REVIEW

Tolerance signatures in transplant recipients

Kenneth A. Newell[a] and Laurence A. Turka[b]

Purpose of review
The intent of this review was to describe biomarkers that predict or identify individuals who exhibit tolerance to a transplanted organ. The identification of tolerance biomarkers would spare some individuals the toxicity of immunosuppressive agents, enhance the safety of studies to induce tolerance, and provide insights into mechanisms of tolerance that may aid in designing new regimens.

Recent findings
Studies of tolerant kidney transplant recipients have revealed an association with B cells. More recent studies have suggested that these B cells may be less mature than those in nontolerant recipients, and especially suited to suppress alloimmune responses. Biomarkers in tolerant liver transplant patients appear to be distinct from those associated renal tolerance. Most reports have identified an association with natural killer and/or γδ T cells rather than B cells. Recent data indicate biomarkers associated with iron homeostasis within the transplanted liver more accurately predict the tolerant state than do biomarkers expressed in the blood, suggesting that the renal allograft itself, which is infrequently sampled, would be informative.

Summary
Given the encouraging progress in identifying tolerance biomarkers, it will be important to validate these markers in larger studies of transplant recipients undergoing prospective minimization or withdrawal of immunosuppression.

Keywords
biomarkers, gene expression, polychromatic flowcytometry, tolerance

INTRODUCTION

The dramatic improvements in short-term solid organ transplant outcomes have not been mirrored by comparable improvements in long-term graft survival [1,2]. Graft loss and recipient death at intermediate and late time points are due to a variable mix of the side-effects of immunosuppression and inadequate control of the alloimmunity leading to progressive graft injury. Many believe that induction of robust, tolerance could address some of the factors that contribute to premature graft loss and patient death. Consistent with this contention two groups recently reported a comparison of outcomes of tolerant and nontolerant kidney transplant recipients. These reports demonstrate that individuals achieving tolerance had reduced incidences of hypertension, hyperlipidemia, de-novo diabetes, malignancy, and infection as well as experiencing improved graft survival versus conventionally treated patients [3",4"].

A major factor limiting the broader clinical application of strategies to induce tolerance is the lack of reliable markers to predict those individuals in whom tolerance could be successfully achieved. Without the existence of a 'tolerance signature' the process of minimizing or withdrawing immunosuppression is one of trial and error in which empiric reductions pose a risk to the recipient of underimmunosuppression, inadequate control of alloimmunity, and premature loss of the transplanted organ. The identification and validation of biomarkers that identify or even predict tolerance would allow a rational and safer approach to the personalized management of immunosuppression including minimization and potentially complete withdrawal. It is further possible that if the identified biomarkers reflect key processes in the development or maintenance of tolerance, this information

[a]Department of Surgery, Emory University, Atlanta, Georgia and [b]Department of Surgery and Immune Tolerance Network, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA

Correspondence to Kenneth A. Newell, Department of Surgery, Emory University, 101 Woodruff Circle, Suite 5105 WMB, Atlanta, GA 30322, USA. Tel: +1 404 727 2489; fax: +1 404 712 2699; e-mail: kanewel@emory.edu

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KEY POINTS

- The identification and validation of biomarkers that predict or identify tolerance to transplanted organs would increase the safety of clinical tolerance studies and potentially provide insights into the mechanism(s) of tolerance that may aid in the design of tolerance inducing immunosuppressive regimens.
- Biomarkers associated with tolerance following kidney transplantation are dominated by increases in B cell numbers and the expression of B cell-related genes in the blood as well as defects in B cell maturation and evidence of active suppression mediated by B cells *in vitro*.
- Biomarkers associated with tolerance following liver transplantation are predominately related to NK cells and γδ T cells in the blood and genes related to iron homeostasis within the graft.
- Validation of these potential biomarkers associated with tolerance will require prospective, longitudinal studies of patients enrolled in studies designed to facilitate the well tolerated withdrawal of immunosuppression.

could be used to aid in the rational design of tolerance-inducing regimens.

OVERVIEW OF CLINICAL TOLERANCE AND BIOMARKERS

Although transplantation tolerance was first induced in experimental models in the mid 1950s, it was not until 1975 that tolerance in clinical transplantation was reported [5]. By the 1990s improvements in immunosuppression, surgical technique, and our understanding of immunology had improved to the point that there was renewed enthusiasm for efforts to induce tolerance in human transplant recipients, and with this has come efforts to define biomarkers of tolerance.

It is important to keep in mind that biomarkers may provide information about the mechanism responsible for the tolerant state, or simply may be associated with the state but not in a causal relationship. In the case of transplantation tolerance, the former would be ideal, as it would not only help inform patient care, but provide new insights into tolerance that would assist in the design and development of novel strategies. The latter can, however, still be of great clinical value. To date, most measures to identify or predict transplantation tolerance have appeared to fall into the latter category as they lack clear evidence that they identify a mechanism of tolerance (a notable exception is Morris *et al.* [6▪▪], see further below).

With respect to the assays themselves, although it would seem intuitively obvious that the demonstration of donor-specific hyporesponsiveness using cellular assays would be of greatest value in assessing tolerance, the development of these types of cell-based assays has proven frustratingly difficult. In fact, much of the success to date in developing biomarkers of tolerance has been achieved using studies of gene expression and phenotyping of immune cells by flow cytometry. At this time, other methodologies such as epigenetics, proteomics, and metabolomics have not been broadly examined in the setting of transplantation. Finally, this overview will focus primarily on biomarkers associated with tolerance following kidney and liver transplantation, as there is a paucity of data pertaining to tolerance following the transplantation of other organs. As will be seen below, kidney and liver biomarkers differ significantly, and it is reasonable to speculate that the biomarkers associated with tolerance for other organs and tissues may be distinct as well [7].

BIOMARKERS IN SPONTANEOUSLY TOLERANT KIDNEY TRANSPLANT RECIPIENTS

One of the earliest attempts to define a tolerance signature in kidney transplant recipients was that of Brouard *et al.* [8] who reported a predictive expression pattern of 33 genes. Of note, 27% of the identified genes were involved in the regulation of the immunomodulatory cytokine TGFβ [8]. These authors also noted greater numbers of $Foxp3^+$ cells in the peripheral blood of tolerant patients relative to patients experiencing chronic rejection. This was ultimately attributed to a relative decrease in $Foxp3^-$ cells in chronic rejection rather than an increase in $Foxp3^+$ cells in tolerant recipients and illustrated the challenges of identifying an appropriate comparison population. Using the combined approaches of gene expression profiling and immune cell phenotyping by flow cytometry, three groups subsequently reported the unexpected finding that tolerant patients (compared with clinically stable patients on standard immunosuppression) had increased numbers of B cells and B cell-related gene expression in peripheral blood [9–11]. Data from the Immune Tolerance Network (ITN) study indicated that not only were B cells and B cell-related transcripts more abundant in tolerant patients but also the B cell repertoire was skewed toward more transitional and naïve B cells and that transcripts for the B cell marker CD20 were increased in the urinary sediment cells of tolerant patients [9]. Unpublished findings from the ongoing ITN study

indicate that our previously reported B cell-based tolerance signature persists over time in tolerant recipients and that individuals rendered tolerant using a protocol of combined kidney and bone marrow transplantation display similar overexpression of the B cell-associated gene most predictive of tolerance (K. A. Newell and L. A. Turka, in preparation).

Lozano *et al.* [7] compared the patterns of gene expression reported to be associated with tolerance in the three studies referenced above and identified 35 genes in common that were predictive of tolerance. Of these 35 genes, 24 are closely related to B cells. Baron and colleagues subsequently performed a meta-analysis of five studies of spontaneously tolerant kidney transplant recipients [7–9,11,12]. Their analysis of 96 tolerant samples from these five studies revealed a panel of 20 genes for which the expression profile predicted tolerance with a 92.5% accuracy [13▪]. Most of these biomarker genes were associated with B cells, but to a lesser extent they were also related to CD4 T cells and CD14 monocytes. An additional piece of evidence suggesting that B cells may be a marker of tolerance is the finding that expression of miR-142-3p, which may modulate the expression of numerous B cell immune response genes, is increased in both the peripheral blood and B cells of tolerant recipients relative to those receiving conventional immunosuppression and that this difference is stable over time and not modulated by immunosuppression [14].

Although most of the data cited above support an association between B cells and tolerance following kidney transplantation without suggesting a mechanistic relationship, using in-vitro assays Chesneau *et al.* [15▪] suggested that B cells from tolerant kidney transplant recipients exhibited a defect in the late stages of differentiation into plasma cells. As a consequence, there were relatively more transitional and naïve B cells and fewer plasma cells. It is interesting to note that in their assay system B cells from tolerant patients produced more IL-10 than did those from nontolerant patients confirming previously published results and suggesting both differences in the maturational status and function of B cells in tolerant patients. This group [16▪▪] recently extended these findings by demonstrating that B cells from tolerant kidney transplant recipients, but not those from recipients with stable function who are receiving immunosuppression nor healthy volunteers, suppress effector T cell function *in vitro* in a dose-dependent fashion through a granzyme B-dependent pathway. Given that many of the other biomarkers of tolerance cannot distinguish between tolerant patients and healthy controls, the finding that B cells from tolerant patients differ functionally when compared with cells from healthy controls is potentially of great importance, as it suggests that the tolerant state may not merely be a 'drug-free' state. Finally, tolerant patients had higher numbers of granzyme B-positive B cells. The observed increase in granzyme B-positive B cells was dependent upon IL-21 causing the authors to hypothesize a feedback loop between B cells and IL-21 producing T cells.

The prevalence of a B-cell-related tolerance signature following renal transplantation is an important and incompletely addressed question. Any biomarker, regardless of its predictive accuracy, will be of little value if it is so rare in patients receiving immunosuppression as to be practically not actionable. Using the expression pattern of 20 genes that they have previously used to identify tolerance, Brouard *et al.* [17] reported that only 3.5% of 144 kidney transplant recipients with stable function receiving conventional immunosuppression at 5 years displayed the tolerance signature. Moreso *et al.* [18▪] have examined the expression of genes related to B cell differentiation, B cell numbers, and the number of transitional and naïve B cells in several cohorts of renal transplant recipients including tolerant patients from the original ITN study, patients with stable function treated with a CNI, patients treated with azathioprine, healthy volunteers and patients with chronic rejection. They confirmed the increased expression of IGKV1D-13 and IGKV4-1 in samples of tolerant patients. Their findings also demonstrated a time-dependent increase in the prevalence of the tolerance signature defined by these molecules in patients with stable function who were treated with a CNI with 0%, 7%, and 25% of the patients in these cohorts displaying the tolerance signature at 1, 5, and 10 years following transplantation. None of the patients with chronic rejection or those treated with azathioprine displayed the markers of tolerance. Although the numbers of patients in each cohort were quite small, these data suggest that the expression of tolerance biomarkers may increase over time following transplantation and vary with the choice of immunosuppressive agents. Consistent with this finding, Benitez *et al.* [19] reported that the success of withdrawing immunosuppression following liver transplantation increased with time since transplantation. We have recently completed enrollment of 249 patients treated with different immunosuppressive protocols in an ITN-sponsored study designed to determine the prevalence of the ITN 'tolerance signature' in patients receiving conventional immunosuppression who demonstrate stable graft function between 1 and 5 years after transplantation.

Two recent studies have suggested a complex relationship between B cells, other immune cell

populations, and tolerance. Roedder *et al.* [20▪▪] used transcriptional profiling as well as flow cytometry to examine samples from HLA-mismatched renal transplant recipients and nontransplant control individuals. Their analysis identified three genes (KLF6, BNC2, and CYP1B1) that predicted operational tolerance and found that 7.3% of a cohort of 150 patients met this three-gene criterion. Phenotypic analysis of sorted cells from the peripheral blood of tolerant patients showed an increase in myeloid-derived dendritic cells and their gene products. Finally, Braza *et al.* [21▪▪] have recently published findings that suggest a role of regulatory T cells in tolerance following kidney transplantation. This study examined differences in the levels of demethylation of the Foxp3 Treg-specific demethylated region (TSDR) in circulating $CD4^+$ T cells in tolerant patients compared with healthy volunteers, patients with stable renal allograft function receiving immunosuppression, and those with chronic rejection [21▪▪]. They noted a higher proportion of $CD4^+$ T cells with demethylated Foxp3 and an expansion of $Foxp3^{hi}$ memory Tregs with greater suppressive properties in tolerant patients as compared with the other groups.

Study of biomarkers associated with the intentional induction of tolerance in kidney transplant recipients

As reviewed elsewhere in this issue, several groups have now reported strategies that promote the development of tolerance in a significant number of selected kidney transplant recipients (their most recent experiences are summarized in [3▪,4▪,22]. Although the individual protocols differ in many ways that may ultimately be important, they share in common the administration of donor antigen in the form of hematopoietic cells, the requirement for nonmyeloablative conditioning, and the gradual tapering of immunosuppression. Although it would seem intuitively obvious that it would be more informative to conduct mechanistic studies to identify biomarkers in these study populations in that one can obtain samples throughout the process of tolerance induction and maintenance, the mechanistic insights gained from these approaches have been hampered by the small numbers of patients in these early exploratory studies.

Both the Stanford and Northwestern/Louisville groups have emphasized that the maintenance of macrochimerism for periods of at least several months is correlated with the development and stability of tolerance. In fact, the Northwestern/Louisville group has reported that persistent chimerism is a better predictor of the maintenance of tolerance than is donor-specific hyporesponsiveness *in vitro* [23]. The group from Stanford has suggested that the relative sparing of Treg relative to Teff cells associated with their regimen contributes to the development of tolerance [24]. The Massachusetts General Hospital investigators found that the tolerant phenotype is associated with the persistence of donor-specific hyporesponsiveness *in vitro* and an increased proportion of $CD4^+CD25^+CD127^-FOXP3^+$ Treg during the early posttransplant period suggesting a suppressive component to tolerance that disappears at later time points [25]. More recently, the MGH group reported evidence for deletion of donor-reactive T cells in tolerant, but not nontolerant patients or those receiving conventional immunosuppression [6▪▪]. Using high-throughput sequencing of the T-cell receptor β chain CDR3 region, they identified donor-reactive T cells prior to transplantation and tracked them following transplantation. They noted a reduction in donor-reactive T cells following transplantation in tolerant patients but not those failed to develop tolerance or those treated with conventional immunosuppression. The deletion of donor-reactive T cell clones correlated better with the tolerant state than did donor-specific hyporesponsiveness as assessed by in-vitro assays. Although the numbers of patients were very small, the results are of great interest and this technique is likely to be used in many upcoming studies.

Biomarkers of tolerance following liver transplantation

There has been a paucity of interventional trials aimed at inducing tolerance following liver transplantation. This is particularly surprising given the widely held belief that relative to other transplanted organs livers are more resistant to the long-term injurious effects of rejection and more predisposed to the development of tolerance. Some investigators have, however, examined spontaneously tolerant patients or conducted trials in which patients have undergone closely supervised drug withdrawal; a scenario in which several studies have suggested that in carefully selected cohorts of liver-transplant recipients the incidence of spontaneous tolerance may approach or even exceed 50% [19,26,27]. These studies have led to the description of a number of potential biomarkers of liver allograft tolerance.

Early studies in spontaneously tolerant pediatric recipients of living donor partial liver allografts reported an increase in the frequency of $CD4^+$ $CD25^{hi}$ T cells, B cells, and the ratio of Vδ1/Vδ2 gamma-delta cells in the peripheral blood relative to

recipients presumed to be nontolerant [28]. This group has subsequently reported that both Foxp3-expressing CD4 T cells and Vδ1 expressing gamma-delta cells accumulate within the liver allografts of tolerant recipients relative to those receiving immunosuppression or with chronic rejection [29,30]. At about the same time, Mazariegos *et al.* [31,32] demonstrated that tolerant recipients of livers from deceased donors had increased numbers of plasmacytoid dendritic cell precursors relative to monocytoid dendritic cell precursors in their blood and that this was independent of time since discontinuation of immunosuppression or the type of immunosuppression used.

More recently, a number of groups have utilized microarrays or PCR methodology to compare the expression of large numbers of genes in tolerant and nontolerant liver-transplant recipients. Martinez-Llordella *et al.* [33] reported an increase in transcripts associated with γδ T cells and natural killer (NK) cells in the peripheral blood of tolerant liver transplant recipients. Consistent with these finding, Li *et al.* [34] defined a set of 13 genes that are highly expressed in NK cells and that were relatively overexpressed in tolerant pediatric and adult transplant recipients. The overexpression of these genes appeared to be independent of clinical or demographic variables. More impressively, a subset of only three genes was highly accurate in distinguishing tolerant from nontolerant recipients in a separate cohort. In the most comprehensive study of its type to date, this group [35] examined peripheral blood cell phenotypes as well as gene expression profiles in the blood and allograft of patients prior to and following attempted weaning of immunosuppression. Similar to previous reports by this group and others, tolerant recipients displayed an increased proportion of NK cells and a decreased proportion of Vδ2 gamma delta T cells in their peripheral blood. An increase in the proportion of $CD4^{-}CD25^{-}CD127^{-}Foxp3^{+}$ T cells in the blood was also noted albeit only after 12 months following discontinuation of immunosuppression. These investigators also noted that gene expression patterns of liver allograft biopsies prior to attempted drug withdrawal were also highly predictive of tolerance. Most strikingly, the gene sets differentially expressed in the liver allograft and blood showed little if any overlap with the allografts of tolerant recipients overexpressing numerous genes related to iron homeostasis. When comparing the predictive power of gene sets derived from blood and liver allograft tissue prior to attempted weaning of immunosuppression, the gene expression signature of the liver allograft more accurately predicted the development of tolerance.

CONCLUSION

Numerous studies utilizing primarily cell phenotyping by flow cytometry or gene expression by microarray or PCR have been conducted in tolerant kidney and liver transplant recipients. Somewhat surprisingly, biomarkers associated with tolerance differ dramatically between the two organ types. Spontaneously tolerant kidney transplant recipients appear to have increased numbers of B cells and overexpress numerous B cell associated genes in their blood. Recent studies have suggested that B cells from tolerant kidney transplant recipients may differ in their capacity to mature and posses immune-suppressive properties not shared by B cells from nontolerant recipients. In contrast, tolerant liver allograft recipients are characterized by an increased prevalence of NK cells and Vδ1 gamma delta T cells in their blood and genes related to iron homeostasis in the liver allograft. Determining which, if any, of these findings are shared by individuals undergoing other types of organ transplantation or treated using protocols specifically designed to induce tolerance remains to be determined.

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Conflicts of interest

K.A.N. currently serves or has served in the past 3 years on scientific advisory boards for Immunovir, Novartis, Oxford Immunotec, and SERC Therapeutics. He also serves as a speaker/moderator for an educational program sponsored by Novartis Pharmaceuticals.

L.A.T. has served as a consultant for Bristol-Myers Squibb and Merck and served on the scientific advisory board of SERC Therapeutics. He receives grant support from Pfizer.

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