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Monitoring Regulatory T-Cells after Transplantation: Is It Useful?

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Abstract

Currently, the gold standard for confirmation of rejection episodes in transplanted organs is histologic analysis of biopsy samples. There is no method to evaluate the risk of rejection in patients or to identify those who might be weaned off immunosuppression entirely. The development of biomarkers for use in transplantation would help achieve this goal of tailored immunosuppressive treatment for individual patients. Markers of regulatory T-cells, with their role in regulating the alloimmune response, have been investigated for their usefulness in this situation. This review will discuss the studies to date on the diagnostic and prognostic potential of regulatory T-cells in organ transplantation. **(Trends in Transplant. 2009;3:119-28)**

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Key words

Regulatory T-cells. FOXP3. Rejection. Graft outcome.

Introduction

Surveillance of solid organ transplants, such as kidney and liver, for rejection and evaluation of graft function is routinely performed through functional parameters such as plasma creatinine and liver enzymes, respectively. Any deterioration in function in the absence of any other obvious causes usually prompts a biopsy to exclude rejection, especially within the first year of transplantation when the risk is high. This process has several disadvantages. Firstly, the biopsy procedure carries risks of complications, most seriously bleeding from the biopsy site, which may lead to graft loss or even death. Secondly, sampling error may result in inaccurate representation of the organ graft as a whole. Thirdly, by the time the transplanted organ is functionally compromised, irreversible tissue damage may have already taken place. The ideal method for monitoring rejection would be noninvasive and could detect rejection early. In the absence of such a monitoring method, histologic examination of biopsy samples remains the gold standard. It would be ideal to develop a more sophisticated screening method for rejection so that unnecessary biopsies could be avoided. In addition, it would be useful to establish the immunologic risks of individual patients after organ transplantation so that immunosuppressive therapy could be tailored accordingly. As

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rejection is primarily an immune-mediated response, measurement of immunologic parameters has the potential to be developed to serve these purposes.

Biomarkers and immunologic risk

A large amount of effort has been devoted to the search for biomarkers (defined as a biological molecule whose presence can indicate a disease state or a high likelihood/ risk of developing a disease or a specific disease phenotype[1]) in many disease states and the field of transplantation is no exception. Initial studies concentrated on analyzing a few candidate genes such as the mediators of cytotoxic T-cell killing perforin and granzyme B[2,3]. More recently, with advances in technology, there has been an increase in microarray based gene expression profiling, which examines the whole genome and does not require the identification of a possible target[4].

The identification of one biomarker, or more likely a panel of biomarkers, to detect a rejection episode would not only allow earlier diagnosis and therefore intervention, but may also identify patients with different levels of risk by providing information on the antidonor response of the recipient.

A percentage of patients who discontinue their immunosuppression do not reject their grafts. This phenomenon is more common in liver transplant patients[5-7], but also occurs in recipients of kidney transplants, although much less frequently[8,9]. Much work has gone into defining a "tolerogenic profile", and this may allow not only the use of lower doses of immunosuppression in patients with tolerogenic profiles, but possibly the weaning of these patients off immunosuppression entirely. In contrast, patients at an increased risk of rejection according to the biomarker profile could be maintained on more intense immunosuppressive regimes to prevent rejection. Currently, there is no way of predicting what level of immunosuppression a patient will need, which inevitably leads to some patients being over-immunosuppressed while others are under-immunosuppressed. The development of biomarkers is the first step to providing tailored immunosuppressive therapy for individual patients.

Regulatory T-cells

The importance of regulatory T-cells (T_{reg}) in tolerance models makes their surface or intracellular markers obvious candidates to provide a biomarker for transplantation. The characteristics of these cells and their role in the induction and maintenance of tolerance have been discussed elsewhere[10-12]. Although the vast majority of organ recipients are not truly tolerant of their grafts, T_{reg} may still play an important role in the prevention of rejection, adjuvant to the immunosuppressive drugs used in the clinic. If so, monitoring their activity in transplant recipients may provide useful immunologic information. This review will focus on their diagnostic potential, both in terms of aiding the diagnosis of rejection and providing an indication of prognosis and information to aid tailoring immunosuppressive therapy for individual patients.

To monitor T_{reg}, a robust marker should ideally be available. Unfortunately, a definitive marker for T_{reg} in humans is yet to be found. The classic T_{reg} are enriched within the $CD4^+CD25^+$ T-cell population[13,14]. To date, the most reliable marker available is forkhead box p3 (FOXP3), a member of the forkhead/ winged helix family of transcription factors[15-17]. In mice, Foxp3 is a specific marker of T_{reg}[15-17], however in humans this transcription factor is also transiently expressed in non-regulatory T-cells upon activation[18]. Other markers used to identify T_{reg} include CD45RB[19,20], CTLA-4[21,22], GITR[23-25], CD122[26], CD103[27], and galectin-10[28], and the absence

Table 1. Summary of the use of FOXP3 mRNA measurement in human solid organ transplant recipients during rejection episodes to aid diagnosis or as a predictor of outcome

Author	Organ	Sample source (factor measured)	No. of patients with rejection/total	Diagnostic of rejection?	Predictive of better/ worse outcome?
Aquino-Dias, et al.[31]	Kidney	Intragraft (mRNA)	20/75	Yes	
		Blood (mRNA)	20/75	Yes	
		Urine (mRNA	20/75	Yes	
Ashton-Chess, et al.[61]	Kidney	Intragraft (mRNA)	14/48	No	
		Blood (mRNA)	15/205	No	
Grimbert, et al.[32]	Kidney	Intragraft (mRNA)	15 BL/11 AR/36 total	Yes	
Mansour, et al.[47]	Kidney	Intragraft (mRNA)	21/46		Better
Bestard, et al.[48]	Kidney	Intragraft (cell no.)	37		Better
Bunnag, et al.[45]	Kidney	Intragraft (mRNA)	31/95	Yes	No
Veronese, et al.[44]	Kidney	Intragraft (cell no.)	41/73	Yes	Worse
Martin, et al.[46]	Kidney	Intragraft (cell no.)	17/17	N/A	Better
Muthukumar, et al.[51]	Kidney	Urine (mRNA)	36/83	Yes	Better
Demirkiran, et al.[33]	Liver	Intragraft (mRNA)	3/20	Yes	
Sakamoto, et al.[49]	Liver	Blood (mRNA)	4/15		Better
Dijke, et al.[34]	Heart	Intragraft (mRNA)	26/41	Yes	
	Heart	Blood (mRNA)	26/41	No	

of CD127 can also indicate a regulatory phenotype[29,30]. A combination of these markers may be the best way of identifying T_{reg}.

The use of FOXP3 as a biomarker has been investigated in several aspects of transplantation: the diagnosis of rejection; the prediction of rejection and the outcome of a rejection episode; the presence of tolerance; and involvement in graft-versus-host disease (GVHD).

Using regulatory T-cells to diagnose rejection

Given their potential to regulate alloreactive T-cells, the presence of T_{reg} in higher numbers should, in theory, exert a graft-protective effect. Conversely, low numbers of T_{reg} may suggest rejection.

Intragraft T_{reg}/FOXP3

Results from these studies are summarized in table 1. Aquino-Dias, et al. studied kidney allografts that had suffered from delayed graft function. The levels of FOXP3 mRNA were paradoxically significantly higher in those patients undergoing acute rejection, and indeed were a more reliable marker of rejection than either of the cytotoxic mediators perforin and granzyme B[31]. A separate comparison of kidney transplant recipients with acute rejection and borderline changes found that again FOXP3 mRNA levels were higher in the rejection group[32].

High FOXP3 mRNA levels have also been found to be predictive of rejection in liver allografts[33]. However, FOXP3 levels were also elevated during hepatitis C virus infection, suggesting that FOXP3 would not provide the

level of specificity required. Levels of FOXP3 mRNA have also been examined in heart transplant recipients[34]. Again, higher FOXP3 mRNA levels were present in the 26 acutely rejecting patients, compared to the 15 patients without rejection.

The increase in T_{reg} in rejection probably reflects an overall increase in the immune response, with T_{reg} being a portion of this, rather than acting in its graft-protective capacity.

There is now increasing evidence that intragraft mRNA levels of FOXP3 do seem to be a good indicator of acute rejection. However, to measure intragraft mRNA, biopsies must still be performed and, so, rejection can be diagnosed by histologic examination, which is still regarded as the gold standard. Therefore, in this respect, it serves little clinical purpose but is likely to remain as a useful research tool. Measuring FOXP3 mRNA levels in peripheral blood or urine (in the case of kidney transplantation) may circumvent this problem.

Peripheral blood and urine T_{reg}/FOXP3

Aquino-Dias, et al., as well as studying intragraft FOXP3 mRNA, measured peripheral blood and urine FOXP3 mRNA levels in their cohort of patients with delayed graft function. They found that, as with intragraft FOXP3, mRNA levels of FOXP3 in blood and urine were significantly higher in the group undergoing acute rejection[31], with sensitivity and specificity in blood of 94 and 95%, respectively, while both corresponding figures in urine were 100%. Satoda, et al. made a similar finding in a miniature swine model of lung transplantation[35].

In contrast, no difference in the levels of FOXP3 mRNA were found in the blood of heart transplant recipients, despite there being a difference in intragraft FOXP3[34]. Similar findings were made in liver transplant recipients[33]. This may be due to the egress of these cells out of the blood and into the graft.

Peripheral blood and urine markers of T_{reg} therefore appear to have potential to be used in the diagnosis of acute rejection of kidney, and potentially lung, transplants, although they have not proven useful so far in heart or liver transplantation. However, the number of patients in the study is small and needs to be confirmed by others. The sensitivity and specificity of 100% in urine observed in the study is very impressive; it is difficult to envisage any tests with such high accuracies. Expansion of the cohort size is likely to reduce these figures. Nonetheless, urinary FOXP3 mRNA level should prove to be a useful marker in this respect.

The development of this method would allow a quick, noninvasive screening test for rejection, and T_{reg} levels could be monitored alongside functional indicators such as plasma creatinine, allowing a patient's immunologic status to be measured over time, and correlated to graft function.

Stem cell transplantation

The possibility of using T_{reg} to diagnose GVHD in recipients of allogeneic stem cell transplants has also been explored. In contrast to the situation with acute rejection of solid organ transplants, FOXP3 mRNA levels were found to be significantly reduced in all forms (acute, chronic, allo, auto) of GVHD in two independent studies[36,37]. These data were contradicted by Meignin, et al., who found no difference in blood mRNA FOXP3 levels between patients with or without GVHD[38]. More work will need to be carried out to clarify the situation, although Rieger, et al. examined $FOXP3^+$ cells in intestinal GVHD lesions and

found evidence to support the hypothesis that lower numbers of FOXP3$^+$ cells (relative to CD8$^+$ cells) is indicative of GVHD[39].

Other studies have been carried out to investigate T_{reg} in GVHD, but these have used CD25 (in combination with CD4) to identify T_{reg}[40-43]. Again, results from these studies have been contradictory, suggesting that CD25, with its expression on activated T-cells, is not a specific enough marker of T_{reg}.

Regulatory T-cells as predictors of rejection/rejection outcome

Intragraft

The presence of T_{reg} in biopsies appears to indicate rejection, presumably by revealing the extent of the alloimmune response. It may be expected that, further to this, higher levels of FOXP3 may indicate a better-regulated immune response and therefore predict a better outcome of the rejection episode. Data acquired so far is summarized in table 1.

This theory was contradicted by data acquired by Veronese, et al., who found by immunohistochemistry that, as expected, the 41 kidney allografts with acute cellular rejection had significantly more FOXP3$^+$ cells than the 32 patients with acute humoral rejection[44]. However, in the group with acute cellular rejection, the higher the number of FOXP3$^+$ cells the worse the outcome. The numbers of CD4$^+$ and CD8$^+$ cells were also higher, suggesting that the higher number of FOXP3$^+$ cells represented merely a more vigorous alloimmune response rather than a disproportionate number of FOXP3$^+$ cells. They also found FOXP3$^+$ T-cells infiltrating tubules, and coined the phrase T_{reg} tubulitis[44]. Indeed, they found that FOXP3$^+$ cells were more likely to infiltrate tubules than CD4$^+$FOXP3$^-$ cells. This infiltration of tubules provides a mechanism for the entry of T_{reg} into the urine (see next section). A separate independent study on FOXP3 mRNA in renal biopsies also found that FOXP3 mRNA levels did not correlate with good graft outcome[45].

Contrasting data came from a much smaller study by Martin, et al., who showed that of 17 kidney transplant biopsies studied, three underwent rejection resulting in graft loss within the first year[46]. These three samples had no FOXP3$^+$ cells when biopsied during the early stages of acute rejection. This study, although small, suggests that it may be worthwhile to conduct further investigations.

Levels of FOXP3 may be able to predict future rejection episodes in certain patients. A study of renal allografts with borderline changes determined that those grafts which did not progress to rejection had significantly higher levels of FOXP3 mRNA than those grafts which did progress[47]. Therefore, in this particular group of patients with borderline changes, FOXP3 may be indicative of the likelihood of progression to rejection. Similarly, in a study of 37 cases of kidney biopsies with subclinical rejection, higher numbers of FOXP3$^+$ predicted better graft function[48]. FOXP3 may be useful in defining the immunologic status in these patients, which are currently not well understood, and predicting the chances of a rejection episode so that immunosuppression can be altered accordingly.

Peripheral blood

A time-course study was carried out on levels of FOXP3 mRNA in blood from liver transplant recipients. In all 15 patients, FOXP3 mRNA levels increased by day 7 posttransplantation, and then returned to baseline by day 28. In the four patients who went on to develop T-cell mediated rejection within 60 days, FOXP3 mRNA levels returned to baseline by day 14 posttransplantation[49]. This study was

small and did not show any statistically significant differences. However, it does suggest that FOXP3, as part of a large panel of biomarkers, may be useful in the prediction of rejection.

Peripheral blood from heart transplant recipients pretransplantation was collected and T_{reg} function examined. The $CD4^+CD25^{hi}$ cells from blood samples taken pretransplantation from heart transplant recipients who went on to develop acute rejection were less suppressive than those from non-rejecters[50]. However, this does limit the usefulness of T_{reg} in blood to predict heart rejection as a functional assay of T_{reg} is probably too time-consuming to be performed clinically.

Urine

Muthukumar, et al. conducted a study into FOXP3 mRNA in the urine of kidney transplant recipients[51]. Again confirming the ability of FOXP3 mRNA levels to diagnose rejection, FOXP3 mRNA was higher in patients with rejection than those without and controls. Increased levels of FOXP3 mRNA within the urine of the rejection group was associated with better graft outcome. It is possible that the difference between the Veronese, et al. and Muthukumar, et al. data is due to the fact that the urinary mRNA levels represent only those T_{reg} that infiltrate tubules within the kidney.

Stem cell transplantation

To predict GVHD after stem cell transplantation, donor cell infiltrates, rather than the peripheral blood of recipients, were analyzed for FOXP3. Two independent studies on donor infiltrate in HLA-identical stem cell transplants showed that patients who received infiltrate with low numbers of $CD4^+FOXP3^+$ cells were more likely to suffer from severe GVHD[52,53]. A separate study used CD4 and CD25 to define T_{reg}, and found, conversely, that GVHD was associated with high numbers of $CD4^+CD25^+$ cells within the donor infiltrate[54]. However, this again may be due to the use of CD25 as a marker of T_{reg}, as it was shown by Rezvani et al. that numbers of $CD4^+FOXP3^+$ and $CD4^+CD25^+$ cells in donor cell infiltrates were inversely correlated[52].

The ability of FOXP3 to predict graft outcome in those patients undergoing rejection remains unclear. It does appear though that FOXP3 has greater potential as a predictor of future rejection episodes, both in kidney and liver transplant recipients, and may help to more successfully diagnose those patients with only slight histologic changes. FOXP3 within the donor infiltrate may also be useful in the prediction of GVHD.

Regulatory T-cells as biomarker of tolerance

As discussed earlier, some patients who discontinue their immunosuppression do not reject their grafts. This is known as spontaneous operational tolerance (SPOT), and great effort has gone into attempts to characterize these patients and define a tolerogenic profile. The results to date are summarized in table 2. This is a difficult task, given the rarity of these patients and the problem of obtaining samples from them.

Intragraft

Sachs, et al. have used a non-myeloablative conditioning regime prior to kidney and bone marrow transplantation, which has allowed them to discontinue immunosuppression at about one year post transplantation[55]. Grafts continued to function well until studied here at 2-5.3 years posttransplantation. Levels of FOXP3 mRNA in kidney biopsies were

Table 2. Summary of the use of FOXP3 mRNA measurement in SPOT patients as a biomarker of tolerance

Author	Organ	Sample source	No. of SPOT patients/total	Marker of tolerance?
Kawai, et al.[55]	Kidney	Intragraft (mRNA)	6/14	Yes
Louis, et al.[59]	Kidney	Blood (cell no. and mRNA)	8/65	No
Alvarez, et al.[67]	Kidney	Blood (mRNA)	3/40	No
Brouard, et al.[60]	Kidney	Blood (mRNA)	17/75	No
Li, et al.[58]	Liver	Intragraft (mRNA)	28/64	No
		Intragraft (cell no.)	28/64	Yes
Pons, et al.[64]	Liver	Blood (cell no. and mRNA)	5/12	Yes

SPOT: spontaneous operational tolerance.

about six-times higher in the immunosuppression-free group than in the group with good graft function that were still receiving immunosuppressive therapy. This indicates that in the absence of immunosuppression, bone marrow transplantation can induce a regulatory phenotype. However, the number of patients is small and needs to be extended to confirm this important finding.

Given the ethical considerations of performing kidney biopsies on patients with good graft function, we[56] and others[57] have used a murine kidney transplant model, in which DBA/2 kidneys are spontaneously accepted by C57BL/6 recipients. High numbers of $Foxp3^+$ cells were found in the graft and spleen of these mice. Although allografts were tolerated initially, some recipients underwent chronic rejection resulting in graft loss, and kidney allografts from these mice contained low numbers of $Foxp3^+$ cells[56].

In liver transplant recipients, FOXP3 mRNA levels in biopsies were higher in grafts from SPOT patients than in those being maintained on immunosuppression but similar to those in chronically rejected grafts[58]. However, when the FOXP3 was examined at the protein level, the number of $FOXP3^+$ cells was significantly higher in SPOT patients than in both those on immunosuppression and those that had been chronically rejected. The reason for this discrepancy is unknown as other studies have found good correlation between mRNA and cell numbers.

Peripheral blood

Louis, et al. investigated $CD4^+CD25^{hi}$ cell numbers and FOXP3 mRNA in SPOT kidney transplant patients[59]. Numbers of $CD4^+CD25^{hi}$ cells and FOXP3 mRNA levels were similar in SPOT patients and those on immunosuppression. However, cell numbers and mRNA levels were lower in chronically rejecting patients. Similar results were obtained in a separate study[59], and Brouard, et al. reached the same conclusion using peripheral blood gene expression profiles[60]. This ability to distinguish chronic rejection using T_{reg} has been contradicted, however, by a study by Ashton-Chess, et al.[61].

A Europe-wide study also analyzed mRNA levels in SPOT kidney transplant recipients[62]. They found that although FOXP3 mRNA levels alone could not be used to distinguish SPOT patients, this group did have a significantly higher ratio of FOXP3 to alpha-mannosidase mRNA than chronically rejecting patients and those with stable function on immunosuppression. This confirms the importance of examining several parameters when judging the immunologic profile of a patient.

Peripheral blood from liver transplant SPOT patients contained higher numbers of $CD4^+CD25^+$ cells than liver transplant recipients on immunosuppression[63]. In addition, $CD4^+CD25^{hi}$ cell numbers and FOXP3 mRNA levels increased and remained high upon withdrawal of immunosuppression in five liver transplant recipients who did not reject their grafts. In the seven patients who underwent rejection upon withdrawal, neither $CD4^+CD25^{hi}$ cell numbers nor FOXP3 mRNA levels increased[64]. Therefore, T_{reg} may be more indicative of tolerance in liver compared to kidney transplant recipients, and could possibly be used to identify the significant percentage of liver transplant recipients currently on immunosuppression who could be candidates for weaning (estimated to be up to 20%[65]), and to determine the success, or failure, of this process.

Conclusions

Although a great deal of conflicting data has been published on the use of FOXP3 as a biomarker in transplantation, it seems that using FOXP3 mRNA levels in peripheral blood could become a reliable method of screening for acute rejection of kidney grafts and GVHD after stem cell transplantation. Larger scale studies involving more patients from multiple centers are urgently needed to establish this. However, it is unlikely that monitoring of T_{reg} will completely replace histologic examination of the transplanted organs, but may provide additional information on the immune status of the recipients during rejection episodes. In those kidney transplant patients with only slight histologic abnormalities, FOXP3 analysis may help to clarify the status of the immune response and predict future rejection episodes. However, the potential of FOXP3 as a predictor of graft outcome is still unclear, given the number of small studies yielding conflicting results. Again, cooperation between different transplant centers to perform a large multicentre study would clarify this issue.

Some of the conflicting data described above may be related to other factors that affect FOXP3 expression. Two studies have shown that FOXP3 is increased with time after transplantation[45,61]. Factors such as these must be considered before FOXP3 is used for clinical diagnostic purposes.

It is very unlikely that one single biomarker, no matter how important in the alloimmune response, will be able to specifically and sensitively predict rejection and graft outcome. A panel of biomarkers from different facets of the immune response is probably the way forward. Genes which have shown promise include those for perforin, granzyme B, and granulysin, which reflect cytotoxic T-cell activity; NKG2D, an activating receptor on natural killer cells and T-cells; cytokines such as interferon-γ; and the chemokine IP-10 (reviewed[66]). It is likely that FOXP3, or more definitive marker(s) of T_{reg}, will be an important component of this panel. Much more work needs to be done in this area, but the ultimate goal is within our grasp. When achieved, the greatest potential for this technique would be the assessment of the individual immunologic risks of transplant recipients, thus enabling the tailoring of immunosuppression to maximize graft outcome while minimizing side effects.

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