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BIOMARKERS OF OPERATIONAL TOLERANCE IN SOLID ORGAN TRANSPLANTATION

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Abstract

Introduction—Long-term immunosuppressive therapy represents a huge burden on transplant recipients, but currently cannot be omitted. Improving long-term transplant outcome by immunosuppressive drug withdrawal may be achieved in patients who have developed (partial) immunological unresponsiveness towards their graft, either spontaneously or through tolerance induction. Reliable biomarkers are essential to define such immunological unresponsiveness and will facilitate controlled immunosuppressive drug weaning as well as provide surrogate end-points for tolerance induction trials.

Areas covered—Tolerance biomarkers have been defined for both liver and kidney transplantation and can accurately identify operationally tolerant transplant recipients retrospectively. These two tolerance fingerprints are remarkably different, indicating the involvement of distinct mechanisms. Limited data suggest that tolerance biomarkers can be detected in immunosuppressed transplant recipients. Whether these patients can safely have their immunosuppressive drugs withdrawn needs to be established.

Expert opinion—Mechanistic interpretation of the kidney transplant tolerance biomarker profile dominated by B cell markers remains a challenge in light of experimental evidence suggesting the pivotal involvement of regulatory T cells. Therefore, defining animal models that resemble human transplant tolerance is crucial in understanding the underlying mechanisms. Additionally, to ensure patient safety while monitoring for tolerance, it is essential to develop biomarkers to non-invasively detect early signs of rejection as well.

Keywords

Tolerance fingerprint; kidney transplantation; liver transplantation; immunosuppressive drug withdrawal; acute rejection

1. Introduction

Solid organ transplantation is a life-saving procedure for various end-stage diseases, but the inherent requirement for life-long immunosuppression for preventing graft rejection comes with many side effects, such as increased risk of infection¹, neoplasms², as well as nephrotoxicity³ and diabetogenicity⁴.

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Therefore, improving long-term transplant outcome by immunosuppressive drug minimization or withdrawal remains a major goal in transplant medicine and may be achieved in patients who have developed tolerance towards their graft, either spontaneously or through active tolerance induction. However, robust parameters to define transplant immunological unresponsiveness and tolerance and monitor its persistence in clinical transplantation need to be established first.

Immunologically, tolerance is defined as the absence of an immune response towards a specific antigen in the absence of immunosuppression. In contrast to clinical transplantation, animal models of organ transplantation allow for straightforward means of detecting immunological tolerance⁵. An animal is formally proven to be tolerant when, in the absence of immunosuppression, a second graft from the same donor is accepted, while a graft from a third-party donor is rejected. Since such unambiguous assays clearly cannot be used in clinical organ transplantation, the surrogate definition of operational tolerance has been established. Operational tolerance is defined as the absence of graft rejection without the use of immunosuppressive drugs⁶.

Biomarkers are characteristics that can be measured objectively and evaluated as an indicator of a normal biological process, a pathogenic process, or a pharmacological response to a therapeutic intervention⁷. At present, the only reliable means of determining the status of the graft is by biopsy⁸, but this invasive procedure is not suitable for monitoring the graft on a regular, sometimes daily basis. For defining a state of operational tolerance, biomarkers should be assessable non-invasively by using, for example, urine or peripheral blood, the latter being minimally invasive. Establishing biomarkers of operational tolerance may provide tools to select patients who are eligible for enrolment in immunosuppressive drug weaning or withdrawal, as well as surrogate end-points for tolerance induction trials.

2. Defining tolerant patient and control groups

In general, operationally tolerant transplant recipients, *i.e.* patients from whom immunosuppressive drugs can be safely withdrawn, are extremely rare and at present cannot be identified prospectively. A limited number of transplant recipients, mostly those of liver grafts, have been described to be free of immunosuppression with good graft function. The reasons for cessation of immunosuppressive drugs vary and include non-compliance, as well as physician supervised weaning due to infections, cancer or other serious side effects. The majority of transplant recipients however, will rely on lifelong immunosuppressive therapy, a situation that is sustained by the lack of biomarkers to guide weaning or cessation of immunosuppressive drugs regardless of the possibility that some patients may have developed a tolerogenic state towards their graft.

The limited number of operationally tolerant transplant recipients poses a major hurdle in reaching sufficient statistical power to perform meaningful analysis in the search of tolerance biomarkers. This is especially problematic in the case of kidney transplantation, where tolerance is a very rare event. The scarcity of operationally tolerant kidney transplant recipients (KTRs) has resulted in a joint effort by two international consortia, the Immune Tolerance Network (ITN, USA) and Indices of Tolerance (IOT, Europe) to identify a relatively large cohort of operationally tolerant KTRs⁹. These consortia have proven to be extremely valuable for the definition of markers of allograft tolerance in kidney transplantation.

Besides the limited number of operationally tolerant transplant recipients that are available to study tolerance biomarkers, defining appropriate control groups poses another major challenge. By comparing operationally tolerant patients to immunosuppressed patients with

stable graft function, one may observe effects of (lack of) immunosuppression rather than tolerance. Additionally, this group may include patients that are actually tolerant or who have developed some degree of specific unresponsiveness to their allograft unknowingly, a situation that has been suggested to occur in both liver and kidney transplantation^{10, 11}. Besides potential effects of immunosuppressive drugs on immunological read-outs such as phenotype and function, patients suffering from acute or chronic allograft rejection will display additional underlying rejection processes, thereby further complicating the definition of tolerance specific markers. Alternatively, healthy individuals do not receive immunosuppressive medication, but also do not bear an allograft, and may therefore not be an appropriate denominator to define operational tolerance.

The best control group to define the immunological profile of operationally tolerant patients would be patients in whom drug weaning or withdrawal has been attempted without success. Since in liver transplantation acute rejection appears to have only limited impact on long-term outcome, intentional weaning has been performed in several transplant centres¹² and has, in some cases, resulted in the ability to include rejecting patients in tolerance biomarker analyses. Whereas immunosuppressive drug withdrawal in kidney transplantation is associated with a significant risk of graft loss¹³, no intentional weaning without further intervention has been performed.

3. Liver transplant tolerance

Operational tolerance in LTRs appears to occur relatively frequent and is probably due to inherent tolerogenic properties of the liver and resident lymphocytes¹⁴. In several immunosuppressive drug withdrawal trials, the percentage of (highly selected) patients that retained stable graft function, and were therefore regarded as operationally tolerant, was around 20%^{10, 15-19}. Great effort has been made to define parameters that could distinguish these operationally tolerant patients from non-tolerant patients (table 1).

By using gene expression analysis, Sánchez-Fueyo and colleagues have been able to develop a 'tolerance signature' in the peripheral blood of LTRs, pointing towards several cell types that are likely to be involved in the development or maintenance of liver transplant tolerance^{10, 20}. In their first study, in which operationally tolerant LTRs (n=16), patients requiring immunosuppression (n=16) and healthy individuals (n=10) were compared, natural killer (NK) cell and $\gamma\delta\text{TCR}^+$ T cell transcripts were upregulated in operationally tolerant LTRs, as determined by microarray and real-time PCR (RT-PCR)²⁰. Additionally, phenotypic analysis of PBMC revealed an increase in the ratio of 'regulatory' V δ 1 T cells over V δ 2 T cells and an increase in CD4⁺CD25⁺ T cells expressing Foxp3 compared with patients receiving immunosuppression or healthy controls; a finding that was consistent with data published by the Kyoto group¹⁹. These data suggest that multiple regulatory cell types are involved in (maintenance) of liver transplant tolerance. A second study by the same group compared the peripheral blood transcriptional profile of a training set of operationally tolerant LTRs (n=17) to those who developed AR upon drug weaning (n=21) and healthy controls (n=16). Confirming their previous data, NK cell and $\gamma\delta\text{TCR}^+$ T cell transcripts were predominantly expressed in operationally tolerant LTRs, determined by microarray. By subsequently defining RT-PCR validated gene sets of 2, 6 or 7 genes, operationally tolerant LTRs from a separate validation cohort (n=11) could be retrospectively identified with high accuracy (error rate between 0.13 and 0.17). Likewise, a recent study on the peripheral blood transcriptional profile both adult (n=17) and paediatric (n=16) operational tolerant LTRs using microarray discovery and RT-PCR validation revealed 13 genes associated with operational tolerance, which were predominantly expressed in NK cells²¹. When tested in independent samples, the sensitivity of this gene set was 100% and the specificity 83%. These studies combined suggest that gene expression profiling by RT-PCR is highly

accurate in identifying tolerant LTRs, but improvement may be possible by including more parameters in the tolerance signature, such as the peripheral blood phenotype.

The possible involvement of $\gamma\delta\text{TCR}^+$ T cells in operational liver tolerance had previously been suggested by a flow cytometric monitoring study on paediatric LTRs^{19, 22}. This study showed a significant increase in the ratio of 'regulatory' V δ 1 T cells over V δ 2 T cells in operationally tolerant LTRs (n=12) compared to patients receiving immunosuppression (n=19) and healthy controls (n=24), suggesting that $\gamma\delta\text{TCR}^+$ T cell composition may be used as a biomarker to identify tolerant LTRs. However, a more detailed analysis on $\gamma\delta\text{TCR}^+$ T cells in adult transplant recipients revealed that the $\gamma\delta\text{TCR}^+$ T cell subset distribution is unlikely to be useful as single marker of operational tolerance. Most immunosuppressed liver and kidney transplant recipients showed an enriched V δ 1 T cell pool, regardless of the presence of a tolerant state²³.

Whereas in the studies from Sánchez-Fueyo and colleagues several NK cell related genes were upregulated in operationally tolerant adult LTRs compared to patients requiring immunosuppression²⁰ or patients who developed acute rejection after immunosuppression withdrawal¹⁰, the percentage of NK cells in the peripheral blood of paediatric LTRs in whom immunosuppression was successfully withdrawn was decreased compared to healthy individuals or stable patients under immunosuppression¹⁹. These discordant results could be attributed to different experimental readouts or due to the involvement of distinct mechanisms of operational tolerance in paediatric and adult LTRs. Paediatric recipients of liver transplants appear to develop tolerance more easily than adult recipients²⁴, which could imply that distinct or additional mechanisms of tolerance development are involved²¹.

Several studies have shown an increase in peripheral CD4⁺CD25^{high} T cells in operationally tolerant LTRs^{19, 20, 22, 25, 26}, which may not be unexpected, since the role of CD4⁺CD25⁺ regulatory T cells (Treg) in rodent models of allograft tolerance has been extensively demonstrated²⁷. Interestingly, Pons *et al.* demonstrated that upon weaning of immunosuppressive drugs, operationally tolerant LTRs (n=5) showed increased frequencies of CD4⁺CD25^{high} cells and FOXP3 transcripts over time, whereas in patients who suffered rejection upon weaning (n=7) no increase in Treg markers was observed²⁵. Koshiba and colleagues showed that the CD4⁺CD25^{high} T cells from operationally tolerant paediatric LTRs were indeed regulatory by demonstrating their capacity of donor-specific suppression in mixed lymphocyte reactions *in vitro*²². With these data in mind, it will be of interest to determine whether Tregs, defined by CD4⁺CD25^{high}CD127^{lo} will provide a better marker for tolerance, since it has been shown that CD4⁺CD25^{high}CD127^{lo} Tregs are more potent in preventing experimental transplant arteriosclerosis than those solely defined by CD4⁺CD25^{high} expression²⁸. Peripheral CD4⁺CD25^{high}CD127^{lo} T cells have already been shown to be elevated in operationally tolerant LTRs (n=26) compared to stable patients under immunosuppression (n=24)²⁹.

The observations indicating that Treg may be involved in liver transplant tolerance were further substantiated by a study showing the presence of CD4⁺Foxp3⁺ cells in liver biopsies of a subset of tolerant LTRs³⁰. Foxp3⁺ cells were present in biopsies of a limited number of stable patients under immunosuppression as well and may be interpreted as that these patients are eligible for immunosuppression withdrawal, although this has not been formally proven. Regardless of the possible predictive value for predicting tolerance, the invasive nature of a biopsy precludes using the presence of intra-graft Foxp3⁺ cells as routine practice to identify patients for immunosuppression withdrawal or to monitor for tolerance stability.

Interestingly, correlating with Treg Foxp3 expression in operationally tolerant LTRs was the level of HLA-G expressed on monocytoïd dendritic cells (mDC), implying that HLA-G

levels expressed by this DC population may be informative to detect operationally tolerant LTRs, possibly in conjunction with Treg markers²⁹. HLA-G is a non-classical HLA molecule that has been described to be involved in maintaining maternal-fetal immune tolerance³¹ and has been suggested to be involved in regulating the alloimmune response as well³². It has been proposed that soluble HLA-G in the serum may also be a measure for tolerance²⁹. Indeed, data on elevated serum HLA-G levels in stable (n=21) and tolerant (n=13) paediatric LTRs suggested that soluble HLA-G in the serum may be a marker for liver allograft acceptance and tolerance³³.

Besides HLA-G expression on mDC, the distribution of plasmacytoid DC (pDC) subsets in the peripheral blood has also been put forward as possible biomarker of liver allograft tolerance^{34, 35}. Since DC are involved in the polarization of immune responses, they are likely candidates to be able to drive the immune system towards a tolerogenic state³⁶. However, the skewed distribution of pDC in tolerant LTRs has not been confirmed by all studies²⁰ and no evidence for the involvement of DC in liver transplant tolerance was derived from microarray analysis¹⁰.

4. Kidney transplant tolerance

Compared to LTRs, kidney transplant recipients (KTRs) even more rarely retain graft function after weaning or cessation of immunosuppressive drugs¹³. This is illustrated by the fact that the ITN and IOT consortia have collectively only identified 36 operationally tolerant KTRs^{37, 38}. Regardless of this limitation, The ITN and IOT consortia have independently developed fingerprints of kidney transplant tolerance that were remarkably similar and could be cross validated in the European and American cohort, respectively. In these studies, operationally tolerant KTRs were compared to stable patients receiving standard immunosuppression and healthy individuals (both studies) as well as patients with chronic rejection and stable function while receiving low-dose steroids only (IOT study). A large number of immunological parameters were determined which resulted in a cross-platform signature of kidney transplant tolerance (figure 1).

Peripheral blood microarrays performed on the 25 operationally tolerant KTRs of the ITN study identified 30 genes that were differentially expressed between tolerant KTRs and those receiving immunosuppression, of which, unexpectedly, 23 genes (77%) were specific for B cells. Validation of these genes, as well as an additional panel of genes involved in immunoregulation by RT-PCR identified 31 genes that were differentially expressed between the two cohorts, of which 26 genes (84%) were B cell specific. Around the same time, the IOT study analysed the gene expression of 11 tolerant KTRs using a custom microarray focusing on transplantation research as well as RT-PCR. Of the 10 most differentially expressed genes between tolerant KTRs compared with immunosuppressed KTRs, those with chronic rejection and healthy controls, 6 genes (60%) were B cell related. When data of three recent microarray studies of operationally tolerant KTRs (ITN, IOT and the Nantes group) were combined, 35 genes were found to be specifically enriched in all three cohorts of operationally tolerant KTRs, despite different microarray platforms, patient characteristics and starting material³⁹. Of these genes, 24 (69%) were B cell related, strongly suggesting that B cells are involved in (the maintenance of) tolerance in KTRs³⁷⁻³⁹. Another interesting finding indicating that the increase of B cell transcripts does not solely occur in the periphery but also in the graft is that CD20 gene expression was significantly increased in urinary sediments of operationally tolerant KTRs compared to those receiving immunosuppression or healthy volunteers³⁸. Strikingly, from the 18 genes that were selected a priori for urinary gene expression, only CD20 was differentially expressed in operationally tolerant KTRs.

Additional to the gene expression analysis that suggested an important role for B cells in kidney transplant tolerance, several studies have shown an increase in the percentage or absolute number of B cells in the peripheral blood of operationally tolerant KTRs^{37, 38, 40, 41}. Contrasting results have been published regarding the phenotype of peripheral B cells in operationally tolerant KTRs. The ITN and IOT studies showed an enrichment of naïve and transitional B cells at the expense of memory B cells^{37, 38}, whereas data from the Nantes group show an increase in CD27⁺ memory B cells with an inhibitory phenotype (discussed below)⁴¹. Interestingly, in several studies on B cell repopulation after rituximab induction in transplantation, the maintenance of a predominant naïve B cell compartment after depletion was associated with prolonged graft function^{42, 43}. Whereas this indicates that a predominance of naïve B cells in the periphery may play a role in unresponsiveness towards the allograft, the exact role of B cell subsets in the development or maintenance of transplant tolerance needs to be studied in more detail.

Functionally, B cells of operationally tolerant KTRs appear to have tolerogenic properties, since stimulated total B cells displayed an increased ratio of TGF- β /IFN- γ ³⁷, whereas the transitional B cell subset, defined by high expression of CD24 and CD38 harboured elevated numbers of IL-10 expressing B cells after stimulation³⁸. Interestingly, human CD24^{hi}CD38^{hi} B cells have recently been described as containing regulatory B cells (Breg)⁴⁴, leading to the speculation that B cells with regulatory properties might be involved in transplant tolerance⁴⁵. However, while the increase in IL-10 producing cells in the transitional B cell subset of operationally KTRs is interesting, the number of IL-10 producing B cells was very low, and should be interpreted with caution³⁸. In addition, the memory B cell population that was enriched in the operationally tolerant KTRs studied by Pallier *et al.* expressed CD1d and CD5, thereby resembling the phenotype of murine Bregs⁴⁶. Interestingly, human CD24^{hi}CD38^{hi} Bregs express CD1d and CD5 as well⁴⁴. Alternatively, the enriched CD27⁺ B cell subset could potentially contain regulatory B10 cells⁴⁷. In addition, the B cell compartment in this study displayed an inhibitory phenotype as shown by a relative increase in the inhibitory Fc receptor Fc γ RIIb and an increase in the negative modulator BANK1⁴¹. No functional data on these B cells in humans have been published yet, although in a rat model of tolerance, in which B cells displayed similar characteristics, transfer of B cells from a tolerant animal into a secondary animal could transfer tolerance⁴⁸.

Another indicator for altered humoral activity in operationally tolerant KTRs is the lack of anti-donor HLA-specific antibodies in the vast majority of patients, determined by using the extremely sensitive Luminex platform³⁷. Since donor non-specific HLA antibodies were present in these patients it is tempting to speculate that at the B cell level some form of transplant-specific tolerance has emerged. Similarly, in a descriptive paper on clinical characteristics of operationally tolerant KTRs, a relatively low proportion of tolerant patients was panel reactive antibody (PRA) positive⁴⁹.

Besides the strong B cell signature of tolerance, other characteristics of operational tolerance in KTRs have been described (table 2). Firstly, the Nantes group showed that operationally tolerant KTRs (n=5) exhibited a skewed T cell receptor V β chain usage, mainly in the CD8⁺ T cell subset⁵⁰. Since these T cells did not have altered cytokine transcripts, these data were interpreted as hyporesponsiveness of donor-specific T cells in operationally tolerant KTRs. In a follow-up study by the same group, the CD8⁺ T cell population of operationally tolerant KTRs (n=6) was shown to consist of an increase proportion of central memory cells and a decreased proportion of effector cells, when compared to patients suffering from chronic rejection (n=14)⁵¹. Together, these data suggest that operationally tolerant KTRs harbour clonally expanded CD8⁺ T cells with altered functional characteristics.

In a third study by the same group, a transcriptional profile of operational tolerance consisting of 49 genes was put forward, which could distinguish operationally KTRs (n=5) from those with chronic rejection (n=11), as well as from healthy controls (n=8)⁵². This profile included the upregulation of many TGF- β regulated genes, as well as downregulation of costimulatory and T cell activation genes, therefore being rather dissimilar to the gene expression profiles described by the ITN and IOT networks, which may be due to the different platforms used. However, as described above, despite many differences, a number of genes (n=35) overlapped between these studies³⁹.

The cross-platform analysis of ITN and IOT indicated some involvement for Treg activity as well. A high ratio of expression of FOXP3 to MAN1A2 (α -1,2-mannosidase) was associated with operational tolerance in KTRs³⁷. Differential expression and the functional relevance of α -1,2-mannosidase in tolerant recipients had been previously described in animal models of tolerance^{53, 54}. Another property of operational tolerance in kidney transplantation that may be related to Treg activity is donor specific T cell unresponsiveness, both in the direct as the indirect pathway of allorecognition. By using an IFN- γ ELISpot assay for assessing direct pathway responses, it was shown that CD4⁺ T cells from operationally tolerant KTRs were hyporesponsive towards donor-antigens³⁷. This was accompanied by lower levels of activated CD4⁺CD25^{int} T cells, when compared with stable patients not on calcineurin inhibitor therapy, healthy controls and patients with chronic rejection³⁷. Additionally, by using the trans-vivo delayed-type hypersensitivity (DTH) assay, Burlingham and colleagues showed a graded donor-specific indirect pathway T cell response with the lowest response in identical twin (n=2) and operationally tolerant patients (n=11), intermediate response in patients under prednisone monotherapy (n=7) and standard immunosuppression (n=18) and the highest response in chronically rejecting patients (n=7)⁵⁵. Whether Tregs are responsible for donor specific unresponsiveness is not clear. Whereas depletion of CD25⁺ cells in the IFN- γ ELISpot assays did not influence responder frequencies³⁷, the DTH assay showed the highest Treg activity in operationally tolerant KTRs⁵⁵.

5. Organ specificity of tolerance biomarkers

From animal studies it is already apparent that different organs display different levels of immunogenicity⁵⁶, and therefore may vary in their requirement of immunoregulation for the development of tolerance. Indeed, as described above, the mechanisms in which tolerance towards liver transplants develops appear to be completely different from those involved in kidney transplant tolerance. Whereas in liver transplant tolerance the major cell types involved appear to be NK cells and $\gamma\delta$ TCR⁺ T cells, in kidney transplantation the predominant cell type appears to be B cells. Obviously, these discrepancies could be due to the use of different assays, technical platforms, as well as centre specific effects. To this end, Lozano *et al.* have investigated the transcriptional and phenotypic characteristics of liver and kidney transplantation tolerance in both operationally tolerant liver and kidney transplant recipients using a single methodology³⁹. Their analysis formally confirmed that there is minimal overlap between the gene signatures of operationally tolerant LTRs and KTRs, and that different cell lineages are involved in the establishment or maintenance of tolerance.

6. Detecting tolerance in stable patients under immunosuppression

Drug-free tolerant transplant recipients constitute a valuable patient group to define immunological parameters associated with operational tolerance. However, the ultimate goal of defining a fingerprint of operational tolerance is to be able to detect potentially tolerant individuals in cohorts of stable patient under immunosuppression. Limited data exist on the

capacity of the currently defined biomarkers of tolerance to identify patients in which immunosuppressive drugs can be withdrawn.

When stable LTRs under immunosuppression who would be eligible for immunosuppressive drug withdrawal according to the most commonly used clinical criteria⁵⁷ were examined for the molecular fingerprint of liver transplant tolerance defined by Sánchez-Fueyo and colleagues, 26% of patients were identified to be potentially operationally tolerant¹⁰. This percentage closely resembles the percentage of patients reported to exert operational tolerance after immunosuppression withdrawal¹². Likewise, Li *et al.* recently showed that 26% of adult and 64% of paediatric LTRs receiving minimal or dual immunosuppression therapy showed high prediction scores for tolerance using an independent gene panel²¹. By using these kinds of analysis prior to immunosuppressive drug weaning or withdrawal to select patients with high probability of being operationally tolerant, the success rate of immunosuppressive drug withdrawal could be significantly increased. However, whether immunosuppressive drugs in these patients have been withdrawn and thus whether the gene set correctly identified operationally tolerant patients has not been reported so far.

In their original paper describing a molecular fingerprint of 49 genes defining operational kidney transplant tolerance, Brouard and colleagues have observed 1 of 12 patients under triple immunosuppression therapy that fulfilled their criteria of operational tolerance⁵². Interestingly, in patients only receiving low-dose steroid monotherapy, 5 of 10 patients fulfilled the criteria of operational tolerance, suggesting that patients who have good graft function under less stringent immunosuppressive therapy may indeed be tolerant. On the other hand, a loss of the tolerance signature was observed in patients who developed rejection after first having been operationally tolerant. In a follow-up study, they used their 49-gene molecular fingerprint of tolerance to estimate the occurrence of tolerance in a group of 144 (highly selected) stable KTRs under immunosuppression¹¹. RT-PCR analysis of 20 most relevant genes revealed that only 5 of 144 patients (3.5%) displayed an operational tolerant gene expression profile. Again, well designed weaning studies on patients with high probability of being tolerant based on biomarker signatures will truly determine whether these signatures are useful.

7. Active induction of transplant tolerance

An alternative route for defining parameters of transplant tolerance is by characterizing the immune profile of patients who have undergone treatment for the active induction of tolerance. A relatively successful route appears to be the induction of mixed chimerism by donor bone marrow transplantation (BMT) in combination with kidney transplantation^{58, 59}. The induction of mixed chimerism has been associated with the development of central tolerance by deletion, as shown by donor-specific unresponsiveness *in vitro* in both mixed lymphocyte reactions and cytotoxicity assays⁵⁸. Central tolerance is unlikely to develop in regular solid organ transplantation and will therefore not contribute to the search of tolerance biomarkers in the routine setting of kidney transplantation. However, despite the survival of the kidney allograft in BMT treated patients, mixed chimerism appears to be only transient⁵⁸, suggesting that peripheral mechanisms of tolerance contribute as well. Indeed, one operationally tolerant KTR in the IOT consortium study had received donor-specific BMT prior to kidney transplantation and showed similar immunological characteristics as KTRs with spontaneous transplant tolerance³⁷. Additionally, intra-graft FOXP3 expression, but not Granzyme B was elevated in chimerism induced tolerant KTRs compared to stable patients receiving immunosuppressive therapy⁵⁸. These results indicate that peripheral tolerance mechanisms may indeed be (partially) involved when applying donor specific BMT to actively induce tolerance. Whether similar mechanisms are involved in spontaneous and induced transplant tolerance by BMT remains to be established.

Alternative means to achieve a tolerant state in transplantation may be by the use of lymphocyte depleting immunosuppressive drugs⁶⁰⁻⁶³. For example, alemtuzumab has been proposed as being able to promote tolerance, or at least allow for minimalisation of immunosuppressive drugs^{64, 65}. Indeed, an increase in the percentage of peripheral CD4⁺CD25⁺ Treg⁶⁶⁻⁶⁸ as well as immunosenescent CD8⁺CD28⁻ cells⁶⁹ has been demonstrated after alemtuzumab treatment. Moreover, novel data from our laboratory suggest that the B cell compartment from KTRs after alemtuzumab treatment resembles that of operationally tolerant KTRs (Heidt *et al.*, in press). Contradictory, the memory T cell compartment appears to be less susceptible to alemtuzumab depletion, potentially resulting in the homeostatic repopulation of these cells^{70, 71}. The net result of alemtuzumab induction appears to be beneficial though, with many patients having stable graft function on minimal maintenance immunosuppressive drugs⁷². This state of 'almost' tolerance has been referred to as *prope* tolerance^{64, 65} and may provide a window of opportunity for further tolerance inducing strategies, such as *ex vivo* cultured donor-specific Tregs, to be deployed.

In theory, peripheral deletion of alloreactive cells should be sufficient for an allograft to remain *in situ* without the need for further immunosuppression. The proteasome inhibitor bortezomib in combination with donor specific transfusion (DST) might be suitable to this end since bortezomib induces apoptosis of highly activated lymphocytes, including plasma cells, B cells and T cells^{73, 74}. In a recent study, pre-transplant DST in combination with bortezomib in 18 KTRs were performed with the aim of minimizing maintenance immunosuppressive drugs⁷⁵. By 264 days after transplantation, four patients (22%) were free of immunosuppression and seven (39%) were on low-dose steroid monotherapy. These encouraging results support the previous notion that clonal deletion through DST can be beneficial to clinical transplant recipients⁷⁶ and warrant further research on the usage of bortezomib to enhance this effect.

Besides the opportunity to define additional biomarkers of tolerance in patients who have undergone active therapy for inducing transplant tolerance, the already established biomarkers may provide surrogate end points for studies on active tolerance induction.

8. Conclusion

Allograft survival in the absence of immunosuppression is one of the remaining major goals of transplantation medicine. Safe weaning of immunosuppressive drugs relies on the availability of robust biomarkers of transplant tolerance. In recent years major steps have been made in the discovery of biomarkers to define operational tolerance in both LTRs and KTRs. These encouraging results have collectively contributed to the establishment of fingerprints of both liver and kidney transplant tolerance. Interestingly, the two fingerprints are very dissimilar, which suggests that distinct mechanisms are involved in the establishment and maintenance of liver and kidney transplant tolerance.

An important unanswered question is why some transplant recipients develop tolerance while other reject their graft upon immunosuppressive drug cessation. One could imagine that differential immunogenicity of allografts could play a role. However, the operationally tolerant KTRs from the IOT cohort received highly mismatched grafts, whereas those from the ITN cohort received well matched grafts. This suggests that, when regarding every HLA mismatch as equally immunogenic, there is no effect of differential immunogenicity on the development of tolerance. However, due to amino acid triplet sharing between HLA molecules, not every mismatch may be immunogenic in the context of the recipient's own HLA repertoire, at least as shown for antibody responses⁷⁷. Additionally, post-transplant immunologic activity towards pathogens that may lead to heterologous immunity could

potentially play a role in whether transplant tolerance may be achieved⁷⁸. In this respect, timing of immunosuppressive drug cessation is likely to be critical.

The difficulty with clinical biomarkers studies remains that, in general, unresponsiveness is not confirmed by biopsy. Understandably, the risks involved in taking a biopsy in a patient who is seemingly doing well without immunosuppressive medication is considered too big. Nevertheless, the next major step to be made is to determine whether these fingerprints can be harnessed for selecting patients for drug minimization and/or withdrawal studies. Only then will the true value of these biomarkers to identify operationally tolerant transplant recipients be revealed. In addition, it will be of interest to see whether tolerance biomarkers will provide surrogate markers of success in attempts to actively induce transplant tolerance. Many novel strategies to actively induce transplant tolerance are currently scrutinized, some of them showing great potential. Finally, a detailed long-term follow up on operationally tolerant patients will be necessary to formally determine whether patients that have stopped taking immunosuppressive drugs without short-term complications will have good long-term graft and patient outcomes.

9. Expert opinion

While rather clear profiles of tolerance markers in both liver and kidney transplantation have been described, the mechanistic interpretation, especially of the B cell dominated profile discovered for kidney transplantation, remains a challenge. Increasing evidence suggests that the phenotypic distribution of the B cell subset can influence long-term graft outcome, supporting the notion that B cells may be involved in transplant tolerance^{42, 43}. However, regardless of the fact that the role for B cells in immune regulation is becoming increasingly clear⁷⁹, a large body of experimental evidence in animal models of transplantation suggests a pivotal role for T cells in the regulation of the alloresponse as well²⁷.

What could be an explanation for the largely dissimilar findings in experimental and clinical transplant tolerance research? Firstly, it may be that separate mechanisms are involved in the establishment and maintenance of transplant tolerance⁵⁵. It is not inconceivable that Tregs play an essential role in the development of tolerance, whereas B cells are involved in the maintenance of tolerance, or their appearance is merely a result of the tolerant state. Since most of the operationally tolerant KTRs that have been studied had been free of immunosuppressive drugs for an extended period of time, the possibly differential requirements for development and maintenance of tolerance are difficult to address.

An alternative explanation would be that operational tolerance may present itself differently in the periphery than locally in the transplanted organ. Being minimally invasive, peripheral fingerprints of liver and kidney transplant tolerance do not exclude the possibility of the involvement of other cell types within the graft, or in the lymph nodes draining the graft.

The occurrence of operational tolerance in transplant recipients is rare, and could in theory be attributed to a lack of immunocompetence in these patients, rather than the development of active immunological tolerance. Although largely unexplored, there is limited data indicating that this is not the case. Ballet *et al.* have used hemagglutination inhibition and ELISpot assays to determine the humoral and cellular response rate of operationally tolerant KTRs to influenza vaccination⁸⁰. They found no difference in response when comparing healthy volunteers with operationally tolerant KTRs, whereas immunosuppressed patients displayed a diminished humoral response. Despite the fact that there are no reports of increased rate of infection of malignancies in operationally tolerant transplant recipients⁴⁹, the possibility of altered immunocompetence should not be ruled out.

Finally, it may be that the development of tolerance under laboratory conditions is fundamentally different from tolerance as seen in the clinic. It is conceivable that the level of immunoregulation required in humans is different from laboratory animals due to heterologous immunity both prior to the development of operational tolerance and once the tolerant state has been established. Indeed, in mouse models of transplantation, the deliberate infection with viruses prohibited the induction of transplant tolerance^{78, 81}. This phenomenon may account, at least partially, for the differences in tolerance markers found in rodent models and clinical transplantation.

It will be crucial to define animal models that more closely resemble human transplant tolerance in the near future to understand the underlying immunological mechanisms that result in transplant tolerance. Possibly, humanised mouse models are a first step in understanding how the human immune system is tolerized in the presence of a human allograft^{28, 82}.

A major point in clinical care for operationally tolerant transplant recipients is the ability to monitor for its stability. Several papers have already described transplant patients that lost their tolerant state towards the allograft and developed a rejection response^{39, 49, 52, 80}. Brouard *et al.* showed that in 2 KTRs the occurrence of rejection followed a loss of the tolerogenic phenotype⁵². However, whether the loss of the tolerance signature is truly predictive for the development of rejection is not known. Weaning immunosuppression will have an inherent risk of acute rejection, despite using selection criteria based in tolerance biomarkers⁸³. Therefore, it is not only important to monitor for markers associated for operational tolerance, but for the early occurrence of graft rejection as well. Since at present no reliable non-invasive early biomarkers for acute rejection that can distinguish between immune activation toward the allograft and infectious agents exist⁸⁴, it may not be wise to embark on drug weaning and withdrawal studies yet. Similar to the multi-platform approach for tolerance biomarkers in KTRs, a combination of markers will be needed to provide sufficient specificity and sensitivity.

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Abbreviations

BMT	bone marrow transplantation
Breg	regulatory B cell
DC	dendritic cell
DST	donor specific transfusion
DTH	delayed type hypersensitivity
KTR	kidney transplant recipient
LTR	liver transplant recipient
NK cell	natural killer cell
PRA	panel reactive antibody
RT-PCR	real-time PCR
Treg	regulatory T cell

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Article Highlights

- Tolerance biomarker profiles have been determined for both liver and kidney transplant recipients.
- Liver and kidney tolerance biomarker profiles are distinct; in liver transplant tolerance mainly NK cells and $\gamma\delta\text{TCR}^+$ T cells are involved, whereas in kidney transplant tolerance a clear B cell signature has been found.
- Limited data suggest that tolerance biomarkers can be detected in immunosuppressed transplant recipients. These patients might benefit from immunosuppressive drug weaning.
- Novel approaches such as donor-specific bone marrow transplantation and pre-transplant alloreactive T cell depletion may allow for the active induction of transplant tolerance.
- Besides monitoring for transplant tolerance, it is essential to develop biomarkers to non-invasively detect early signs of rejection as well.
- It will be crucial to define animal models that closely resemble human transplant tolerance to understand the immunological mechanisms that result in transplant tolerance.

Figure 1.

Cross-platform biomarker signature of kidney transplant recipients determined by the IOT consortium. The signature is dominated by B cell features such as elevated expression of mainly B cell specific genes, the absence of donor-specific HLA antibodies in the serum and elevated levels of (naïve and transitional) peripheral B cells. Other markers that make up the tolerance signature are the ratio of FOXP3 to α -1,2-mannosidase gene expression, a low donor-specific direct T cell response and decreased levels of activated T cells.

Table 1**Biomarkers for operational tolerance in liver transplantation**

Parameter	Technique	Description	Reference
<i>NK cells</i>			
NK cell transcripts	microarray/RT-PCR	NK cell related gene expression upregulated	10, 20, 21
Peripheral NK cells	flow cytometry	Percentage of peripheral NK cells downregulated	19
<i>$\gamma\delta$TCR⁺ T cells</i>			
$\gamma\delta$ TCR ⁺ T cell transcripts	microarray/RT-PCR	$\gamma\delta$ TCR ⁺ T cell related gene expression upregulated	10, 20
V δ 1/V δ 2 T cell ratio	flow cytometry	Elevated V δ 1/V δ 2 T cell ratio	19, 20, 22
		V δ 1/ V δ 2 T cell ratio upregulated in all transplant recipients regardless of tolerant state	23
<i>Regulatory T cells</i>			
CD4 ⁺ CD25 ^{high} T cells	flow cytometry	Percentage of peripheral CD4 ⁺ CD25 ^{high} cells upregulated	19, 20, 22, 25, 26
		Increase of CD4 ⁺ CD25 ^{high} cells over time after weaning	25
CD4 ⁺ CD25 ^{high} CD127 ^{lo} T cells	flow cytometry	Percentage of peripheral CD4 ⁺ CD25 ^{high} CD127 ^{lo} cells upregulated	29
<i>Dendritic cells</i>			
pDC/mDC ratio	flow cytometry	Elevated pDC/mDC ratio	34, 35
		No difference in pDC/mDC ratio from operationally tolerant LTRs compared to controls	20
DC HLA-G expression	flow cytometry	Elevated HLA-G expression on mDC	29
Serum HLA-G	ELISA	Elevated serum HLA-G levels	33
<i>B cells</i>			
Peripheral B cells	flow cytometry	Percentage of peripheral B cells upregulated	19

Table 2**Biomarkers for operational tolerance in kidney transplantation**

Parameter	Technique	Description	Reference
<i>NK cells</i>			
Peripheral NK cells	Flow cytometry	Elevated percentage and number of peripheral CD56 ⁺ CD3 ⁻ NK cells	37
<i>T cells</i>			
Peripheral T cells	Flow cytometry	Percentage of peripheral CD3 ⁺ T cells downregulated	37
		Lower percentage of activated CD4 ⁺ CD25 ^{int} T cells	37
T cell alloreactivity	IFN- γ ELISpot	Donor specific hyporesponsiveness	37
	Trans-vivo DTH assay	Low donor specific response, comparable to identical twin transplant	55
Whole blood gene expression	RT PCR	Elevated FOXP3/ α -1,2-mannosidase ratio	37
<i>B cells</i>			
Peripheral B cell phenotype	Flow cytometry	Elevated percentage of peripheral CD19 ⁺ B cells	37, 38, 41
		Increased percentage of naïve and transitional B cells	37, 38
		Increased percentage and number of memory B cells	41
		Absolute number of total (and naïve) B cells	38, 40
B cell cytokine production	IC cytokine staining	Skewed cytokine response total B cells towards TGF- β production	37
		Increase in IL-10 producing cells within the transitional B cell subset	38
PBMC/B cell gene expression	RT-PCR	Decreased CD32a/CD32b mRNA ratio, increased BANK1 expression	41
Whole blood gene expression	Microarray/RT-PCR	B cell specific gene expression upregulated	38, 39, 41
Urinary gene expression	RT-PCR	Elevated CD20 expression in urinary sediments	38
Serum HLA antibodies	Luminex	Majority of operationally tolerant KTRs had no serum anti-donor HLA antibodies	3