TRANSPLANT INTERNATIONAL

Transplant International ISSN 0934-0874

REVIEW

The use of novel diagnostics to individualize immunosuppression following transplantation

Stephan Schlickeiser,[1] David Boës,[1] Mathias Streitz[1] and Birgit Sawitzki[1,2]

1 Institute of Medical Immunology, CCM, Charité University Berlin, Berlin, Germany
2 Berlin-Brandenburg Center for Regenerative Therapies (BCRT), CVK, Charité University Berlin, Berlin, Germany

Keywords
B cell, Elispot, gene marker, HLA antibodies, plasma cell, transplantation.

Correspondence
Birgit Sawitzki, Institute of Medical Immunology, Charité University Medicine, 10117 Berlin, Germany.
Tel.: +49 30 450 524136;
fax: +49 30 450 524 907;
e-mail: birgit.sawitzki@charite.de

Conflicts of interest
The authors have declared no conflicts of interest.

Received: 10 November 2014
Revision requested: 19 December 2014
Accepted: 16 January 2015
Published online: 4 February 2015

doi:10.1111/tri.12527

Summary

Despite major improvements in short-term survival of organ allografts, long-term graft survival has not changed significantly. It is also known that toxic side effects of current immunosuppressive drugs (IS) especially calcineurin inhibitors (CNI) contribute to the unsatisfactory graft and patient survival following transplantation. Thus, clinicians strive to reduce or wean IS in potentially eligible patients. Research in the last 10 years has focussed on identification of biomarkers suitable for patient stratification in minimization or weaning trials. Most of the described biomarkers have been run retrospectively on samples collected within single-centre trials. Thus, often their performance has not been validated in other potentially multicentre clinical trials. Ultimately, the utility of biomarkers to identify potential weaning candidates should be investigated in large randomized prospective trials. In particular, for testing in such trials, we need more information about the accuracy, reproducibility, stability and limitations of the described biomarkers. Also, data repositories summarizing crucial information on biomarker performance in age- and gender-matched healthy individuals of different ethnicity are missing. This together with improved bioinformatics tools might help in developing better scores for patient stratification. Here, we will summarize the current results, knowledge and limitations on biomarkers for drug minimization or weaning trials.

Introduction

Solid organ transplantation has evolved to an effective treatment of most end-stage organ failures [1–3]. Improvements in organ procurement, surgical techniques but especially IS have led to impressively increased short-term organ and patient survival [4,5]. In particular, use of CNIs such as Cyclosporin A (CsA) or tacrolimus has reduced occurrence of biopsy proven acute rejection rates within the first months post-transplant to sometimes below 10% [6]. This immunosuppressive effectiveness comes with some major drawbacks. The chronic use of IS, for example CNIs leads to toxic side effects such as nephrotoxicity, increased susceptibility to tumour formation and infections, development of diabetes and hypertension [7,8]. Indeed, these side effects contribute to the so far unsatisfactory long-term graft and patient survival [9]. Although scientists and also industry continuously search for novel immunomodulatory therapies with similar effectiveness but decreased toxicity, it is generally accepted that IS minimization or even complete withdrawal might help to improve long-term outcomes in a significant proportion of patients [10,11]. Thus, over the last 10–15 years, many researchers worldwide have tried to identify biomarkers or functional assays suitable for an identification of patients eligible for IS minimization or weaning [12–14]. Surprisingly, many biomarkers and assays have been described, but very often their performance was not tested in other and especially prospective randomized clinical trials involving different clinical centres. In the following paragraphs, we will summarize the current knowledge on potential biomarker and assays. We will also point out their limitations

and missing gaps before taking such diagnostic approaches into daily clinical decision-making.

Potential markers and assays suitable for patient stratification in minimization approaches

When aiming at identification of biomarkers for IS minimization, it is important to consider when and to what degree medications are planned to be reduced. In principle, three scenarios can be envisaged: (i) patients will be enrolled into tolerance inducing trials with CNI avoidance or transient use; (ii) early post-transplant minimization (up to 1 year post-transplant) in patients receiving standard IS treatment regimens or combination therapies with immunomodulatory agents such as belatacept; (iii) late post-transplant minimization (> 1 year post-transplant) or complete withdrawal in potentially 'operationally' tolerant patients (see also Fig. 1). We will focus mainly on the latter two as tolerance inducing protocols such as induction of chimerism by combined hematopoetic stem cell (HSC) and solid organ transplantation have been only recently approached in a limited number of patients and thus a longer follow-up and more data are required to draw meaningful conclusions [15–20].

Potential markers or functional assays suitable for identification of patients eligible for IS minimization should fulfil certain criteria. They should indicate the global or even better antigen-specific immune reactivity of the patient and should be highly reproducible across different centres. Furthermore, the sample material required to run the analysis should be representative and potentially easy accessible. Therefore, researchers have searched for quantitative and qualitative differences between eligible and noneligible patient groups of immune cells, their function or products in blood, plasma or serum samples, biopsy material or fluids draining the graft. Examples for biomarkers and assays tested for their suitability in IS minimization approaches are listed in Table 1. With results from preclinical studies showing the importance of regulatory cell populations such as $CD4^+CD25^+Foxp3^+$ T cells for long-term graft acceptance investigators have studied their proportions and numbers in intragraft or peripheral samples in relation to occurrence of acute or chronic rejection, development of tolerance and success or failure of IS minimization [21–27]. Contrary predominance of effector cell populations may identify patients likely to develop acute rejections or deterioration of graft function upon IS reduction [24,28,29]. Alternatively, quantification of inflammatory chemokines directing intragraft infiltration of effector cells such as CXCL9 or CXCL10 (IP-10) in serum or urine samples may substitute the analysis of their target cells [30–35]. In addition, a few functional assays allowing quantification of donor-reactive memory or effector T or B cells such as IFNg Elispot have been established and partially tested in IS minimization trials [36–39]. We will discuss the suitability of each of the markers and assays according to clinical question or trial design as mentioned above in the following paragraphs.

In addition to biomarkers characterizing the patient's immune reactivity towards the allograft, other biomarkers of IS drug toxicity and efficacy (e.g. pharmacodynamic, pharmacokinetic or pharmacogenetic biomarkers) should be included into an immune monitoring programme of transplant patients. Together with biomarkers of

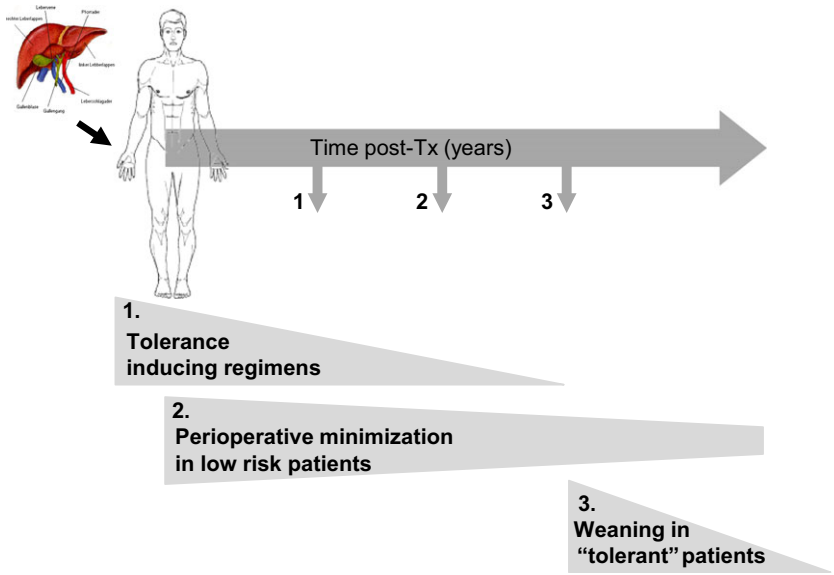

Figure 1 Clinical approaches leading to drug minimization or withdrawal.

Table 1. Overview on potential biomarkers for patient selection in minimization approaches.

Objective	Serum/plasma/urine	Blood leucocytes	Biopsy	Functional assays
Pretransplant patient selection for early peri- or postoperative drug minimization	Antibodies: Anti-HLA and / or DSA predict AMR [76–78] Autoantibodies predict acute rejection [79] Chemokines: Increased serum CXCL9 and CXCL10 expression predict acute rejection and CAN [32,80,81]	Flow cytometry: Frequency of memory or effector T cells predictive of acute rejection [28] qRT-PCR: Low TCAIM expression in acute rejection [82]		ELISPOT: Donor-specific IFN-γ ELISPOT predictive for acute rejection [37,39] Activation marker: Donor-induced CD154/CD137 expression [45]
Post-transplant patient selection for drug minimization or weaning of operationally tolerant patients	Antibodies: Relevance of de-novo DSA for acute and chronic rejection [83–90] Association of autoantibodies with acute and chronic rejection [91,92] Chemokines: Urinary CXCL9 and CXCL10 indicate acute rejection [31,33,47,93] CCL2:Cr predicts fibrosis [94] Urinary qRT-PCR: Diagnosis of acute rejection and fibrosis by CXCL10, PRF, GZB [35,48,49,95,96] miRNAs: Urinary expression of miR-210 indication of acute rejection [97] Urinary miRNA profile diagnosis progression of CAD [98]	Flow cytometry: High frequency of CD4+ CD25+ /Foxp3+ Tregs identifies tolerant patients [21,25] High frequency of naive, transitional or regulatory B cells identifies tolerant patients [13,14,68] Epigenetics: Increased TSDR demethylation in tolerant patients (Braza F JASN in press) RNA microarray / qRT-PCR: Low TCAIM expression prior to and at acute rejection [29,60,82] High TLR4 expression in chronic rejection [61] Gene marker of reduced costimulation in tolerant patients [12] B-cell gene marker in tolerance [13,14,67] DUSP1, PBEF1, PSEN1, MAPK9, NKTR as a gene set of acute rejection [62,63] miRNAs: Increased miR142-3p expression in PBMCs/B cells of tolerant patients [99]	RNA microarray / qRT-PCR: Increased CXCL10 and RANTES expression in 3-months protocol biopsies predicts early graft loss [46] Increased or decreased Foxp3 expression at acute rejection and fibrosis [27] Molecular score for progressive chronic diseases [100] Molecular score for AMR [52] Association of CXCL13 with chronic AMR [55] Tribbles-1 as a marker of chronic AMR [56] Decreased tubular PI3K and c-Rel expression in tolerance [101] miRNAs: Expression of miR142-5p, miR155 and miR223 predictive of acute rejection [57] CAD signature in paired biopsy and urine samples [59]	ELISPOT: Donor-specific IFN-γ ELISPOT [38,39] Self-Ag-specific IFN-γ ELISPOT [102] Activation marker: Donor-induced CD154/CD137 expression at acute rejection [44] Cytokine production: IL-10 production of B cells in tolerance [68]

over-immunosuppression, they will help clinicians in individualizing IS treatment. These topics are not the focus of the current review but have been excellently reviewed elsewhere [40–42].

Patient selection for tolerance inducing regimens or novel immunomodulatory therapies

To date, as mentioned earlier, only induction of chimerism by combined HSC and solid organ transplantation has actively achieved drug-free long-term graft acceptance in patients [15–20,43]. The patient numbers enrolled into such clinical trials so far are very small and the success awaits worldwide validation in larger patient groups. So far, the limited results indicate that successful drug withdrawal in a majority of patients is more likely to happen with conditioning favouring induction of durable chimerism [15,17]. Unfortunately, nothing is known about the special pretransplant features of patients in whom chimerism induction using a reduced conditioning could be achieved. Identifying such important features would make induction of chimerism more applicable to a broader transplant community.

Reduced perioperative or early post-transplant immunosuppression

It is assumed that a significant proportion of transplant recipients would achieve stable graft function in the absence of acute rejection episodes with a less intensive standard of care IS regimen. Testing this hypothesis in clinical trials requires their pre- or early post-transplant identification. It has been described several times that patients characterized by a high frequency of memory or effector T cells have an increased risk of developing acute rejections following transplantation [24,28,29]. Indeed, pre- and early post-transplant quantification CD4+ effector memory or effector T cells prior to transplantation allowed identification of patients developing acute cellular and humoral rejection following liver transplantation [28]. Here, a global quantification of all memory/effector T cells in recipient PBMCs has been carried out by flow cytometry, which allowed capturing the patient's general immune competence. Whether such a more global analysis of T-cell subset composition probably in conjunction with analysis of other leucocyte subpopulations allows patient pretransplant risk stratification needs to be tested in future prospective randomized trials. More specific and appropriate are probably functional assays quantifying the frequency of antigen-reactive T cells responding with an increase in expression of activation markers, for example CD40L (CD154) or effector cytokines, for example IFNg upon stimulation with donor cells [44,45]. In particular, the latter approach, when used as an IFNg Elispot, was selectively applied by several groups to identify rejection prone recipients [38,39]. Indeed, the IFNg Elispot is the only 'biomarker' so far, which was applied in a prospective clinical trial for patient stratification into an either intensive or reduced IS treatment arm [37]. The results indicate that it might be safe to treat Elispot negative patients early on with a CNI sparing protocol. However, those results need further validation, as this was a nonrandomized clinical trial performed at a single transplant centre. Such a randomized multicentre trial is currently designed within the EU consortium BIO-DrIM (www.biodrim.eu). Although very informative, functional assays have also their limitations. They are error-prone and require a high level of standardization to achieve comparable results across different transplant centres [36]. These aspects will be addressed later in much more detail. In addition, they require live donor leucocyte material either from blood in case of living donation or spleen in case of deceased donation. Furthermore, recipients need to donate a large blood volume (> 30 ml) per analysis, which all hampers their frequent use in clinical routine. Thus, scientists and clinicians need to carefully discuss and decide when to implement functional assays into patient immune monitoring.

As pointed out earlier quantification of inflammatory chemokines directing intragraft infiltration of effector cells could also be informative in drug minimization trials. Investigators have detected an increased expression of CXCL10 and RANTES in biopsies of patients with acute rejection [46]. However, more importantly, analysis of serum or urinary CXCL9 and CXCL10 allows a noninvasive monitoring of acute or even chronic rejection [30–35,47,48]. Recent evidence from a multicentre validation trial indicates that low CXCL9 protein concentration in 6-month post-transplant urines obtained from stable allograft recipients was associated with a reduced probability to develop a decline in renal function as estimated by eGFR [49]. The study results have their limitations as the positive predictive value was rather low, but were obtained in a multicentre setup, which makes it an attractive biomarker to be incorporated into a carefully designed monitoring programme.

In contrast to a hypothesis-driven approach in quantifying chemokine expression, scientists have also performed intragraft and whole blood gene expression screens of mRNAs but lately also miRNAs to identify expression patterns, which help to diagnose or predict acute rejection. Intragraft transcriptome analysis enabled the establishment of molecular classifiers for T-cell-mediated rejection [50], antibody-mediated rejection [51,52] or acute kidney injury [53,54]. Furthermore, such analyses resulted in the identification of mediators driving chronic antibody-mediated rejection, such as CXCL13 or Tribbles-1 [55,56]. Lately, micro RNA profiles predictive for occurrence of acute rejection and development of chronic allograft dysfunction have been identified [57–59].

In addition, peripheral mRNA profiling resulted in identification of gene markers being down- or up-regulated prior to or at the time of acute rejection [60,61].

The group of Minnie Sarwal has done tremendous work in developing over a series of validation steps a qRT-PCR-based diagnostic kit incorporating quantification of five mRNAs (DUSP1, PBEF1, PSEN1, MAPK9 and NKTR) and defining a score for the identification of acute rejection [62,63].

It has to be said that some of the above-mentioned biomarkers can be probably used for patient selection regardless of organ transplanted such as CXCL10, whereas other biomarkers are specific for, for example kidney transplant patients or have not been investigated in recipients of other organ grafts. Also as depicted in Table 1, some biomarkers especially gene marker sets vary dependent on the sample type and are, for example different between peripheral blood leucocytes and biopsy samples.

Late post-transplant minimization of immunosuppression

As mentioned earlier at later stages following transplantation (> 1 year post-transplant), it is assumed that a significant proportion of kidney (ca. 10%) and especially liver transplant patients (up to 30%) could maintain good and stable graft function with dramatically reduced (partial weaning) or even without (complete weaning) IS [64,65]. The later stage is also described as 'operational' tolerance and was first observed in transplant patients in whom IS treatment was stopped for clinical indications, for example PTLD or by the patient himself [14,65,66]. The underlying mechanisms for induction and maintenance of operational tolerance in transplant patients still remains to be defined. Thus, researchers have strived to identify biomarkers for monitoring the development and stability of operational tolerance, which would help to safely select transplant patients in whom IS could be withdrawn [12–14,25]. With more results being published, it became clear that mechanisms leading to spontaneous tolerance of liver and kidney transplants may be distinct [67]. Whereas, tolerant liver transplant patients seem to be characterized by a peripheral expansion of gd T cells and NK cells [25], tolerant kidney transplant patients have increased peripheral proportions and numbers of naïve and transitional B cells [13,14,68]. The expansion of potentially 'less pathogenic' B cells is associated with a B-cell specific gene expression profile and increased IL-10 production [68]. Although several groups have reported this expansion of B cells in tolerant kidney transplant patients, the B-cell subpopulations described to be increased in such patients and the associated gene expression pattern do not completely overlap. This raises an issue about the reproducibility and validity of the published findings.

What are the missing gaps before we can implement biomarker-driven patient stratification into clinical practice?

As exemplified in the previous paragraphs, many biomarkers for an early or late post-transplant selection of patients for drug minimization and withdrawal have been described. However, it is important to note that nearly none of the published biomarkers have been implemented in patient stratification yet. Also, no biomarker information is used to broadly manage IS treatment of transplant patients. So what are the reasons and missing gaps before we can implement such biomarkers or functional assays into clinical practice? Vary often findings on biomarker profiles could not be reproduced by other groups in different patient cohorts. This may be in part due to insufficiently standardized methodologies and too small patient cohorts. In the next paragraphs, we will therefore discuss open issues, which should be investigated or established to implement safe and efficient biomarker-driven patient stratification.

Biomarker/Assay standardization

Biomarkers for patient selection have been identified in research laboratories in an environment, which is not used to follow strict diagnostic guidelines. However, biomarker measurement requires accuracy and reproducibility of analytical methods [69]. A biomarker is defined as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, or pharmacological responses to a therapeutic intervention' [70]. The term 'objectively measured' already indicates that a biomarker needs to be thoroughly validated and the performance standardized before it can be introduced into clinical decision-making. Unfortunately, this is very rarely the case with many of the biomarker described in the context of transplantation. Already in the discovery process certain rules should be followed to allow fast transition into clinical application. Before initiating a clinical trial for biomarker discovery for each method used standard operating procedures (SOPs) should be filed, which not only define the steps of sample analysis (e.g. incubation time and temperature, buffer supplies), but also define certain rules for the kind of sample material, the age of the sample material and their storage conditions [71]. Following those strict rules already during the discovery process will increase the power and specificity of discovered biomarkers but also minimize the risk for failures during afterwards performed validation studies. Furthermore, the variability or reproducibility of the biomarker performance should be tested as early as possible. Thus, the following tests should be performed prior to biomarker application: interassay variability (analyse the same samples simultaneously), intra-assay variability (analyse the samples on several consecutive days), interoperator variability (analysis of the same samples by different operators = people) and as an application of such biomarkers in multicentre centre clinical trials is most likely also an intercentre or interlab variability (analysis of the same samples at different laboratories/centres) [36,49,71]. In particular, estimating the variability (coefficient of variation) of the biomarker performance when the same samples are analysed at different centres will really allow you to determine a certain cut-off for meaningful and reproducible differences between different clinical entities. Along the same line additional assay parameters need to be analysed to assess the biomarker performance [69]. This refers especially to the lowest reliably measurable concentration

of a biomarker, often termed as lower limit of detection (LoD) or lower limit of quantitation (LoQ). This again will be important for defining cut-offs for biomarker differences between certain clinically interesting patient groups.

Data validation in independent larger patient cohorts at different transplant centres

In addition to a carful characterization of biomarker performance regarding their variability or reproducibility an independent validation of the biomarker suitability to distinguish between different clinical entities in different transplant centres at larger patient numbers needs to be performed. As such an investigation is very expensive only carefully validated biomarkers should be considered and most likely support through collaborative network grants or industry is needed.

Data repositories and reference values for healthy individuals and patients according to different age groups, gender and ethnicity

As transplant rejection or acceptance is a result of continuous immunological responses very often as outlined earlier, the discovered biomarkers relevant for IS minimization are reflecting differences in numbers or function of certain immune cell subpopulations. Baring that in mind, we should not forget that those parameters are not only dependent on the clinical status of the patient but also on their age, gender and ethnicity. Thus, in order to define inflammatory alterations in a given patient it will be important to create secured data repositories of data from patients but also healthy individuals of various age and ethnic background.

Bioinformatics tools

With the acquisition of large data sets from single or very often multiple parameters, it is evident that in order to define statistical significant but also meaningful differences between different clinical entities such as patients suitable or not for IS minimization new bioinformatics tools need to be applied or even for some methods such as flow cytometry to be established. This should be performed with collaborative network grants.

Prospective clinical trials testing minimization strategies according to biomarker performance

The best biomarker will always be pure association as long as its results are not used for clinical decision-making. This requires that the suitability of a biomarker for decision-making has been proven in prospective controlled randomized clinical trials. Recently, Bestard and colleagues could show in a prospective clinical trial that treating potentially 'low risk' kidney transplant patients, who showed a negative pretransplant donor-specific IFN-γ Elispot response, with a calcineurin inhibitor free regimen is safe with regard to, for example occurrence of acute rejection [37]. Those results are clearly superior to historic findings treating all, 'low' and 'high' risk, patients with a calcineurin inhibitor free regimen [72–75]. However, although the results are very positive, we have to be still cautious as the study design did not include a control arm. Thus, there is a huge demand for such validation trials most likely performed as multicentre clinical trials as currently performed within the EU-granted project BIO-DrIM (www.biodrim.com).

Conclusions

The results summarized within this review are very encouraging. The described biomarkers and functional assays may help us to stratify patients into those being eligible or non-eligible for drug minimization or weaning approaches. However, they also highlight that we as a community need to put enormous efforts into the analysis of biomarker performance including their variability/reproducibility. In addition, it is time to now validate the suitability for clinical decision-making of the most promising biomarkers within large prospective controlled trials.

Funding

The authors have declared no funding.

Acknowledgement

We acknowledge the support of our work by the EU consortia 'ONE Study' and 'BIO-DrIM'.

References

1. Grinyo JM. Why is organ transplantation clinically important? *Cold Spring Harb Perspect Med* 2013; **3**: a014985.
2. Tonelli M, Wiebe N, Knoll G, *et al.* Systematic review: kidney transplantation compared with dialysis in clinically relevant outcomes. *Am J Transplant* 2011; **11**: 2093.
3. Wyld M, Morton RL, Hayen A, Howard K, Webster AC. A systematic review and meta-analysis of utility-based quality of life in chronic kidney disease treatments. *PLoS Med* 2012; **9**: e1001307.
4. Moreso F, Hernandez D. Has the survival of the graft improved after renal transplantation in the era of modern immunosuppression? *Nefrologia* 2013; **33**: 14.

5. Frei U, Daloze P, Vitko S, *et al.* Acute rejection in low-toxicity regimens: clinical impact and risk factors in the Symphony study. *Clin Transplant* 2010; **24**: 500.
6. Ekberg H, Tedesco-Silva H, Demirbas A, *et al.* Reduced exposure to calcineurin inhibitors in renal transplantation. *N Engl J Med* 2007; **357**: 2562.
7. Marcen R. Immunosuppressive drugs in kidney transplantation: impact on patient survival, and incidence of cardiovascular disease, malignancy and infection. *Drugs* 2009; **69**: 2227.
8. Ekberg H, Bernasconi C, Noldeke J, *et al.* Cyclosporine, tacrolimus and sirolimus retain their distinct toxicity profiles despite low doses in the Symphony study. *Nephrol Dial Transplant* 2010; **25**: 2004.
9. Womer KL, Kaplan B. Recent developments in kidney transplantation–a critical assessment. *Am J Transplant* 2009; **9**: 1265.
10. Casey MJ, Meier-Kriesche HU. Calcineurin inhibitors in kidney transplantation: friend or foe? *Curr Opin Nephrol Hypertens* 2011; **20**: 610.
11. Brar JE, Nader ND. Immune minimization strategies in renal transplantation. *Immunol Invest* 2014; **43**: 807.
12. 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.
13. Newell KA, Asare A, Kirk AD, *et al.* Identification of a B cell signature associated with renal transplant tolerance in humans. *J Clin Invest* 2010; **120**: 1836.
14. Sagoo P, Perucha E, Sawitzki B, *et al.* Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans. *J Clin Invest* 2010; **120**: 1848.
15. Leventhal J, Abecassis M, Miller J, *et al.* Chimerism and tolerance without GVHD or engraftment syndrome in HLA-mismatched combined kidney and hematopoietic stem cell transplantation. *Sci Transl Med* 2012; **4**: 124ra128.
16. Kawai T, Cosimi AB, Spitzer TR, *et al.* HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med* 2008; **358**: 353.
17. Leventhal J, Abecassis M, Miller J, *et al.* Tolerance induction in HLA disparate living donor kidney transplantation by donor stem cell infusion: durable chimerism predicts outcome. *Transplantation* 2013; **95**: 169.
18. 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.
19. Scandling JD, Busque S, Dejbakhsh-Jones S, *et al.* Tolerance and withdrawal of immunosuppressive drugs in patients given kidney and hematopoietic cell transplants. *Am J Transplant* 2012; **12**: 1133.
20. Kawai T, Sachs DH, Sprangers B, *et al.* Long-term results in recipients of combined HLA-mismatched kidney and bone marrow transplantation without maintenance immunosuppression. *Am J Transplant* 2014; **14**: 1599.
21. Braudeau C, Racape M, Giral M, *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.
22. Cirocco RE, Carreno MR, Mathew JM, *et al.* FoxP3 mRNA transcripts and regulatory cells in renal transplant recipients 10 years after donor marrow infusion. *Transplantation* 2007; **83**: 1611.
23. Dummer CD, Carpio VN, Goncalves LF, Manfro RC, Veronese FV. FOXP3+ regulatory T cells: from suppression of rejection to induction of renal allograft tolerance. *Transpl Immunol* 2012; **26**: 1.
24. Giaretta F, Bussolino S, Beltramo S, *et al.* Different regulatory and cytotoxic CD4+ T lymphocyte profiles in renal transplants with antibody-mediated chronic rejection or long-term good graft function. *Transpl Immunol* 2013; **28**: 48.
25. 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.
26. Zuber J, Brodin-Sartorius A, Lapidus N, *et al.* FOXP3-enriched infiltrates associated with better outcome in renal allografts with inflamed fibrosis. *Nephrol Dial Transplant* 2009; **24**: 3847.
27. Zuber J, Grimbert P, Blancho G, *et al.* Prognostic significance of graft Foxp3 expression in renal transplant recipients: a critical review and attempt to reconcile discrepancies. *Nephrol Dial Transplant* 2013; **28**: 1100.
28. Gerlach UA, Vogt K, Schlickeiser S, *et al.* Elevation of CD4+ differentiated memory T cells is associated with acute cellular and antibody-mediated rejection after liver transplantation. *Transplantation* 2013; **95**: 1512.
29. Donckier V, Craciun L, Miqueu P, *et al.* Expansion of memory-type CD8+ T cells correlates with the failure of early immunosuppression withdrawal after cadaver liver transplantation using high-dose ATG induction and rapamycin. *Transplantation* 2013; **96**: 306.
30. Hu H, Kwun J, Aizenstein BD, Knechtle SJ. Noninvasive detection of acute and chronic injuries in human renal transplant by elevation of multiple cytokines/chemokines in urine. *Transplantation* 2009; **87**: 1814.
31. Jackson JA, Kim EJ, Begley B, *et al.* Urinary chemokines CXCL9 and CXCL10 are noninvasive markers of renal allograft rejection and BK viral infection. *Am J Transplant* 2011; **11**: 2228.
32. Rotondi M, Rosati A, Buonamano A, *et al.* High pretransplant serum levels of CXCL10/IP-10 are related to increased risk of renal allograft failure. *Am J Transplant* 2004; **4**: 1466.
33. Schaub S, Nickerson P, Rush D, *et al.* Urinary CXCL9 and CXCL10 levels correlate with the extent of subclinical tubulitis. *Am J Transplant* 2009; **9**: 1347.
34. Suthanthiran M, Schwartz JE, Ding R, *et al.* Urinary-cell mRNA profile and acute cellular rejection in kidney allografts. *N Engl J Med* 2013; **369**: 20.

35. Tatapudi RR, Muthukumar T, Dadhania D, *et al.* Noninvasive detection of renal allograft inflammation by measurements of mRNA for IP-10 and CXCR3 in urine. *Kidney Int* 2004; **65**: 2390.
36. Bestard O, Crespo E, Stein M, *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.
37. Bestard O, Cruzado JM, Lucia M, *et al.* Prospective assessment of antidonor cellular alloreactivity is a tool for guidance of immunosuppression in kidney transplantation. *Kidney Int* 2013; **84**: 1226.
38. Bestard O, Nickel P, Cruzado JM, *et al.* Circulating alloreactive T cells correlate with graft function in longstanding renal transplant recipients. *J Am Soc Nephrol* 2008; **19**: 1419.
39. Nickel P, Presber F, Bold G, *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.
40. Budde K, Matz M, Durr M, Glander P. Biomarkers of over-immunosuppression. *Clin Pharmacol Ther* 2011; **90**: 316.
41. Millan O, Urtasun N, Brunet M. Biomarkers of the immunomodulatory effect of immunosuppressive drugs in transplant recipients. *Transplant Rev* 2009; **23**: 120.
42. Wieland E, Olbricht CJ, Susal C, *et al.* Biomarkers as a tool for management of immunosuppression in transplant patients. *Ther Drug Monit* 2010; **32**: 560.
43. Leventhal JR, Mathew JM, Salomon DR, *et al.* Genomic biomarkers correlate with HLA-identical renal transplant tolerance. *J Am Soc Nephrol* 2013; **24**: 1376.
44. Sindhi R, Ashokkumar C, Higgs BW, *et al.* Allospecific CD154+ T-cytotoxic memory cells as potential surrogate for rejection risk in pediatric intestine transplantation. *Pediatr Transplant* 2012; **16**: 83.
45. Schoenbrunn A, Frentsch M, Kohler S, *et al.* A converse 4-1BB and CD40 ligand expression pattern delineates activated regulatory T cells (Treg) and conventional T cells enabling direct isolation of alloantigen-reactive natural Foxp3+ Treg. *J Immunol* 2012; **189**: 5985.
46. Matl I, Hribova P, Honsova E, Brabcova I, Viklicky O. Potential predictive markers in protocol biopsies for premature renal graft loss. *Kidney Blood Press Res* 2010; **33**: 7.
47. Hu H, Aizenstein BD, Puchalski A, Burmania JA, Hamawy MM, Knechtle SJ. Elevation of CXCR3-binding chemokines in urine indicates acute renal-allograft dysfunction. *Am J Transplant* 2004; **4**: 432.
48. Schaub S, Hirt-Minkowski P. Urinary-cell mRNA and acute kidney-transplant rejection. *N Engl J Med* 2013; **369**: 1858.
49. Keslar KS, Lin M, Zmijewska AA, *et al.* Multicenter evaluation of a standardized protocol for noninvasive gene expression profiling. *Am J Transplant* 2013; **13**: 1891.
50. Halloran PF, Pereira AB, Chang J, *et al.* Potential impact of microarray diagnosis of T cell-mediated rejection in kidney transplants: The INTERCOM study. *Am J Transplant* 2013; **13**: 2352.
51. Sellares J, Reeve J, Loupy A, *et al.* Molecular diagnosis of antibody-mediated rejection in human kidney transplants. *Am J Transplant* 2013; **13**: 971.
52. Halloran PF, Pereira AB, Chang J, *et al.* Microarray diagnosis of antibody-mediated rejection in kidney transplant biopsies: an international prospective study (INTERCOM). *Am J Transplant* 2013; **13**: 2865.
53. Famulski KS, de Freitas DG, Kreepala C, *et al.* Molecular phenotypes of acute kidney injury in kidney transplants. *J Am Soc Nephrol* 2012; **23**: 948.
54. Halloran PF, Reeve JP, Pereira AB, Hidalgo LG, Famulski KS. Antibody-mediated rejection, T cell-mediated rejection, and the injury-repair response: new insights from the Genome Canada studies of kidney transplant biopsies. *Kidney Int* 2014; **85**: 258.
55. Siepert A, Brosel S, Vogt K, *et al.* Mechanisms and rescue strategies of calcineurin inhibitor mediated tolerance abrogation induced by anti-CD4 mAb treatment. *Am J Transplant* 2013; **13**: 2308.
56. Ashton-Chess J, Giral M, Mengel M, *et al.* Tribbles-1 as a novel biomarker of chronic antibody-mediated rejection. *J Am Soc Nephrol* 2008; **19**: 1116.
57. 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.
58. Mas VR, Dumur CI, Scian MJ, Gehrau RC, Maluf DG. MicroRNAs as biomarkers in solid organ transplantation. *Am J Transplant* 2013; **13**: 11.
59. Scian MJ, Maluf DG, David KG, *et al.* MicroRNA profiles in allograft tissues and paired urines associate with chronic allograft dysfunction with IF/TA. *Am J Transplant* 2011; **11**: 2110.
60. Sawitzki B, Bushell A, Steger U, *et al.* Identification of gene markers for the prediction of allograft rejection or permanent acceptance. *Am J Transplant* 2007; **7**: 1091.
61. Braudeau C, Ashton-Chess J, Giral M, *et al.* Contrasted blood and intragraft toll-like receptor 4 mRNA profiles in operational tolerance versus chronic rejection in kidney transplant recipients. *Transplantation* 2008; **86**: 130.
62. Li L, Khatri P, Sigdel TK, *et al.* A peripheral blood diagnostic test for acute rejection in renal transplantation. *Am J Transplant* 2012; **12**: 2710.
63. Li L, Khush K, Hsieh SC, *et al.* Identification of common blood gene signatures for the diagnosis of renal and cardiac acute allograft rejection. *PLoS ONE* 2013; **8**: e82153.
64. Chandrasekharan D, Issa F, Wood KJ. Achieving operational tolerance in transplantation: how can lessons from the clinic inform research directions? *Transpl Int* 2013; **26**: 576.
65. Dugast E, Chesneau M, Soulillou JP, Brouard S. Biomarkers and possible mechanisms of operational tolerance in kidney transplant patients. *Immunol Rev* 2014; **258**: 208.

66. Roussey-Kesler G, Giral M, Moreau A, *et al.* Clinical operational tolerance after kidney transplantation. *Am J Transplant* 2006; **6**: 736.
67. Lozano JJ, Pallier A, Martinez-Llordella M, *et al.* Comparison of transcriptional and blood cell-phenotypic markers between operationally tolerant liver and kidney recipients. *Am J Transplant* 2011; **11**: 1916.
68. Chesneau M, Pallier A, Braza F, *et al.* Unique B cell differentiation profile in tolerant kidney transplant patients. *Am J Transplant* 2014; **14**: 144.
69. Christenson RH, Duh SH. Methodological and analytic considerations for blood biomarkers. *Prog Cardiovasc Dis* 2012; **55**: 25.
70. Salter H, Holland R. Biomarkers: refining diagnosis and expediting drug development - reality, aspiration and the role of open innovation. *J Intern Med* 2014; **276**: 215.
71. Streitz M, Miloud T, Kapinsky M, *et al.* Standardization of whole blood immune phenotype monitoring for clinical trials: panels and methods from the ONE study. *Transplant Res* 2013; **2**: 17.
72. Budde K, Becker T, Arns W, *et al.* Everolimus-based, calcineurin-inhibitor-free regimen in recipients of de-novo kidney transplants: an open-label, randomised, controlled trial. *Lancet* 2011; **377**: 837.
73. Flechner SM, Gurkan A, Hartmann A, *et al.* A randomized, open-label study of sirolimus versus cyclosporine in primary de novo renal allograft recipients. *Transplantation* 2013; **95**: 1233.
74. Helal I, Chan L. Steroid and calcineurin inhibitor-sparing protocols in kidney transplantation. *Transplant Proc* 2011; **43**: 472.
75. Vincenti F. Are calcineurin inhibitors-free regimens ready for prime time? *Kidney Int* 2012; **82**: 1054.
76. Ziemann M, Schonemann C, Bern C, *et al.* Prognostic value and cost-effectiveness of different screening strategies for HLA antibodies prior to kidney transplantation. *Clin Transplant* 2012; **26**: 644.
77. Yamanaga S, Watarai Y, Yamamoto T, *et al.* Frequent development of subclinical chronic antibody-mediated rejection within 1 year after renal transplantation with pretransplant positive donor-specific antibodies and negative CDC crossmatches. *Hum Immunol* 2013; **74**: 1111.
78. Fidler SJ, Irish AB, Lim W, Ferrari P, Witt CS, Christiansen FT. Pre-transplant donor specific anti-HLA antibody is associated with antibody-mediated rejection, progressive graft dysfunction and patient death. *Transpl Immunol* 2013; **28**: 148.
79. Giral M, Foucher Y, Dufay A, *et al.* Pretransplant sensitization against angiotensin II type 1 receptor is a risk factor for acute rejection and graft loss. *Am J Transplant* 2013; **13**: 25670.
80. Lazzeri E, Rotondi M, Mazzinghi B, *et al.* High CXCL10 expression in rejected kidneys and predictive role of pretransplant serum CXCL10 for acute rejection and chronic allograft nephropathy. *Transplantation* 2005; **79**: 1215.
81. Rotondi M, Netti GS, Lazzeri E, *et al.* High pretransplant serum levels of CXCL9 are associated with increased risk of acute rejection and graft failure in kidney graft recipients. *Transpl Int* 2010; **23**: 465.
82. Hutchinson JA, Riquelme P, Sawitzki B, *et al.* Cutting Edge: immunological consequences and trafficking of human regulatory macrophages administered to renal transplant recipients. *J Immunol* 2011; **187**: 2072.
83. Gerlach UA, Lachmann N, Sawitzki B, *et al.* Clinical relevance of the de novo production of anti-HLA antibodies following intestinal and multivisceral transplantation. *Transpl Int* 2014; **27**: 280.
84. Halloran PF, de Freitas DG, Einecke G, *et al.* An integrated view of molecular changes, histopathology and outcomes in kidney transplants. *Am J Transplant* 2010; **10**: 2223.
85. de Souza PS, David-Neto E, Panajotopolous N, *et al.* Dynamics of anti-human leukocyte antigen antibodies after renal transplantation and their impact on graft outcome. *Clin Transplant* 2014; **20**: 12451.
86. DeVos JM, Gaber AO, Knight RJ, *et al.* Donor-specific HLA-DQ antibodies may contribute to poor graft outcome after renal transplantation. *Kidney Int* 2012; **82**: 598.
87. Everly MJ, Rebellato LM, Haisch CE, *et al.* Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. *Transplantation* 2013; **95**: 410.
88. Kaneku H, O'Leary JG, Banuelos N, *et al.* De novo donor-specific HLA antibodies decrease patient and graft survival in liver transplant recipients. *Am J Transplant* 2013; **13**: 1541.
89. Lachmann N, Terasaki PI, Budde K, *et al.* Anti-human leukocyte antigen and donor-specific antibodies detected by luminex posttransplant serve as biomarkers for chronic rejection of renal allografts. *Transplantation* 2009; **87**: 1505.
90. Lachmann N, Terasaki PI, Schonemann C. Donor-specific HLA antibodies in chronic renal allograft rejection: a prospective trial with a four-year follow-up. *Clin Transpl* 2006; **52**: 171.
91. Dragun D, Muller DN, Brasen JH, *et al.* Angiotensin II type 1-receptor activating antibodies in renal-allograft rejection. *N Engl J Med* 2005; **352**: 558.
92. Taniguchi M, Rebellato LM, Cai J, *et al.* Higher risk of kidney graft failure in the presence of anti-angiotensin II type-1 receptor antibodies. *Am J Transplant* 2013; **13**: 2577.
93. Hricik DE, Nickerson P, Formica RN, *et al.* Multicenter validation of urinary CXCL9 as a risk-stratifying biomarker for kidney transplant injury. *Am J Transplant* 2013; **13**: 2634.
94. Ho J, Wiebe C, Gibson IW, *et al.* Elevated urinary CCL2: Cr at 6 months is associated with renal allograft interstitial fibrosis and inflammation at 24 months. *Transplantation* 2014; **98**: 39.
95. 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.

96. van Ham SM, Heutinck KM, Jorritsma T, *et al.* Urinary granzyme A mRNA is a biomarker to diagnose subclinical and acute cellular rejection in kidney transplant recipients. *Kidney Int* 2010; **78**: 1033.
97. Lorenzen JM, Volkmann I, Fiedler J, *et al.* Urinary miR-210 as a mediator of acute T-cell mediated rejection in renal allograft recipients. *Am J Transplant* 2011; **11**: 2221.
98. Maluf DG, Dumur CI, Suh JL, *et al.* The urine microRNA profile may help monitor post-transplant renal graft function. *Kidney Int* 2014; **85**: 439.
99. Danger R, Pallier A, Giral M, *et al.* Upregulation of miR-142-3p in peripheral blood mononuclear cells of operationally tolerant patients with a renal transplant. *J Am Soc Nephrol* 2012; **23**: 597.
100. Famulski KS, Reeve J, de Freitas DG, Kreepala C, Chang J, Halloran PF. Kidney transplants with progressing chronic diseases express high levels of acute kidney injury transcripts. *Am J Transplant* 2013; **13**: 634.
101. Becker LE, de Oliveira Biazotto F, Conrad H, *et al.* Cellular infiltrates and NFkappaB subunit c-Rel signaling in kidney allografts of patients with clinical operational tolerance. *Transplantation* 2012; **94**: 729.
102. Angaswamy N, Klein C, Tiriveedhi V, *et al.* Immune responses to collagen-IV and fibronectin in renal transplant recipients with transplant glomerulopathy. *Am J Transplant* 2014; **14**: 685.