the context of kidney transplantation, it has been shown that that higher antidonor immune responses correlate with acute rejection[18], chronic rejection[19], and reduced allograft function[19,20].

Histological evaluation of biopsy material is currently considered the gold standard for diagnosing the cause of allograft dysfunction. Unfortunately, the biopsy procedure is expensive, prone to sampling errors and interobserver variation, and carries a significant risk of morbidity and mortality. Nevertheless, various biomarkers have been evaluated in biopsy specimens in order to improve interpretation of the biopsy. Some groups have studied patterns of T-cell receptor (TCR) usage in T-cells infiltrating explanted kidney allografts, and these appear to be distinct in grafts lost to acute rejection and those lost to chronic rejection[21]. Other groups have used high-density microarrays to analyze gene expression in biopsy tissues and have identified patterns associated with particular types of acute rejection and different clinical outcomes[22,23]. Indeed, microarray analysis of kidney transplant biopsies reveals a molecular heterogeneity of allograft rejection that is not evident by light microscopy alone[24].

Recently, expression profiles of microRNA (miRNA), small abundant noncoding RNA that regulate gene expression, have been used to predict human kidney allograft status[25]. This study showed that intra-graft miRNA profiles distinguish patients with acute rejection from patients with normal allograft biopsy results, and that acute rejection can be diagnosed with a high degree of accuracy from intra-graft levels of miRNA. Furthermore, miRNA profiles were also predictive of kidney allograft function. This study also showed that that miRNA overexpressed in biopsies of patients with acute rejection are highly expressed in peripheral blood mononuclear cells, further evidence that findings in peripheral blood may correlate well enough with what is happening in the allograft to support the use of peripheral blood as a source of potential biomarkers. More extensive validation of such methods, in correlation with new and established blood and urine biomarkers and with defined clinical endpoints, will facilitate the introduction of some of these techniques into clinical practice.

To date, most experience of immune monitoring in clinical transplantation has been

in assessing donor-specific immune responsiveness in order to predict graft rejection. This has been of particular benefit in hematopoietic cell transplantation in the era before high-resolution tissue typing techniques[26]. Identifying biomarkers of donor-specific tolerance presents a different set of challenges[27]. First and foremost, most solid organ transplants worldwide are carried out to treat end-stage kidney disease, but tolerant recipients of kidney allografts are very scarce. Most kidney transplant recipients will reject their allografts if immunosuppression is inappropriately tapered or discontinued. Patients who have successfully been weaned from immunosuppression, either by their own initiative due to noncompliance or under medical supervision due to life-threatening comorbidities, are few and far between. Secondly, if the mechanism of tolerance requires loss of donor-specific immune reactivity due to T-cell depletion[28], recognition of this would require assessment of an absent immune response. The challenge, therefore, would be to ensure sufficient sensitivity of the assays used. Alternatively, if the mechanism of tolerance requires active regulation of genes or effector immune responses, recognition of this should be an achievable goal.

Biomarkers of tolerance in liver transplantation

Clinically, evaluation of tolerance in liver transplantation has been limited to assessment of allograft function, supplemented by monitoring through repeated biopsy and peripheral blood sampling for biomarker analysis. Current biomarkers under evaluation include dendritic cell subsets, regulatory T-cells, antidonor antibodies, and gene polymorphisms[29]. While it has long been recognized that complete freedom from immunosuppression is sporadically possible in long-surviving recipients of liver and kidney allografts, the first serious attempt at clinician-led, prospective, immunosuppressive drug weaning began in pediatric and adult liver transplant recipients in Pittsburgh in 1992[30]. Cytokine gene polymorphisms were studied in a cohort of pediatric recipients. All of the immunosuppression-free children and the majority of those on minimal immunosuppression displayed low tumor necrosis factor (TNF)-α and high/intermediate interleukin (IL)-10 profiles in comparison with control patients on maintenance immunosuppression[31]. In addition, there was a difference in dendritic cell subset ratios between the two groups of patients. In comparison with patients on maintenance immunosuppression, circulating levels of plasmacytoid dendritic cells (pDC2), reported to selectively induce T-helper (Th) 2 responses, were more prevalent relative to monocytoid dendritic cells (pDC1), which induce Th1-type responses, in the immunosuppression-free or minimally immunosuppressed patients[32].

The best attempts at identifying biomarkers of tolerance in adult liver transplant recipients have emerged from a group based in Barcelona, Spain. Using peripheral blood gene expression profiling and extensive blood cell immunophenotyping, Martinez-Llordella, et al. demonstrated that operationally tolerant patients could be identified with a signature of genes that encoded $\gamma\delta$ T-cell and natural killer (NK) cell receptors, as well as genes involved in cell proliferation arrest[33]. They also found, in the tolerant patients, greater numbers of circulating potentially regulatory T-cell subsets, $CD4^+CD25^+$ T-cells and $\gamma\delta$ T-cells, in particular the $V\delta1^+$ subtype that has been implicated in immunoregulatory processes in epithelial tissues. Interestingly, previously observed differences in ratios of dendritic cell subsets could not be replicated in this patient cohort. Additionally, significant upregulation of proinflammatory genes was noted in hepatitis C virus (HCV)-infected recipients, suggesting that immunosuppression-induced nonspecific inflammation could be related to the worsened prognosis of HCV-positive liver

transplant recipients. The same group have studied gene expression profiles in the peripheral blood of liver transplant recipients, comparing patients where immunosuppression weaning was successful (tolerant: n = 28) with those where the weaning process was attempted but led to acute rejection, requiring reintroduction of immunosuppression (non-tolerant: n = 33) and with healthy controls[34]. They identified three distinct gene signatures incorporating a modest number of genes (between 2-7) that accurately discriminated tolerant and non-tolerant liver allograft recipients and healthy non-transplanted controls. This genomic footprint of operational tolerance has been validated in an independent cohort of 23 additional liver transplant recipients and is mainly characterized by upregulation of genes encoding for a variety of cell-surface receptors expressed by NK, $CD8^+$, and $\gamma\delta$ T-cells. The previously observed expansion of putative regulatory T-cells ($CD4^+CD25^+Foxp3^+$, $\gamma\delta TCR^+$, and $\delta 1TCR^+$ T-cells) in peripheral blood was replicated in this new set of tolerant recipients.

Taken together, it appears that a combination of transcriptional profiling and flow cytometry in peripheral blood may accurately identify liver transplant recipients who are able to accept their grafts in the absence of pharmacological immunosuppression. Validation of these findings in prospective immunosuppression weaning trials is now required.

Biomarkers of tolerance in kidney transplantation

Whereas the liver has a strong regenerative capacity and there are few or no adverse consequences of liver allograft rejection, provided that it is diagnosed and treated promptly, the situation is very different for kidney transplantation, in that graft rejection inevitably causes some degree of irreversible parenchymal damage that comprises graft function and longevity. For this reason, the prospective immunosuppression weaning studies that have been carried out in liver transplant recipients have not been replicated in kidney transplantation. Most observations of operational tolerance in kidney transplantation involve those rare patients who either of their own accord, due to nonadherence with immunosuppressive medication, or under medical supervision, due to life-threatening infection, malignancy, or drug toxicity, discontinue immunosuppression but do not suffer allograft rejection. These patients represent a highly selected group who may not be representative of the total kidney allograft recipient population.

A collaboration between a group in Nantes, France, and another group in Stanford, UK, used expression arrays to identify a set of 33 genes that could correctly distinguish, with high specificity, operationally tolerant kidney transplant recipients from patients with acute and chronic allograft rejection and healthy age-matched volunteers[35]. Expression of co-stimulatory genes and markers of early and late T-cell activation were reduced in tolerant patients compared with controls, and although expression of the anti-inflammatory cytokine transforming growth factor β (TGF-β) was not upregulated in tolerant patients, many TGF-β-regulated genes were. The same group have analyzed blood cell phenotypes and transcriptional patterns in a group of eight operationally tolerant kidney allograft recipients, and demonstrated higher absolute numbers of circulating B-cells and regulatory T-cells ($CD25^{hi}CD4^+$) in comparison with a control group of patients with chronic rejection and a significant decrease in FOXP3 transcript levels in the recipients with chronic rejection[36]. Interestingly in this study, the blood cell phenotype of clinically tolerant patients did not differ from that of healthy individuals, suggesting that operational tolerance is not due to an increased pool of regulatory T-cells, but may be due to maintenance of a natural state that is lacking in patients with chronic rejection. By

contrast, a different group reported a more variable TCR-Vβ repertoire and a higher percentage of $CD4^{+}CD25^{high}$ in long-term, stable, kidney transplant recipients, two of whom were immunosuppression free, in comparison with patients with chronic rejection, dialysis patients, and healthy controls[37].

In parallel, two initiatives were established to undertake multi-parameter biomarker analyses in tolerant allograft recipients; these results have been recently published. The Indices of Tolerance study based in Europe and the Immune Tolerance Network based in the USA have recently identified and validated a set of biomarkers that identify operationally tolerant, immunosuppression-free kidney transplant recipients. The common findings from both studies are a somewhat unexpected lymphocyte subset distribution, with expansion of peripheral B lymphocytes, and a gene expression profile that concomitantly indicates an enrichment of B-cell-related genes[38,39]. In the Indices of Tolerance study, a combination of cross-platform biomarkers was more sensitive and more specific than individual assay biomarkers for identifying tolerance[38].

Translation into clinical practice

Once a biomarker or set of biomarkers has been found to be associated with a particular biological process or disease, the next challenge is to validate this association prior to adoption of the candidate biomarkers into routine clinical practice. From a practical perspective, this requires access to high throughput and standardized clinical laboratory protocols. It also requires broad characterization of the biomarkers across a wider population, studying variation with gender, age, and ethnicity, as well as with unrelated comorbid conditions. It is therefore mandatory to assess and validate biomarkers in large patient cohorts. For most disease processes, this is likely to require large-scale, prospective, multicentre studies. In the context of clinical transplantation, if the aim is to discriminate tolerant individuals from healthy controls, it will be important to ensure that candidate biomarkers are not simply identifying patients who are not taking immunosuppressive drugs. In this regard, an important comparator group would be non-transplanted patients who receive courses of immunosuppressive therapy for other conditions, as this would help determine the contribution of the drugs versus the allograft in dictating peripheral blood cell phenotypes and gene expression profiles. If these challenges can be overcome, the next and most important validation step will be to determine whether immunosuppressive drugs can be withdrawn from transplant recipients who display the tolerant phenotype.

Human liver allografts are less susceptible to rejection than other organs, and it appears that approximately 20% of liver transplant recipients can successfully be weaned from immunosuppression[6]. This is associated with acute cellular rejection in between 12-76% of cases, but this is generally mild and often resolves after restoration of baseline immunosuppression. Reassuringly, only two cases of graft loss have been reported[40,41]. In renal transplants, however, the situation is somewhat less optimistic. It is well known that nonadherence with immunosuppressive therapy is prevalent in the renal transplant population, with an overall rate of over 35 cases per 100 persons/year. There is significant correlation between adherence and rejection-free survival in the first six months posttransplantation, whereas early nonadherence correlates with late nonadherence and subsequent adverse clinical events[42]. It is not currently possible to accurately estimate the risk that an individual patient undertakes by attempting supervised or unsupervised weaning from immunosuppression, but the prevailing view is that immunosuppression-free stable allograft function is more difficult to attain in kidney allograft

recipients than in liver allograft recipients. The obvious implication is that prospective studies of immunosuppression weaning guided by biomarkers of tolerance in kidney transplant recipients will require careful patient selection, counseling, and close supervision. Concomitant use of peripheral blood markers predictive of rejection will enable closer monitoring of these patients and may facilitate safe conduct of prospective immunosuppression weaning studies in kidney transplantation.

In conclusion, collaborative efforts, such as the European transplantation network RISET and the Immune Tolerance Network in the US, have given access to an extensive network of standardized laboratory facilities and assays, which has, in turn, enabled rapid progress in identification of candidate biomarkers to aid better evaluation of the immune status of the organ transplant recipient. The current intensification of efforts to identify and validate biomarkers of tolerance and rejection will mean that reliable biomarkers may soon be available to predict both with high accuracy. Individualized therapy for solid organ transplant recipients might then underpin clinical practice for the coming decade.

Acknowledgements

EU (QLRT–2002–02127) and Immune Tolerance Network (ITN503ST) grants. RISET consortium (Reprogramming the Immune System for the Establishment of Tolerance, 512090 IP, of EU FP6). MRC (G0801537/ID: 88245) and Guy's & St Thomas's Charity Grants (080530). Authors acknowledge financial support from the Department of Health via NIHR comprehensive Biomedical Research Centre award to Guy's & St Thomas' NHS Foundation Trust in partnership with KCL and King's College Hospital NHS Foundation Trust.

References

1. Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. Nature. 1953;172:603-6. **The first and pivotal report demonstrating that immune tolerance could be induced de novo.*
2. Kingsley CI, Nadig SN, Wood KJ. Transplantation tolerance: lessons from experimental rodent models. Transpl Int. 2007;20:828-41.
3. Kawai T, Cosimi AB, Spitzer TR, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. N Engl J Med. 2008;358:353-61. **This study suggests that immune regulation may be more important than deletion of effector T cells for tolerance induction in humans.*
4. Scandling JD, Busque S, Dejbakhsh-Jones S. et al. Tolerance and chimerism after renal and hematopoietic-cell transplantation. N Engl J Med. 2008;358:362-8.
5. Ashton-Chess J, Giral M, Soulillou JP, Brouard S. Using biomarkers of tolerance and rejection to identify high- and low-risk patients following kidney transplantation. Transplantation. 2009;87(Suppl):S95-9.
6. Lerut J, Sanchez-Fueyo A. An appraisal of tolerance in liver transplantation. Am J Transplant. 2006;6:1774-80.
7. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther. 2001;69: 89-95.
8. Bellomo R, Kellum JA, Ronco C. Defining acute renal failure: physiological principles. Intensive Care Med. 2004;30:33-7.
9. Tarantino G. From bed to bench: which attitude towards the laboratory liver tests should health care practitioners strike? World J Gastroenterol. 2007:13:4917-23.
10. Li B, Hartono C, Ding R, et al. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. N Engl J Med. 2001;344:947-954 ***This study was the first to demonstrate that mRNA analysis of urine from kidney transplant recipients may be used as a noninvasive marker of rejection.*
11. Muthukumar T, Dadhania D, Ding R, et al. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. N Engl J Med. 2005;353:2342-51. ***This study demonstrates the remarkable ability of FOXP3 transcript levels in urine to predict clinical outcomes.*
12. Hartono C, Muthukumar T, Suthanthiran M. Noninvasive diagnosis of acute rejection of renal allografts. Curr Opin Organ Transplant. 2010;15:35-41.
13. Shin GT, Kim SJ, Lee TS, Oh CK, Kim H. Gene expression of perforin by peripheral blood lymphocytes as a marker of acute rejection. Nephron Clin Pract. 2005;100:c63-70.
14. Simon T, Opelz G, Wiesel M, Ott RC, Susal C. Serial peripheral blood perforin and granzyme B gene expression measurements for prediction of acute rejection in kidney graft recipients. Am J Transplant. 2003;3:1121-7.
15. Simon T, Opelz G, Wiesel M, et al. Serial peripheral blood interleukin-18 and perforin gene expression measurements for prediction of acute kidney graft rejection. Transplantation. 2004;77:1589-95.
16. Aquino-Dias EC, Joelsons G, da Silva DM, et al. Non-invasive diagnosis of acute rejection in kidney transplants with delayed graft function. Kidney Int. 2008;73:877-84.
17. Hernandez-Fuentes MP, Warrens AN, Lechler RI. Immunologic monitoring. Immunol Rev. 2003;196:247-64. **This is a comprehensive review of the available tools to monitor the immune response in organ transplant recipients*
18. Hricik DE, Rodriguez V, Riley J, et al. Enzyme linked immunosorbent spot (ELISPOT) assay for interferon-gamma independently predicts renal function in kidney transplant recipients. Am J Transplant. 2003;3:878-84.
19. Baker RJ, Hernandez-Fuentes MP, Brookes PA; et al. Loss of direct and maintenance of indirect alloresponses in renal allograft recipients: implications for the pathogenesis of chronic allograft nephropathy. J Immunol. 2001;167; 7199-206.
20. Najafian N, Salama AD, Fedoseyeva EV, Benichou G, Sayegh MH. Enzyme-linked immunosorbent spot assay analysis of peripheral blood lymphocyte reactivity to donor HLA-DR peptides: potential novel assay for prediction of outcomes for renal transplant recipients. J Am Soc Nephrol. 2002;13:252-9.

21. Gagne K, Brouard S, Giral M, et al. Highly altered V beta repertoire of T cells infiltrating long-term rejected kidney allografts. J Immunol. 2000;164:1553-63.
22. Sarwal M, Chua MS, Kambham N, et al. Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. N Engl J Med. 2003;349:125-38.
23. Zarkhin V, Sarwal MM. Microarrays: monitoring for transplant tolerance and mechanistic insights. Clin Lab Med. 2008;28:385-410.
24. Shah S, Sarwal MM. Microarrays: interrogating the transplant transcriptosome. Clin Transpl. 2004:261-7.
25. Anglicheau D, Sharma VK, Ding R, et al. MicroRNA expression profiles predictive of human renal allograft status. Proc Natl Acad Sci USA. 2009;106:5330-5.
26. Sharrock CE, Kaminski E, Man S. Limiting dilution analysis of human T cells: a useful clinical tool. Immunol Today. 1990;11:281-6.
27. Newell KA, Larsen CP. Tolerance assays: measuring the unknown. Transplantation. 2006;81:1503-9.
28. Turka LA, Lechler RI. Towards the identification of biomarkers of transplantation tolerance. Nat Rev Immunol. 2009;9:521-6.
29. Castellaneta A, Thomson AW, Nayyar N, de Vera M, Mazariegos GV. Monitoring the operationally tolerant liver allograft recipient. Curr Opin Organ Transplant. 2010;15:28-34.
30. Mazariegos GV, Reyes J, Marino IR, et al. Weaning of immunosuppression in liver transplant recipients. Transplantation. 1997;63:243-9.
31. Mazariegos GV, Reyes J, Webber SA, et al. Cytokine gene polymorphisms in children successfully withdrawn from immunosuppression after liver transplantation. Transplantation. 2002;73:1342-5.
32. Mazariegos GV, Zahorchak AF, Reyes J, et al. Dendritic cell subset ratio in peripheral blood correlates with successful withdrawal of immunosuppression in liver transplant patients. Am J Transplant. 2003;3:689-96.
33. Martinez-Llordella M, Puig-Pey I, Orlando G, et al. Multiparameter immune profiling of operational tolerance in liver transplantation. Am J Transplant. 2007;7:309-19. ***This was the first study to demonstrate the feasibility of non-invasive identification of tolerant liver transplant recipients employing peripheral blood and gene expression profiling.*
34. Martinez-Llordella M, Lozano JJ, Puig-Pey I, et al. Using transcriptional profiling to develop a diagnostic test of operational tolerance in liver transplant recipients. J Clin Invest. 2008;118:2845-57.
35. Brouard S, Mansfield E, Braud C, et al. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. Proc Natl Acad Sci USA. 2007;104:15448-53. ***This microarray evaluation of operational tolerance in transplant recipients was one of the first studies of its kind.*
36. Louis S, Braudeau C, Giral M, et al. Contrasting CD25hiCD4+T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance. Transplantation. 2006;81:398-407.
37. Velasquez SY, Arias LF, Garcia LF, Alvarez CM. T cell receptor beta chain (TCR-Vbeta) repertoire of circulating CD4(+) CD25(-), CD4(+) CD25(low) and CD4(+) CD25(high) T cells in patients with long-term renal allograft survival. Transpl Int. 2010;23:54-63.
38. Sagoo P, Perucha E, Sawitzki B , et al. Development of a crossplatform biomarker signature to detect renal transplant tolerance in humans. J Clin Invest 2010;120(6):1848-61.
39. Newell KA, Asare A, Kirk A, et al. Identification of a B cell signature associated with renal transplant tolerance in humans. J Clin Invest 2010;120(6):1836-47.
40. Devlin J, Doherty D, Thomson L, et al. Defining the outcome of immunosuppression withdrawal after liver transplantation. Hepatology. 1998;27:926-33.
41. Tryphonopoulos P, Tzakis AG, Weppler D; et al. The role of donor bone marrow infusions in withdrawal of immunosuppression in adult liver allotransplantation. Am J Transplant. 2005;5:608-13.
42. Fine RN, Becker Y, De Geest S, et al. Nonadherence consensus conference summary report. Am J Transplant. 2009;9:35-41.