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MONITORING T CELL ALLOREACTIVITY

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Abstract

Currently, immunosuppressive therapy in kidney transplant recipients is center-specific, protocol-driven, and adjusted according to functional or histological evaluation of the allograft and/or signs of drug toxicity or infection. As a result, a large fraction of patients are likely to receive too much or too little immunosuppression, exposing them to higher rates of infection, malignancy and drug toxicity, or increased risk of acute and chronic graft injury from rejection, respectively. To help clinicians individualize a immunosuppression regimens requires the development of assays able to reliably quantify and/or predict the magnitude of the recipient's immune response towards the allograft. As alloreactive T cells are central mediators of allograft rejection, monitoring T cell alloreactivity has become a priority for the transplant community. Among available assays, flow cytometry based phenotyping, T cell proliferation, T cell cytokine secretion, and ATP release (ImmuKnow), have been the most thoroughly tested. While numerous cross-sectional studies have found associations between the results of these assays and the presence of clinically relevant post-transplantation outcomes, data from prospective studies are still scanty, thereby preventing widespread implementation in the clinic. Future studies are required to test the hypothesis that tailoring immunosuppression on the basis of results offered by these biomarkers leads to better outcomes than current standard clinical practice.

Keywords

acute rejection; biomarker; ELISPOT; ImmuKnow; MLR; Kidney transplantation

Introduction

Despite major advancements in the understanding of the alloimmune response and improvements in organ preservation and surgical techniques, life-long immunosuppression is still required to prevent acute rejection of a transplanted organ. In general, immunosuppression is titrated according to center-specific, pre-defined protocols based on clinical assessment of risk, and adjusted on the basis of immunosuppressive drug levels and allograft function. Because there is no reliable indicator for risk of acute rejection or infection, managing immunosuppression is quite challenging. Current techniques to determine risk include the use of clinical factors (age, race, history of sensitizing events,

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etc.), human leukocyte antigen (HLA) typing, and donor-specific alloantibody (DSA) screening.

In kidney transplantation, the diagnosis of acute rejection, still a major risk factor for poor long-term graft outcome [1], is defined by renal allograft biopsy, a procedure that is costly, time consuming, and carries a small, but genuine risk of complications [2]. While a rise in serum creatinine is most commonly the indication for allograft biopsy, a change in serum creatinine is a relatively insensitive marker of renal dysfunction that increases only in the late phases of injury when substantial functional loss has occurred [3]. Subclinical rejections, *i.e.* histological evidence of acute cellular rejection in the absence of changes in serum creatinine, are detectable in 3–60% kidney allograft recipients [4–6]. These subclinical rejections can negatively impact long-term graft outcomes in the absence of therapy [7]. Surveillance allograft biopsies can detect graft abnormalities when serum creatinine is still unchanged, but they are inconvenient and impractical to perform in a serial nature on all patients. By contrast, over-immunosuppression exposes patients to an increased risk of opportunistic infections and cancer, but no reliable indicator for these complications exists.

The identification of biomarkers of immune reactivity is a true priority in transplantation research [8, 9]. Immune biomarkers could identify patients at higher risk for developing acute rejection, identify transplant recipients at risk for infection and/or malignancy due to over-immunosuppression, and guide decision-making regarding the need for transplant biopsies (Figure 1). These biomarkers would guide immunosuppressant withdrawal in patients at minimal risk and would direct specific therapeutic strategies to limit injury in patients at higher risk, together resulting in prolonged graft survival and improved patient health. Moreover, some of these assays may be useful predictors not only of acute rejection, but of long-term outcomes, which are increasingly dissociated from the excellent results enjoyed in the first 3 years after transplantation [10] (Figure 1). This review provides an overview of biomarkers useful for assessing the alloreactivity of T cells, key mediators of both acute and chronic allograft injury.

T cell alloreactivity

Development of T cell alloreactivity biomarkers starts with the understanding of anti-donor T-cell immunity. There are two main pathways of HLA alloantigen recognition: the direct and the indirect pathway. The ‘direct’ pathway requires the recognition of intact donor HLA alloantigens on the surface of donor cells, whereas the indirect pathway of HLA allorecognition involves the internalization, processing, and presentation of alloantigens as peptides bound to recipient HLA molecules [11, 12]. Since professional donor antigen presenting cells (APCs) disappear after the first weeks or months after transplantation, priming by the direct pathway has classically been thought to be important for the pathogenesis of acute rejection in the early post-transplantation period [13–15]. Priming via the indirect pathway has long been considered to play a pathogenic role in late graft failure [16–18], but recent studies suggest that both pathways persist during the entire life of the graft and can contribute to the pathogenesis of chronic injury [19]. A set of assays have been developed to quantify T cell alloreactivity (Figure 2). Most assays test the direct

alloreactivity pathway, but modifications in culture conditions can be made for almost all assays to measure indirect pathway responses.

Mixed lymphocyte reaction

Measurement of the primary *in vitro* response to direct recognition of allogeneic molecules occurs in the mixed lymphocyte reaction (MLR), in which recipient T cells are tested for reactivity to donor cells. In an MLR, peripheral blood lymphocytes from two individuals are mixed together in tissue culture for several days; donor lymphocytes are inactivated, thereby allowing only the recipient lymphocytes to proliferate in response to foreign histocompatibility antigens (one-way MLR) (Table 1) [20]. This reaction was first described in the 1960s using 3Hthy incorporation as a read-out, and has been extensively used to study anti-donor T cell responses. Low levels of T cell expansion are considered a sign of over-immunosuppression, whereas high T cell expansion in response to donor antigens is read as increased risk of allograft rejection. In 19 recipients of cadaveric renal allografts, donor-specific hypo-responsiveness assessed by MLR at 3 and 6 months after transplantation was associated with a better graft outcome at 1 year [21]. However, in this conventional form, MLR is poorly reproducible and has a limited predictive value in clinical transplantation [22].

To improve the reproducibility of the 3HThy based proliferative MLR, the readout of the assay has been modified by labelling the T cells with carboxyfluorescein diacetate, succinimidyl ester (CFSE). Since CFSE is partitioned equally among daughter cells with each division, measurement of CFSE dilution by flow cytometry provides information on the rate of cell division. Titration of immunosuppression according to the results of CFSE-based MLR led to a lower incidence of acute rejection and superior graft survival at one year compared to a standard immunosuppression protocol in 115 liver transplant recipients [23]. Though details of patient allocation were not provided, this report supports further studies testing the use of CFSE-based MLR to tailor immunosuppression in organ transplant patients, including kidney transplant recipients. However, despite the fact that the assay is relatively easy and inexpensive to perform, it requires 5 days, a timeframe that makes it impractical for use in routine clinical care.

Ashokkumar *et al.* tried to overcome the issues of prolonged stimulations and blood sample requirements common in MLR assays, by testing expression of the early activation marker CD154⁺ (CD40L) by flow cytometry on recipient T cells as a measure of rejection risk [24]. This assay requires < 24 h of stimulation and only 3 mL of blood. These authors showed that CD154⁺ cytotoxic T memory cell responses before liver transplantation were associated with a significantly increased risk for rejection. This assay can be ordered as PlexImmune™ (Plexison, Pittsburgh, United States) with results in the United States available two days after obtaining blood samples. The assay requires extraction of PBMCs not only from the recipient but also the donor. When donor cells are insufficient or unavailable, “surrogate PBMCs” may be used [24], but the accuracy of the assay in this setting needs to be proven. Furthermore, data supporting the use of this assay as a tool in kidney transplantation are lacking.

ELISPOT

Among notable advances in transplantation immunology research is the recognition that a proportion of the alloreactive T cell repertoire derives from the memory pool [25], whose unique properties indicate that they may be detrimental to transplant outcome [26, 27], and consequently, measurements of memory alloimmunity could be used as biomarkers for post-transplantation outcomes [28]. Donor-reactive memory T cells are detectable by cytokine enzyme-linked immunospot (ELISPOT). This assay combines the features of the MLR with the concept of an ELISA assay (Table 1). Recipient peripheral lymphocytes are cultured in the presence of inactivated donor or third-party cells in tissue culture wells coated with a capture antibody against the cytokine of interest. The ELISPOT plates are then incubated for 18–24 h to allow responding cells to recognize alloantigens and to release cytokines, which are captured directly at their source of secretion, before they are diluted, degraded, or absorbed by receptors on nearby cell surfaces. The spot that develops represents a cell that had been primed to the stimulating antigen(s) *in vivo*. To increase reproducibility of the assay and reduce the time associated with visual counting, computerized plate readers using digital cameras have been developed. Thus, ELISPOT provides a measure of the frequency of previously activated or memory T cells that respond to specific antigens by producing a selected cytokine [29].

Pre-transplantation measurements of recipient T-cell alloreactivity to donor antigen via the IFN- γ ELISPOT assay are consistently correlated with acute rejection after kidney transplantation. The first study to evaluate this assay reported that 7 of 9 kidney transplant recipients with pre-transplantation donor-specific IFN- γ spots $\geq 25/300,000$ cells had acute rejection after transplantation, while none of 10 recipients with < 25 spots per 300,000 cells developed acute rejection [30]. In a larger cohort of 37 African-American recipients of deceased donor transplants with pre-transplantation testing, 50% of IFN- γ ELISPOT positive recipients had a biopsy-proven acute rejection versus only 17% of IFN- γ ELISPOT negative recipients [31]. Expanding on this experience, the same group reported the association between a positive pre-transplantation IFN- γ ELISPOT response and acute rejection in a separate, larger cohort of 100 kidney transplant recipients [32]. This association was independent of HLA matching, delayed graft function, donor source, ethnicity, and dialysis vintage [32]. In studies outside of the United States, Nickel *et al.* described a correlation between acute rejection and pre-transplantation IFN- γ ELISPOTs in 42 German patients [33], and others have described similar findings in Korean and Polish kidney transplant recipients [34, 35]. ELISPOT results have also been used to predict graft function. In 55 kidney transplant recipients, IFN- γ ELISPOT levels during the first 6 months after surgery correlated significantly with graft function at 6 and 12 months following transplantation [36]. Similar results were reported by a subsequent study in 23 kidney transplant recipients, showing that pre-transplantation anti-donor IFN- γ ELISPOT had a significant inverse correlation with allograft function at 6 and 12 months [37].

Given evidence suggesting that ELISPOT may quantify recipient risk for rejection, it has been used to tailor immunosuppression. Sixty kidney transplant patients were assigned a calcineurin inhibitor-based or a calcineurin inhibitor-free immunosuppressive regimen based on pre-transplantation anti-donor IFN- γ ELISPOT assay results classified as “high” vs.

“low”, respectively [38]. High ELISPOT levels were frequent even in the absence of anti-HLA antibodies and represented a major risk factor for acute rejection and graft dysfunction, despite the use of more potent immunosuppression with calcineurin inhibitors. Though further investigations are needed, this study suggests that evaluating antidonor T-cell sensitization before transplantation may help define immunosuppressive regimens on a patient-to-patient basis.

Given the appreciable rejection risk associated with greater pre-transplant ELISPOT results, this assay may be used to identify patients appropriate for antibody induction therapy. In a retrospective analysis of 130 kidney transplant patients, individuals with higher pre-transplantation ELISPOT levels were at increased risk of acute rejection. However, patients with high ELISPOT levels who received antibody induction therapy (anti-CD25 or thymoglobulin) had a significantly lower risk of acute rejection than their counterparts who did not received induction. Post-transplant conversion to a negative ELISPOT assay occurred in 86% of patients who received induction therapy vs. 35% of patients who did not [39]. A subsequent study from the same group showed that thymoglobulin induces a prolonged reduction in anti-donor IFN- γ ELISPOT response, whereas anti-CD25 depleting antibodies do not affect it [40].

The ELISPOT procedure takes 24–36 hours, which makes it unsuitable to assess the pre-transplant immunological risk in deceased donor recipients. To address this issue and the that of patients with no donor cell availability as well as to provide information on the overall T cell immunoreactivity of transplant patients, Reinke’s group identified a T-cell reactivity index (panel of reactive T-cells, or PRT) based on the frequency of positive ELISPOT responses prior to transplantation using a pool of donor antigens that may be reflective of potential organ donors [41]. Similar to the panel-reactive antibody (PRA) test for identifying individuals with elevated levels of anti-HLA antibodies, the PRT may identify patients at risk for post-transplantation cellular immune-mediated graft injury. In another study of 30 kidney transplant patients, six of the seven (86%) patients with acute rejection were PRT-positive whereas only one had low PRT before transplantation [42]. Development of beads coated with single HLA antigens able to stimulate memory T cells could allow for the assessment of pre-transplant T cell alloreactivity and identification of suitable donors, limiting acute rejection risk.

Importantly, recent work by the Fairchild group identified a subpopulation of alloreactive memory CD28⁻CD8⁺T cells are not detected by the above-noted approaches [43–47]. Addition of IL-15 to the cultures rescues the proliferating capacity of these cells, making them detectable [48]. Since renal epithelial cells are able to produce IL-15 during inflammation, quantification of these cells might be relevant in quantifying the immune risk of kidney transplant recipients. Future investigations using MLR and ELISPOT may require the addition of IL-15 to cultures to more accurately quantify the memory cell population and their contribution to graft injury.

Monitoring indirect alloreactivity

Most of the aforementioned assays are designed to evaluate the direct alloimmune response. However, evidence that indirect alloreactive CD4⁺ T-cells are the only cells that can provide help to alloreactive B-cells, leading to the formation of anti-HLA antibodies, prompted an increased interest in the development of novel assays capable of quantify this pathway [49–51]. Costs and complexity of the procedures, however, make the assessment of indirect alloresponses more challenging. Three approaches to address the indirect alloimmune response include: peptides from polymorphic regions of HLA, lysates of donor cells, and non-polymorphic peptides.

By using peptides from polymorphic regions of HLA, a cross-sectional study of 45 kidney transplant patients showed that increased IFN- γ ELISPOTs in both the direct and indirect pathways were associated with abnormal graft function [52]. This method has higher reproducibility, and other retrospective and cross-sectional studies have shown similar results [53–55], but no prospective study has been done yet. Moreover, this approach is challenged by the difficulties in creating large libraries of peptides that encompass the wide number of HLA polymorphisms.

An alternative approach is the use of donor cell lysates or fragments, which in theory should allow the testing of the full repertoire of alloantigen available. A study in mice showed that donor cell lysates generated through cell freezing and thawing were able to induce a T cell response in skin transplanted animals through the indirect pathway [56]. A similar approach was tested in humans by measuring the IFN- γ production of PBMC from kidney transplant patients with or without chronic rejection in response to donor cells undergone through multiple cycles of freezing and thawing [16]. Indirect response alloreactivity was significantly lower in stable patients compared to those with chronic rejection, a difference that was not captured by evaluating the direct pathway only. In another cross-sectional study of 34 longstanding living donor renal transplant recipients, indirect IFN- γ ELISPOT response against cell fragments correlated with proteinuria, a major predictor of graft failure [19]. However, to date, no prospective studies have been done to validate this approach.

Alloimmune responses are frequently associated with intramolecular epitope spreading, and in experimental models, this includes responses to cryptic self-epitopes [57, 58]. In support of this concept, a cross-sectional study in 110 kidney transplant recipients showed that IFN- γ production by PBMC stimulated with nonpolymorphic HLA-derived peptides was associated with chronic rejection [59]. The assessment of responses to such peptides might, therefore, be used to provide evidence of alloimmunization without the need to account for donor or recipient HLA type, because the response to “cryptic self-epitopes” could equally meet these criteria [60, 61]. As with the other approaches above, however, prospective studies are lacking, and will be required to validate the utility of these assays in the prediction of allograft outcomes.

The problem of standardization

While ELISPOT assays are promising, the complexities of these multistep assays make them difficult to standardize. Standardization is essential, however, in order to implement them clinically. Two large investigative transplant medicine groups have investigated ways to standardize ELISPOT for everyday use. Lin *et al.* established cellular reagent standards isolated from blood bank buffy coats and 8 transplant recipients from the Clinical Trials of Organ Transplantation (CTOT) study. When standardized cellular reagents were supplied to participating labs that then used their own protocols, ELISPOT results were strikingly different [62]. A standard operating procedure (SOP), normalizing variables such as cell thawing, cell plating, assay procedures, and reagents, was tested at the central laboratory, and successfully normalized results among assays. When tested amongst participating labs, a correlation coefficient of 0.7 was achieved. If analysis of completed assays was performed by the central lab, the correlation increased to 0.851. Analysis of stored CTOT transplant recipient samples achieved a similar degree of concordance to the standardized cellular reagents, demonstrating clinical relevance of the SOP. A similar endeavor was undertaken by investigators and laboratories involved in the European Union's RISE (Reprogramming the Immune System for Establishment of Tolerance) network. After the introduction of an SOP for processing and analysis of samples, a low coefficient of variance (<30%) was calculated for results of assays performed at any one of three participating centers [63]. Strong concordance of results was seen in a group of 8 samples from kidney transplant recipients with evidence of acute rejection. Taken together, these investigations suggest that standardization and validation of ELISPOT assays is possible between large institutions and can faithfully identify highly alloreactive patients. Furthermore, these data support the initiation of prospective interventional studies to test the utility of ELISPOT in tailoring immunosuppression in kidney transplant patients. If results from these trials support the clinical utility of ELISPOT, creative industry collaborations and reimbursement strategies will be important to bring these tests to the clinic, considering the relatively high cost, assay complexity and labor intensity required for accurate performance.

Characterizing Regulatory T cells

Regulatory T cells (Treg) consist of a heterogeneous population of T cells with the ability to suppress immune responses. There are two main types of Treg: thymus-derived CD4⁺CD25⁺FoxP3⁺ natural Treg (nTreg) and adaptive or inducible Treg (iTreg) that develop from naive T cells in the periphery under tolerogenic conditions [64]. It has long been established that FoxP3 is the major transcription factor that determines the fate, identity, and function of Treg, and Treg regulate immune functions by producing cytokines such as TGF- β , IL-10, and IL-35 [65, 66]. However, there are subsets of Treg that do not express Foxp3. For example, TGF- β -producing Th3 and IL-10-secreting Tr1 Treg, which do not express Foxp3, can be potent suppressors in some experimental systems.

Given the vast evidence demonstrating the contribution of Tregs regulating immune responses in different animal models, great hopes have flourished on the potential use of Tregs as markers of tolerance, transplant rejection, or prediction of graft outcomes. However, while murine studies consistently showed a relationship between increased

numbers of FoxP3⁺ Tregs and tolerance [67], the induction of CD25 and FoxP3 upon activation of human Teffs has led to far more variable clinical data [68]. Findings of reduced numbers of Tregs in patients with acute or chronic rejection have not been consistent [69–75], nor have findings of increased numbers of Tregs in tolerant recipients [69, 76–78]. Despite the growing list of markers useful for identifying human Tregs, none is able to clearly identify T cells with suppressive capacity [79]. Thus, pure enumeration of immunophenotypic Tregs needs to be accompanied by an assessment of Treg suppressive function. Using autologous cells as responders in Treg assays of kidney transplant patients can be misleading, since immunosuppression can decrease their proliferative capacity, which may explain why Tregs from patients with rejection had impaired suppressive function in some studies but not others [72, 74, 75, 80, 81]. To address this issue, Akimova *et al.* developed a standardized suppression assay where CD25, CD127, CTLA4 and FoxP3⁺ Treg function was tested against T effector (Teff) cells from healthy donors, so as to ensure that the suppression assays reflected effects of immunosuppression on Treg alone and not conventional Teff cells. By using this strategy, they found that patients with tacrolimus levels >3.6 ng/mL had weaker Treg function than those with levels <3.6 ng/mL, whereas rapamycin therapy positively correlated with Treg numbers and their expression of CTLA4 [82]. Whether this new strategy to quantify Treg activity correlates with the risk of developing acute rejection is still unknown. Following a similar approach to an MLR, Canavan *et al.* developed a 7-hour flow-cytometric assay to assess Treg suppressive function, by measuring CD154 and CD69 expression on T effector cells in a suppression assay [83]. Expression levels of these markers correlated excellently with gold-standard assays involving inhibition of CFSE dilution and cytokine production. This test could allow rapid evaluation of Treg suppressive function in clinical trials of cell therapy, enabling the translation of the large body of preclinical data from bench to bedside. Analysis of the epigenetic status (*e.g.* degree of methylation) of FoxP3 can also provide helpful information on the suppressive function of Tregs. An additional way to characterize the suppressive function of Tregs is to measure the ratio between T effector/Treg. Lower values have been associated with better graft outcomes [84].

Altogether, these assays provide a new approach to monitor the immune status of transplant patients. However, no existing evidence suggests that measuring Treg activity can predict the risk of future acute/chronic rejection or infections. Similarly, conflicting evidence exists regarding an increased Treg presence in transplant patients with cancer [85]. The entire picture may be complicated by evidence that increased Tregs associated with rapamycin are associated with higher risk of acute and chronic rejection and lower risk of tumors compared to cyclosporine, possibly secondary to differential effects of the two drugs on effector T and actively proliferating tumor cells [86, 87]. *Ad hoc* prospective studies are therefore needed to validate the utility of Treg characterization in the management of transplant patients.

ImmuKnow

ImmuKnow (Cylex Ltd, United States) was developed as a biomarker to guide immunosuppressant drug dosing following solid organ transplantation and was approved by the United States Food and Drug Administration (FDA) for clinical use in 2002. ImmuKnow measures adenosine triphosphate produced after stimulation of T-cells with a non-specific

stimulus, plant lectin phytohemagglutinin (PHA) mitogen [88]. In an effort to simulate *in vivo* conditions, whole blood is used to ensure that immunosuppressive drugs are maintained during cell stimulation. After overnight incubation, CD4 T cells are isolated and ATP consumption is measured [88] (Table 1).

Initial reports showed that ImmuKnow values decrease in the setting of active, clinically apparent infection, and increase after recovery [89–91]. However, these studies measured the ImmuKnow values at the time of disease rather than before its onset, which prevented any analysis on the predictive value of the assay. Other investigators tried to address this issue and reported that low ImmuKnow values are associated with an increased incidence of infections in stable transplant recipients on maintenance immunosuppression [89, 92–95]. Yet, these studies were small with relatively few infection episodes and short follow-up periods. A subsequent large, retrospective analysis of 1,330 ImmuKnow values in 583 adult renal transplant recipients at a single center failed to detect any association between ImmuKnow levels and future development of opportunistic infections or acute rejections in the following 3 months [96]. Similarly, a small retrospective study of 31 pediatric kidney transplant recipients failed to show an association between single time point ImmuKnow assay values and the subsequent development of an adverse event in the subsequent 90 days [97]. A meta-analysis reached a similar conclusion challenging the power of ImmuKnow to predict the occurrence of opportunistic infections or acute rejections in solid organ transplantation, and also provided statistical evidence of publication bias related to the under-reporting of studies showing low ImmuKnow predictive capacity [98].

Recently, a prospective cohort study measured longitudinal changes in ImmuKnow levels in 55 kidney transplant recipients over 3-year follow-up (534 visits) and analyzed their relationship with the risk of developing infections or acute rejection [99]. ImmuKnow levels were transiently up-regulated during the first 3 months after kidney transplantation with a tendency to become stable below baseline values during the following 3 years. No association was found between increased ImmuKnow levels and the risk of developing acute rejection, *de novo* donor-specific antibodies (DSA), or infections. Similar results were found by another prospective study in 67 kidney transplant patients, where absolute ATP values postoperatively had poor predictive value with regard to rejection or infection/malignancy. However, patients with pre-transplant ATP values <300 ng/mL had significantly less rejection episodes vs. those with ATP values >300 ng/mL [100].

Overall, while the assay appears to be highly reproducible, with a rapid turnaround time and a relatively low cost, its clinical utility is limited. A single value may be difficult to interpret, particularly after induction with lymphocyte-depleting therapy, where the number of CD4⁺ T cells is severely reduced. Moreover, the impact of homeostatic proliferation on the level of ATP produced has not been documented. A possible explanation for the poor sensitivity of ImmuKnow in detecting rejection may be that it relies on the nonspecific PHA mitogen. Changes in the assay protocol that may include specific stimulation with donor antigens could improve its sensitivity and specificity in early detection of acute rejections or infections, but, in its present form, it cannot be recommended to be implemented in routine clinical practice.

Conclusions and Future Directions

The development of noninvasive, accurate, and reliable assays to monitor alloreactivity in kidney transplant recipients could potentially lead to a new treatment paradigm in transplantation, with a conceivably safer, more tolerable, and cost-effective approach to immunosuppression. Over the last decade, many assays have been developed to noninvasively quantify the level of T cell alloreactivity in transplant patients (Table 1). While some of these assays have been validated prospectively, no studies have formally tested whether or not these assays can be used as an alternative to, or in combination with an allograft biopsy to improve kidney transplant outcomes and to reduce the morbidity and mortality associated with over- and under-immunosuppression. To this end, future randomized clinical trials should assess whether titrating immunosuppressive therapy according to the results of one or some combination of these assays provides better outcomes than the conventional approach based on serum creatinine levels, allograft biopsies and immunosuppressive drug levels. It is unlikely that a single immune-monitoring assay will provide adequate information representative of the complex pathophysiology of an individual's alloresponse. To overcome this major limitation, multiple tools could be combined to measure the immune response from different perspectives, including not only T cell alloreactivity, but also antibody profile and gene-expression pattern. A systems approach, where the focus is moved from analysis of individual cell types towards more integrated studies of the entire immune system, has been also proposed as a way to better measure alloreactivity and to personalize immunosuppressive therapy [101].

The task of translating immune-monitoring assays into day-to-day clinical practice is challenging, but will be the key to individualized immunosuppression in the future. Notably, the relevance of these assays extends beyond transplantation, since they could provide vital information also pertinent for the management of patients with autoimmune disease.

References

1. Tantravahi J, Womer KL, Kaplan B. Why hasn't eliminating acute rejection improved graft survival? *Annu Rev Med* 2007;58:369–385. [PubMed: 17002551]
2. Schwarz A, Gwinner W, Hiss M, Radermacher J, Mengel M, Haller H. Safety and adequacy of renal transplant protocol biopsies. *Am J Transplant* 2005;5:1992–1996. [PubMed: 15996250]
3. Mannon RB, Kirk AD. Beyond histology: novel tools to diagnose allograft dysfunction. *Clin J Am Soc Nephrol* 2006;1:358–366. [PubMed: 17699232]
4. Rush D Protocol transplant biopsies: an underutilized tool in kidney transplantation. *Clin J Am Soc Nephrol* 2006;1:138–143. [PubMed: 17699200]
5. Gloor JM, Cohen AJ, Lager DJ, Grande JP, Fidler ME, Velosa JA, et al. Subclinical rejection in tacrolimus-treated renal transplant recipients. *Transplantation* 2002;73:1965–1968. [PubMed: 12131699]
6. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. Natural history, risk factors, and impact of subclinical rejection in kidney transplantation. *Transplantation* 2004;78:242–249. [PubMed: 15280685]
7. Rush D, Winnipeg Transplant G. Insights into subclinical rejection. *Transplant Proc* 2004;36:71S–73S. [PubMed: 15041310]
8. Biomarkers Definitions Working G. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther* 2001;69:89–95. [PubMed: 11240971]

9. Cravedi P, Heeger PS. Immunologic monitoring in transplantation revisited. *Curr Opin Organ Transplant* 2012,17:26–32. [PubMed: 22186089]
10. Lodhi SA, Lamb KE, Meier-Kriesche HU. Solid organ allograft survival improvement in the United States: the long-term does not mirror the dramatic short-term success. *Am J Transplant* 2011,11:1226–1235. [PubMed: 21564524]
11. Lechler RI, Lombardi G, Batchelor JR, Reinsmoen N, Bach FH. The molecular basis of alloreactivity. *Immunol Today* 1990,11:83–88. [PubMed: 2186745]
12. Shoskes DA, Wood KJ. Indirect presentation of MHC antigens in transplantation. *Immunol Today* 1994,15:32–38. [PubMed: 8136009]
13. Lechler RI, Batchelor JR. Immunogenicity of retransplanted rat kidney allografts. Effect of inducing chimerism in the first recipient and quantitative studies on immunosuppression of the second recipient. *J Exp Med* 1982,156:1835–1841. [PubMed: 6757374]
14. Lechler RI, Batchelor JR. Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. *J Exp Med* 1982,155:31–41. [PubMed: 7033437]
15. van Besouw NM, Zijderwijk JM, Vaessen LM, Balk AH, Maat AP, van der Meide PH, et al. The direct and indirect allogeneic presentation pathway during acute rejection after human cardiac transplantation. *Clin Exp Immunol* 2005,141:534–540. [PubMed: 16045744]
16. Baker RJ, Hernandez-Fuentes MP, Brookes PA, Chaudhry AN, Cook HT, Lechler RI. Loss of direct and maintenance of indirect alloresponses in renal allograft recipients: implications for the pathogenesis of chronic allograft nephropathy. *J Immunol* 2001,167:7199–7206. [PubMed: 11739543]
17. Vella JP, Spadafora-Ferreira M, Murphy B, Alexander SI, Harmon W, Carpenter CB, et al. Indirect allorecognition of major histocompatibility complex allopeptides in human renal transplant recipients with chronic graft dysfunction. *Transplantation* 1997,64:795–800. [PubMed: 9326400]
18. Sayegh MH, Carpenter CB. Role of indirect allorecognition in allograft rejection. *Int Rev Immunol* 1996,13:221–229. [PubMed: 8782743]
19. Bestard O, Nickel P, Cruzado JM, Schoenemann C, Boenisch O, Sefrin A, et al. Circulating alloreactive T cells correlate with graft function in longstanding renal transplant recipients. *J Am Soc Nephrol* 2008,19:1419–1429. [PubMed: 18417724]
20. Thomas FT, Lee HM, Lower RR, Thomas JM. Immunological monitoring as a guide to the management of transplant recipients. *Surg Clin North Am* 1979,59:253–281. [PubMed: 155890]
21. Ghobrial II, Morris AG, Booth LJ. Clinical significance of in vitro donor-specific hyporesponsiveness in renal allograft recipients as demonstrated by the MLR. *Transpl Int* 1994,7:420–427. [PubMed: 7865106]
22. Hernandez-Fuentes MP, Warrens AN, Lechler RI. Immunologic monitoring. *Immunol Rev* 2003,196:247–264. [PubMed: 14617209]
23. Tanaka Y, Tashiro H, Onoe T, Ide K, Ishiyama K, Ohdan H. Optimization of immunosuppressive therapy based on a multiparametric mixed lymphocyte reaction assay reduces infectious complications and mortality in living donor liver transplant recipients. *Transplant Proc* 2012,44:555–559. [PubMed: 22410068]
24. Ashokkumar C, Talukdar A, Sun Q, Higgs BW, Janosky J, Wilson P, et al. Allospecific CD154+ T cells associate with rejection risk after pediatric liver transplantation. *Am J Transplant* 2009,9:179–191. [PubMed: 18976293]
25. Page AJ, Ford ML, Kirk AD. Memory T-cell-specific therapeutics in organ transplantation. *Curr Opin Organ Transplant* 2009,14:643–649. [PubMed: 19779342]
26. Woodland DL, Kohlmeier JE. Migration, maintenance and recall of memory T cells in peripheral tissues. *Nat Rev Immunol* 2009,9:153–161. [PubMed: 19240755]
27. Pearl JP, Parris J, Hale DA, Hoffmann SC, Bernstein WB, McCoy KL, et al. Immunocompetent T-cells with a memory-like phenotype are the dominant cell type following antibody-mediated T-cell depletion. *Am J Transplant* 2005,5:465–474. [PubMed: 15707400]
28. Dinavahi R, Heeger PS. T-cell immune monitoring in organ transplantation. *Curr Opin Organ Transplant* 2008,13:419–424. [PubMed: 18685339]

29. Mashishi T, Gray CM. The ELISPOT assay: an easily transferable method for measuring cellular responses and identifying T cell epitopes. *Clin Chem Lab Med* 2002;40:903–910. [PubMed: 12435107]
30. Heeger PS, Greenspan NS, Kuhlenschmidt S, DeJelo C, Hricik DE, Schulak JA, et al. Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. *J Immunol* 1999;163:2267–2275. [PubMed: 10438971]
31. Augustine JJ, Siu DS, Clemente MJ, Schulak JA, Heeger PS, Hricik DE. Pre-transplant IFN-gamma ELISPOTs are associated with post-transplant renal function in African American renal transplant recipients. *Am J Transplant* 2005;5:1971–1975. [PubMed: 15996247]
32. Augustine JJ, Poggio ED, Clemente M, Aeder MI, Bodziak KA, Schulak JA, et al. Hemodialysis vintage, black ethnicity, and pretransplantation antidonor cellular immunity in kidney transplant recipients. *J Am Soc Nephrol* 2007;18:1602–1606. [PubMed: 17389735]
33. Nickel P, Presber F, Bold G, Biti D, Schonemann C, Tullius SG, et al. Enzyme-linked immunosorbent spot assay for donor-reactive interferon-gamma-producing cells identifies T-cell presensitization and correlates with graft function at 6 and 12 months in renal-transplant recipients. *Transplantation* 2004;78:1640–1646. [PubMed: 15591953]
34. Kim SH, Oh EJ, Kim MJ, Park YJ, Han K, Yang HJ, et al. Pretransplant donor-specific interferon-gamma ELISPOT assay predicts acute rejection episodes in renal transplant recipients. *Transplant Proc* 2007;39:3057–3060. [PubMed: 18089321]
35. Koscielska-Kasprzak K, Drulis-Fajdasz D, Kaminska D, Mazanowska O, Krajewska M, Gdowska W, et al. Pretransplantation cellular alloreactivity is predictive of acute graft rejection and 1-year graft function in kidney transplant recipients. *Transplant Proc* 2009;41:3006–3008. [PubMed: 19857662]
36. Hricik DE, Rodriguez V, Riley J, Bryan K, Tary-Lehmann M, Greenspan N, et al. Enzyme linked immunosorbent spot (ELISPOT) assay for interferon-gamma independently predicts renal function in kidney transplant recipients. *Am J Transplant* 2003;3:878–884. [PubMed: 12814480]
37. Nather BJ, Nickel P, Bold G, Presber F, Schonemann C, Pratschke J, et al. Modified ELISPOT technique--highly significant inverse correlation of post-Tx donor-reactive IFN-gamma-producing cell frequencies with 6 and 12 months graft function in kidney transplant recipients. *Transpl Immunol* 2006;16:232–237. [PubMed: 17138059]
38. Bestard O, Cruzado JM, Lucia M, Crespo E, Casis L, Sawitzki B, et al. Prospective assessment of antidonor cellular alloreactivity is a tool for guidance of immunosuppression in kidney transplantation. *Kidney Int* 2013;84:1226–1236. [PubMed: 23783240]
39. Augustine JJ, Poggio ED, Heeger PS, Hricik DE. Preferential benefit of antibody induction therapy in kidney recipients with high pretransplant frequencies of donor-reactive interferon-gamma enzyme-linked immunosorbent spots. *Transplantation* 2008;86:529–534. [PubMed: 18724221]
40. Cherkassky L, Lanning M, Lalli PN, Czern J, Siegel H, Danziger-Isakov L, et al. Evaluation of alloreactivity in kidney transplant recipients treated with antithymocyte globulin versus IL-2 receptor blocker. *Am J Transplant* 2011;11:1388–1396. [PubMed: 21564525]
41. Andree H, Nickel P, Nasiadko C, Hammer MH, Schonemann C, Pruss A, et al. Identification of dialysis patients with panel-reactive memory T cells before kidney transplantation using an allogeneic cell bank. *J Am Soc Nephrol* 2006;17:573–580. [PubMed: 16396965]
42. Poggio ED, Augustine JJ, Clemente M, Danzig JM, Volokh N, Zand MS, et al. Pretransplant cellular alloimmunity as assessed by a panel of reactive T cells assay correlates with acute renal graft rejection. *Transplantation* 2007;83:847–852. [PubMed: 17460554]
43. Pawlik A, Florczak M, Masiuk M, Dutkiewicz G, Machalinski B, Rozanski J, et al. The expansion of CD4+CD28- T cells in patients with chronic kidney graft rejection. *Transplant Proc* 2003;35:2902–2904. [PubMed: 14697933]
44. Azuma M, Phillips JH, Lanier LL. CD28- T lymphocytes. Antigenic and functional properties. *J Immunol* 1993;150:1147–1159. [PubMed: 8381831]
45. Brzezinska A, Magalska A, Szybinska A, Sikora E. Proliferation and apoptosis of human CD8(+)CD28(+) and CD8(+)CD28(-) lymphocytes during aging. *Exp Gerontol* 2004;39:539–544. [PubMed: 15050288]

46. Scheuring UJ, Sabzevari H, Theofilopoulos AN. Proliferative arrest and cell cycle regulation in CD8(+)CD28(-) versus CD8(+)CD28(+) T cells. *Hum Immunol* 2002;63:1000–1009. [PubMed: 12392852]
47. Tortorella C, Pisconti A, Piazzolla G, Antonaci S. APC-dependent impairment of T cell proliferation in aging: role of CD28- and IL-12/IL-15-mediated signaling. *Mech Ageing Dev* 2002;123:1389–1402. [PubMed: 12297341]
48. Traitanon O, Gorbachev A, Bechtel JJ, Keslar KS, Baldwin WM 3rd, Poggio ED, et al. IL-15 induces alloreactive CD28(-) memory CD8 T cell proliferation and CTLA4-Ig resistant memory CD8 T cell activation. *Am J Transplant* 2014;14:1277–1289. [PubMed: 24842641]
49. Taylor AL, Negus SL, Negus M, Bolton EM, Bradley JA, Pettigrew GJ. Pathways of helper CD4 T cell allorecognition in generating alloantibody and CD8 T cell alloimmunity. *Transplantation* 2007;83:931–937. [PubMed: 17460565]
50. Conlon TM, Saeb-Parsy K, Cole JL, Motallebzadeh R, Qureshi MS, Rehakova S, et al. Germinal center alloantibody responses are mediated exclusively by indirect-pathway CD4 T follicular helper cells. *J Immunol* 2012;188:2643–2652. [PubMed: 22323543]
51. Steele DJ, Laufer TM, Smiley ST, Ando Y, Grusby MJ, Glimcher LH, et al. Two levels of help for B cell alloantibody production. *J Exp Med* 1996;183:699–703. [PubMed: 8627185]
52. Poggio ED, Clemente M, Riley J, Roddy M, Greenspan NS, DeJelo C, et al. Alloreactivity in renal transplant recipients with and without chronic allograft nephropathy. *J Am Soc Nephrol* 2004;15:1952–1960. [PubMed: 15213286]
53. Illigens BM, Yamada A, Anosova N, Dong VM, Sayegh MH, Benichou G. Dual effects of the alloresponse by Th1 and Th2 cells on acute and chronic rejection of allotransplants. *Eur J Immunol* 2009;39:3000–3009. [PubMed: 19658090]
54. Reznik SI, Jaramillo A, SivaSai KS, Womer KL, Sayegh MH, Trulock EP, et al. Indirect allorecognition of mismatched donor HLA class II peptides in lung transplant recipients with bronchiolitis obliterans syndrome. *Am J Transplant* 2001;1:228–235. [PubMed: 12102256]
55. Stegmann S, Muller A, Zavazava N. Synthetic HLA-A2 derived peptides are recognized and presented in renal graft recipients. *Hum Immunol* 2000;61:1363–1369. [PubMed: 11163094]
56. Benichou G, Valujskikh A, Heeger PS. Contributions of direct and indirect T cell alloreactivity during allograft rejection in mice. *J Immunol* 1999;162:352–358. [PubMed: 9886406]
57. Ciubotariu R, Liu Z, Colovai AI, Ho E, Itescu S, Ravalli S, et al. Persistent allopeptide reactivity and epitope spreading in chronic rejection of organ allografts. *J Clin Invest* 1998;101:398–405. [PubMed: 9435312]
58. Womer KL, Stone JR, Murphy B, Chandraker A, Sayegh MH. Indirect allorecognition of donor class I and II major histocompatibility complex peptides promotes the development of transplant vasculopathy. *J Am Soc Nephrol* 2001;12:2500–2506. [PubMed: 11675428]
59. Smith HJ, Hanvesakul R, Bentall A, Shabir S, Morgan MD, Briggs D, et al. T Lymphocyte responses to nonpolymorphic HLA-derived peptides are associated with chronic renal allograft dysfunction. *Transplantation* 2011;91:279–286. [PubMed: 21183869]
60. Boisgerault F, Anosova NG, Tam RC, Illigens BM, Fedoseyeva EV, Benichou G. Induction of T-cell response to cryptic MHC determinants during allograft rejection. *Hum Immunol* 2000;61:1352–1362. [PubMed: 11163093]
61. Lovegrove E, Pettigrew GJ, Bolton EM, Bradley JA. Epitope mapping of the indirect T cell response to allogeneic class I MHC: sequences shared by donor and recipient MHC may prime T cells that provide help for alloantibody production. *J Immunol* 2001;167:4338–4344. [PubMed: 11591757]
62. Ashoor I, Najafian N, Korin Y, Reed EF, Mohanakumar T, Ikle D, et al. Standardization and cross validation of alloreactive IFN-gamma ELISPOT assays within the clinical trials in organ transplantation consortium. *Am J Transplant* 2013;13:1871–1879. [PubMed: 23710568]
63. Bestard O, Crespo E, Stein M, Lucia M, Roelen DL, de Vaal YJ, et al. Cross-validation of IFN-gamma Elispot assay for measuring alloreactive memory/effector T cell responses in renal transplant recipients. *Am J Transplant* 2013;13:1880–1890. [PubMed: 23763435]
64. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. *Immunity* 2009;30:616–625. [PubMed: 19464984]

65. Maynard CL, Harrington LE, Janowski KM, Oliver JR, Zindl CL, Rudensky AY, et al. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. *Nat Immunol* 2007;8:931–941. [PubMed: 17694059]
66. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 2007;450:566–569. [PubMed: 18033300]
67. Joffre O, Santolaria T, Calise D, Al Saati T, Hudrisier D, Romagnoli P, et al. Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes. *Nat Med* 2008;14:88–92. [PubMed: 18066074]
68. Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* 2007;37:129–138. [PubMed: 17154262]
69. Louis S, Braudeau C, Giral M, Dupont A, Moizant F, Robillard N, et al. Contrasting CD25hiCD4+T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance. *Transplantation* 2006;81:398–407. [PubMed: 16477227]
70. He Q, Fan H, Li JQ, Qi HZ. Decreased circulating CD4+CD25highFoxp3+ T cells during acute rejection in liver transplant patients. *Transplant Proc* 2011;43:1696–1700. [PubMed: 21693260]
71. Perrella O, Sbreglia C, Arenga G, Perrella A, Ferrara A, D'Antonio A, et al. Acute rejection after liver transplantation: Is there a specific immunological pattern? *Transplant Proc* 2006;38:3594–3596. [PubMed: 17175341]
72. Demirkiran A, Kok A, Kwekkeboom J, Kusters JG, Metselaar HJ, Tilanus HW, et al. Low circulating regulatory T-cell levels after acute rejection in liver transplantation. *Liver Transpl* 2006;12:277–284. [PubMed: 16447185]
73. Ashton-Chess J, Dugast E, Colvin RB, Giral M, Foucher Y, Moreau A, et al. Regulatory, effector, and cytotoxic T cell profiles in long-term kidney transplant patients. *J Am Soc Nephrol* 2009;20:1113–1122. [PubMed: 19357258]
74. Dijke IE, Korevaar SS, Caliskan K, Balk AH, Maat AP, Weimar W, et al. Inadequate immune regulatory function of CD4+CD25bright+FoxP3+ T cells in heart transplant patients who experience acute cellular rejection. *Transplantation* 2009;87:1191–1200. [PubMed: 19384166]
75. Akl A, Jones ND, Rogers N, Bakr MA, Mostafa A, El Shehawy el M, et al. An investigation to assess the potential of CD25highCD4+ T cells to regulate responses to donor alloantigens in clinically stable renal transplant recipients. *Transpl Int* 2008;21:65–73. [PubMed: 17887959]
76. Koshiba T, Li Y, Takemura M, Wu Y, Sakaguchi S, Minato N, et al. Clinical, immunological, and pathological aspects of operational tolerance after pediatric living-donor liver transplantation. *Transpl Immunol* 2007;17:94–97. [PubMed: 17306739]
77. Martinez-Llordella M, Puig-Pey I, Orlando G, Ramoni M, Tisone G, Rimola A, et al. Multiparameter immune profiling of operational tolerance in liver transplantation. *Am J Transplant* 2007;7:309–319. [PubMed: 17241111]
78. Martinez-Llordella M, Lozano JJ, Puig-Pey I, Orlando G, Tisone G, Lerut J, et al. Using transcriptional profiling to develop a diagnostic test of operational tolerance in liver transplant recipients. *J Clin Invest* 2008;118:2845–2857. [PubMed: 18654667]
79. Chen X, Oppenheim JJ. Resolving the identity myth: key markers of functional CD4+FoxP3+ regulatory T cells. *Int Immunopharmacol* 2011;11:1489–1496. [PubMed: 21635972]
80. Braudeau C, Racape M, Giral M, Louis S, Moreau A, Berthelot L, et al. Variation in numbers of CD4+CD25highFOXP3+ T cells with normal immuno-regulatory properties in long-term graft outcome. *Transpl Int* 2007;20:845–855. [PubMed: 17854443]
81. Kreijveld E, Koenen HJ, van Cranenbroek B, van Rijssen E, Joosten I, Hilbrands LB. Immunological monitoring of renal transplant recipients to predict acute allograft rejection following the discontinuation of tacrolimus. *PLoS One* 2008;3:e2711. [PubMed: 18628993]
82. Akimova T, Kamath BM, Goebel JW, Meyers KE, Rand EB, Hawkins A, et al. Differing effects of rapamycin or calcineurin inhibitor on T-regulatory cells in pediatric liver and kidney transplant recipients. *Am J Transplant* 2012;12:3449–3461. [PubMed: 22994804]
83. Canavan JB, Afzali B, Scotta C, Fazekasova H, Edozie FC, Macdonald TT, et al. A rapid diagnostic test for human regulatory T-cell function to enable regulatory T-cell therapy. *Blood* 2012;119:e57–66. [PubMed: 22219224]

84. Vallotton L, Hadaya K, Venetz JP, Buehler LH, Ciuffreda D, Nseir G, et al. Monitoring of CD4+CD25highIL-7Ralphahigh activated T cells in kidney transplant recipients. *Clin J Am Soc Nephrol* 2011;6:2025–2033. [PubMed: 21757642]
85. Hope CM, Grace BS, Pilkington KR, Coates PT, Bergmann IP, Carroll RP. The immune phenotype may relate to cancer development in kidney transplant recipients. *Kidney Int* 2014;86:175–183. [PubMed: 24429406]
86. Ruggenenti P, Perico N, Gotti E, Cravedi P, D'Agati V, Gagliardini E, et al. Sirolimus versus cyclosporine therapy increases circulating regulatory T cells, but does not protect renal transplant patients given alemtuzumab induction from chronic allograft injury. *Transplantation* 2007;84:956–964. [PubMed: 17989600]
87. Mathew T, Kreis H, Friend P. Two-year incidence of malignancy in sirolimus-treated renal transplant recipients: results from five multicenter studies. *Clin Transplant* 2004;18:446–449. [PubMed: 15233824]
88. Kowalski R, Post D, Schneider MC, Britz J, Thomas J, Deierhoi M, et al. Immune cell function testing: an adjunct to therapeutic drug monitoring in transplant patient management. *Clin Transplant* 2003;17:77–88. [PubMed: 12709071]
89. Kowalski RJ, Post DR, Mannon RB, Sebastian A, Wright HI, Sigle G, et al. Assessing relative risks of infection and rejection: a meta-analysis using an immune function assay. *Transplantation* 2006;82:663–668. [PubMed: 16969290]
90. Borade SM, Janata K, Vigneswaran WT, Alex CG, Garrity ER. Cylex ImmuKnow assay levels are lower in lung transplant recipients with infection. *J Heart Lung Transplant* 2008;27:990–994. [PubMed: 18765191]
91. Kobashigawa JA, Kiyosaki KK, Patel JK, Kittleson MM, Kubak BM, Davis SN, et al. Benefit of immune monitoring in heart transplant patients using ATP production in activated lymphocytes. *J Heart Lung Transplant* 2010;29:504–508. [PubMed: 20133166]
92. Millan O, Sanchez-Fueyo A, Rimola A, Guillen D, Hidalgo S, Benitez C, et al. Is the intracellular ATP concentration of CD4+ T-Cells a predictive biomarker of immune status in stable transplant recipients? *Transplantation* 2009;88:S78–84. [PubMed: 19667966]
93. Sanchez-Velasco P, Rodrigo E, Valero R, Ruiz JC, Fernandez-Fresnedo G, Lopez-Hoyos M, et al. Intracellular ATP concentrations of CD4 cells in kidney transplant patients with and without infection. *Clin Transplant* 2008;22:55–60. [PubMed: 18217906]
94. Perez-Flores I, Sanchez-Fructuoso A, Santiago JL, Fernandez-Arquero M, Calvo N, de la Concha EG, et al. Intracellular ATP levels in CD4+ lymphocytes are a risk marker of rejection and infection in renal graft recipients. *Transplant Proc* 2009;41:2106–2108. [PubMed: 19715845]
95. Nobuoka Y, Mizuno S, Nishikawa K, Nakatani K, Muraki Y, Yamada T, et al. Immune response following liver transplantation compared to kidney transplantation: usefulness of monitoring peripheral blood CD4+ adenosine triphosphate activity and cytochrome P450 3A5 genotype assay. *Clin Dev Immunol* 2013;2013:936063. [PubMed: 24454479]
96. Huskey J, Gralla J, Wiseman AC. Single time point immune function assay (ImmuKnow) testing does not aid in the prediction of future opportunistic infections or acute rejection. *Clin J Am Soc Nephrol* 2011;6:423–429. [PubMed: 21088287]
97. Ryan CM, Chaudhuri A, Concepcion W, Grimm PC. Immune cell function assay does not identify biopsy-proven pediatric renal allograft rejection or infection. *Pediatr Transplant* 2014;18:446–452. [PubMed: 24930482]
98. Ling X, Xiong J, Liang W, Schroder PM, Wu L, Ju W, et al. Can immune cell function assay identify patients at risk of infection or rejection? A meta-analysis. *Transplantation* 2012;93:737–743. [PubMed: 22357178]
99. Libri I, Gnappi E, Zanelli P, Reina M, Giuliadori S, Vaglio A, et al. Trends in immune cell function assay and donor-specific HLA antibodies in kidney transplantation: A 3-year prospective study. *Am J Transplant* 2013;13:3215–3222. [PubMed: 24266972]
100. Myslik F, House AA, Yanko D, Warren J, Caumartin Y, Rehman F, et al. Preoperative Cylex assay predicts rejection risk in patients with kidney transplant. *Clin Transplant* 2014;28:606–610. [PubMed: 24628326]

101. Brodin P, Valentini D, Uhlin M, Mattsson J, Zumla A, Maeurer MJ. Systems level immune response analysis and personalized medicine. *Expert Rev Clin Immunol* 2013,9:307–317. [PubMed: 23557267]

Figure 1.

The Role of T cell alloreactivity biomarkers in patient management: biomarkers of T cell alloreactivity provide clinicians with information in both the pre- and post-transplantation periods to target immunosuppression and prevent iatrogenic complications and acute rejection.

Figure 2.

Biomarkers of T cell alloreactivity: common assays measuring the magnitude of direct or indirect activation of recipient T cells include the mixed lymphocyte reaction (left), cytokine ELISPOT (center), and detection of activation markers (right). In a mixed lymphocyte reaction (MLR) stimulated T cells are labeled with a fluorescent dye (left). The degree of fluorescence dilution is directly related to the extent of T cell proliferation. ELISPOT assays (center) use plate-bound antibody to detect cytokines (e.g. IFN- γ) secreted by an individual cell, manifest as a "spot" after use of a secondary antibody and enzymatic developer. Activation markers on alloreactive T cells (right) are expressed within 24–36h after incubation with alloantigen, allowing for their identification.

Table 1.

Options for monitoring T cell alloimmunity

Assay	Description	Pros/Cons
Mixed lymphocyte reaction (MLR)	Lymphocytes from donor and recipient are combined and co-cultured; dye-dilution assays with CFSE are used to measure T cell proliferation in the recipient in response to donor antigen presentation	Pros: inexpensive, relatively easy to perform Cons: poorly reproducible and time-consuming; older technique, so limited data in tacrolimus era
ImmunKnow®	Measures early response to stimulation by detecting intracellular ATP synthesis in CD4 cells selected from blood by monoclonal antibody coated magnetic beads; the amount of ATP present in stimulated blood specimens is a measure of lymphocyte activity	Pros: highly reproducible, rapid turnaround time, and relatively low cost Cons: poor sensitivity/specificity, no large prospective studies to support its predictive value
Enzyme-linked immunospot (ELISPOT)	Recipient lymphocytes are cultured in the presence of inactivated donor or third-party cells in tissue culture wells coated with a capture antibody against the cytokine of interest; plates are incubated for 18–24h; spots that develop represent a cells that had been primed to the stimulating antigen(s) <i>in vivo</i> , and are quantified using computerized plate readers	Pros: high throughput and functional readout; faster than MLR and amenable to standardization; multiple prospective studies to support its predictive value Cons: expensive (compared to MLR) and labor-intensive

Abbreviations: CFSE - carboxyfluorescein diacetate succinimidyl ester; ATP - adenosine triphosphate