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REVIEW

Reconsidering the detection of tolerance to individualize immunosuppression minimization and to improve long-term kidney graft outcomes

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Summary

In kidney transplantation, minimizing the side effects of the immunosuppressive regimen and inducing tolerance to allograft are the two main objectives to improve outcome. At present, these objectives are far from being achieved and remain elusive for the majority of transplant recipients. Rejection rate and mortality on the long term are still unacceptable. There is thus a pressing need to improve this situation. Therefore, some spontaneously tolerant kidney recipients are described in clinics, and recent advances in immunological and molecular techniques have led to a resurgence of interest in studying those rare transplanted recipients through coordinated efforts from international consortia. Indeed, they offer, on the one hand, the possibility to develop specific biomarkers indicative of this state that would constitute a major advantage in the care of the patients allowing personalized minimization of drugs, so reducing related costs and side effects. On the other hand, they represent a unique model of study to understand the mechanisms of regulation implicated in this state that may help the development of inducing therapies. Recent efforts, concentrated on noninvasive analyses of peripheral blood, identified a predominance of several B-cell subsets, some of which harbouring regulatory functions, and related marker genes. These findings, validated in independent multicentric cohorts, led credence to an unsuspected role for the B-cell compartment in tolerance to kidney allograft. The identification of patients, harbouring these markers, among immunosuppressed recipients with stable graft function and the existence of drugs with selective effect on B cell pave the way for the possibility to improve long-term graft outcomes. Therefore, before routine application, these findings need to be confirmed in large prospective studies in the context of planned reduced immunosuppression.

Preamble

Advances in our understanding of human immunological processes and developments in new therapeutic and diagnostic agents make the detection and/or induction of graft tolerance a real possibility in the near future. Many therapeutic agents with potential tolerogenic properties have been described, and some of them are currently undergoing clinical trials [1]. The recent characterization of well-defined biomarkers of tolerance represents a considerable help for the development of tolerance therapeutics. Identifying patients in whom donor-specific tolerance has developed would constitute a major advance in the care of transplant recipients. This ability would allow the minimization or even the withdrawal of immunosuppressive (IS) therapy in selected patients, thus reducing the number of adverse effects and costs and optimizing long-term graft outcomes. With these tools, studies will benefit from an

appropriate clinical endpoint to define the operational tolerance state. Long-term prospective studies could address the generation of tolerance but only in the context of IS withdrawal protocols. The only chance for a weaning study to be successful will be a carefully designed one, in which weaning is considered in the presence of increased surveillance and using validated biomarkers of tolerance.

In the setting of liver transplantation, results from the group headed by Dr Sanchez-Fueyo have led to the characterization of biomarkers predictive of tolerance [2]. These findings have led to the design of the first protocol in clinical transplantation, in which a tolerance signature has been used to monitor disease and inform decisions on drug withdrawal [2]. Notwithstanding, in kidney transplantation, drug withdrawal trials are not acceptable as such protocol represents a real risk of graft loss. Final validation of the biomarkers will need to be performed much more cautiously. One approach would be to test their predictive capacity in drug minimization trials conducted in subgroups of kidney recipients with low immunological risk. This is currently under process in a clinical study of calcineurin inhibitor (CNI) weaning (http://clinicaltrials.gov/ct2/show/NCT01292525) conducted with the centre of the CENTAURE network (http://www.fondation-centaure.org/). As an alternative, biomarker-guided minimization trials could be proposed. It might be preferable, however, to gain deeper understanding of the pathogenic role of circulating B-cell subsets in the development of kidney allograft tolerance before biomarker-guided minimization studies are conducted. Until these studies are completed, investigators should be discouraged from conducting immunosuppression weaning attempts on the unique basis of transcriptional biomarkers whose validity has not been confirmed signatures.

This review discusses recent advances in the identification of the B-cell subsets and related stable biomarkers indicative of tolerance in the setting of kidney transplantation. These findings may contribute to reduce immunosuppressive (IS) treatments and serve to guide new therapeutic approaches. The routine clinical use of these markers, once validated, would bring the possibility of personalized medicine.

Introduction

Transplantation is the treatment of choice for end-stage renal diseases. It is one of the revolutionary fields in modern medicine that has saved thousands of lives. According to current estimates, 18 000 recipients live with a transplanted kidney in Europe and up to 2700 patients are transplanted each year in France. As quality of life is improved and length of life is also significantly prolonged after kidney transplantation [3], its application has been progressively and successfully extended to new indications, particularly in aged patients. Furthermore, there is compelling evidence of continuous improvement in kidney recipient and transplant survival over the last two decades [4] that are attributed to not only a general improvement in surgical techniques and clinical management, diagnostic tools and control of infectious and neoplasia [5], but also in a better control of the alloimmune response thanks to immunosuppression (IS) [6,7]. Modern IS, classically based on maintenance triple-drug regimen (including a calcineurin or mammalian target of rapamycin inhibitor, an antiproliferative agent and corticosteroids), has drastically decreased acute rejection incidence to drop below 10% in most transplant centres [8]. However, these drugs have a marginal effect on chronic rejection as long-term graft loss rates remain unmodified [6,9–11]. The cost for IS drugs is about 1110 Euros monthly for a patient [12]. Lifelong IS is far from being harmless as it is associated with numerous side effects including infectious complications [13–18], malignancies [5,19] and metabolic disorders [20] that contribute substantially to morbidity and mortality among transplant recipients [21]. Of major concern, cardiovascular diseases [22], opportunistic infections [13] and malignancies [23] are especially underscored as particularly deleterious accounting for 70% of deaths in patients with well-functioning kidney allograft. In addition to these concerns, calcineurin inhibitors, which form the backbone of most commonly used IS regimens, are nephrotoxic, a side effect that likely contributes to both the premature failure of renal allografts and the development of end-stage renal disease in individuals who have received nonrenal transplants [24,25]. Histological lesions compatible with long-term calcineurin inhibitor exposure are observed in virtually all transplants in the long term [11]. Thus ironically, long-term survival of kidney transplants, which initially benefited from modern IS treatments, may now be principally limited by the effects of long-term exposure to these drugs [6,11,15,16]. Importantly, these side effects and also the functional modifications of the graft are reversible upon IS weaning [26,27]. Face to the burden of chronic IS [28], these observations have progressively shifted the attention of clinicians towards a need for IS minimization. This question is undoubtedly challenging as it requires to achieve a balance of adequate graft protection while minimizing the consequences of excessive IS [29]. Such task implies to weight the risks of precipitating acute rejection or chronic allograft dysfunction while minimizing IS [30]. Identifying 'low-risk' patients among kidney-transplanted cohorts using relevant biomarkers is therefore crucial to be able to more precisely understand how to assess rejection risk in recipients who could be selected for safe IS minimization. Ideally, a situation of long-term graft acceptance in the complete absence of IS drugs, a situation known as

Table 1. Common side effects of main immunosuppressive drugs.

	Medication	Side Effects
Medication used for maintenance therapy	Calcineurin inhibitors (CNI)	Nephrotoxicity, neurotoxicity, hypertension, hyperlipidemia and hyperkalemia, diabetes mellitus, increased bone resorption, hirsutism, gingival hyperplasia, hearing i mpairment and cholestatic syndrome, post-transplant malignancies and skin cancers
	Azathioprine	Hepatic nodular hyperplasia, portal sclerosis, myelosuppression, post-transplant malignancies and skin cancers
	Mycophenolate	GI disturbance, myelosuppression growth retardation
	Corticosteroids	Cushingoid appearance, fluid retention, diabetes mellitus, hypertension, growth impairment, hyperlipidemia, osteopenia, impairment in wound healing, failure to thrive
Medication used for induction therapy	Anti-CD25 receptor antibodies (basiliximab, daclizumab)	Anaphylaxis, allergic reaction
	Anti-CD52 monoclonal antibody (alemtuzumab)	Profound lymphocyte depletion, which increases the risk of infection, in particular CMV reactivation
	Antithymocyte globulin (ATG)	Lymphopenia, serum sickness, anaphylactic reaction, shock, bronchospasm

'tolerance' is thus increasingly regarded as an ideal solution [31] in kidney transplantation. This 'Holy Grail' of transplantation [32] is not fiction as recent advances in transplant immunology suggest that this clinical state exists and may be achievable in near future. Identifying these 'low-risk' patients as candidate for IS minimization and understanding the mechanisms to induce this state are important objectives to face IS problems. But, even more importantly, study of such a unique process may help to understand how the follow-up of patients under classical IS may be improved.

Current strategies to reduce the burden of lifelong is

As a result of the success of effective IS, many more transplant recipients live now longer after transplant compared with decades ago and have time to manifest the long-term effects of chronic IS. Indeed, it is becoming increasingly clear that if an effective control of rejection on the one hand protects the graft function and prolongs patient survival, at the same time the patient is exposed to the risk of complications of prolonged IS and also to new post-transplant disease, even in the presence of excellent graft function. These complications result from either persistently low immune defences as a result of IS therapy (infections and malignancies) or as a result of side effects of IS drugs, which affect virtually every organ system (renal function impairment, diabetes, cardiovascular disease among all). Some of these main side effects associated with the most commonly used drugs are detailed (see Table 1). Current clinical trials are now concentrating on how to reduce, prevent or antagonize the burden of chronic IS. Strategies to limit the impact of chronic IS include reduction of full drug dose, development of new non-nephrotoxic agents and trials of tolerance induction.

Alternatives to full drug dose

To balance efficacy while limiting side effects of existing drugs, four alternative approaches to full dose have emerged [33] (see Table 2) that may help to guide protocols towards individualization of specific IS regimens.

The first one is drug minimization which reduces the amount of the drug administered. This alternative may be undertaken from the time of transplant (*de novo*), or later post-transplant (elective) as a result of an adverse event. It can be defined as the attainment of a state in which, thanks to routine monitoring, drug is decreased down to levels that do not cause clinically significant side effects yet prevent rejection [34]. The second one is drug conversion, which tapers drug dosing at any time post-transplant until achieving full replacement with alternative immunosuppressants. This alternative may be undertaken at any time post-transplant and is usually a result of an unacceptable drug-related adverse event. The third one is drug withdrawal, which slowly eliminates the amount of drug administered early or late post-transplant. The fourth one is drug avoidance, which substitutes other drugs.

All of these alternatives to full drug usage also involve the use of concurrent immunosuppressant agents in standard or low doses (triple therapy). Indeed, most used IS protocol is traditionally composed by a triple therapy maintenance regimen consisting of corticosteroid, an antimetabolite and either a calcineurin inhibitor (CNI) or a mammalian target of rapamycin (mTOR) antagonist. Many patients continue using this triple regimen at medium and even long term from transplant [35]. Thus several studies in the literature had analysed the effects of minimization of IS regimens or the avoidance of some drugs from therapeutic protocols [30,33,36], especially CNIs and steroids. Reduction or suspension of steroids, previously during long-term follow-up, seems to be related to a higher

Table 2. Alternatives to full dose drug regimens.

Strategy	Definition	Timing
Minimization	Lower dosage of drug	Planned *de novo*, or result of adverse event
Conversion	Tapering of drug dose until eliminated and replaced with other immunosuppressant	Usually result of adverse event
Withdrawal	Tapering of drug dose until eliminated, may be replaced with other immunosuppressant	Planned *de novo* or result of adverse event
Avoidance	No drug given, other immunosuppressant used	Planned *de novo*

incidence of acute rejection, while this modification is safer if performed during the first weeks after transplantation. Suspension of calcineurin inhibitors (CNI) is related to a higher incidence of rejection and an improved renal function. The introduction of mTOR antagonist (e.g. sirolimus and everolimus) is characterized by increased levels of lipids, and a long-term observation is required.

In spite of these efforts, if modern IS is now manageable in the short term, it still remains a major hurdle for long-term outcomes in renal transplantation [37]. Nowadays, no clear consensus exists about the comparative efficacy and safety of these alternatives to full-dose drug regimens [30,33,36]. Reasons for the lack of rational approaches are manifold [30,33,36], but most of the studies were performed using selected populations of recipients, as first transplants, patients without previous episodes of acute rejections or with stability of renal function. In addition, the absolute low volume of renal transplantation, the lack of valid surrogate markers for long-term outcomes including patient and graft survival, unclear reference range plasma levels in therapeutic drug monitoring of immunosuppressant combination therapy over time as well as the lack of biomarkers for the patients' humoral and cellular immune response status also contribute to the difficulty of these strategies.

These alternatives also imply, especially for conversion, the development of new IS agents to achieve adequate immunosuppression with minimal toxicity [20]. Ongoing attempts have lead to discovery of several newer promising agents with different mechanisms of actions as exemplified by belatacept that might be used without maintenance steroids or calcineurin inhibitors [38]. These protocols may also include induction agents (monoclonal antibodies) to maintain sufficient therapeutic effectiveness. Another area of current study is formed by formulation of specific protocols for induction of tolerance acquired after transplantation and creation of tests that could demonstrate it.

Is minimization

Up to now, the combination of multiple drugs has markedly increased the efficacy of the IS regimen. Therefore, the intensification of these treatments has also resulted in over-IS-associated side effects (e.g. opportunistic infections and malignancies) and in the emergence of new complications. Because these treatments have also negative impacts on the quality of life, physicians have to struggle for reducing these deleterious side effects [39]. This is exemplified by the emergence of a previously rare infection, BK virus nephropathy, which may account for irreversible graft loss in 3 to 5% of renal transplant recipients [40]. Since 1990, a new IS strategy has gained much credit in the transplant community, named 'minimal IS' [41,42]. The rationale was that new IS combinations with fewer and lower doses of drugs may be effective yet less toxic [43]. Drug minimization regimens are thus being explored in select patient populations to improve the safety of current IS protocols while preserving their efficacy. This strategy is based on the concept that, over time, the risk of rejection decreases and, at the same time, the cumulative risk for toxicity increases. Indeed, IS is usually heavier in the perioperative period and early post-transplant (induction) when the risk of rejection is higher due to a number of factors including preservation injury of the graft and sudden exposure of the recipient immune system to a load of foreign antigen. Later, depending on graft function and tolerability, IS doses are gradually reduced (maintenance) to levels adequate to prevent rejection and avoid toxicity. In theory, it is not far-fetched to imagine that drug minimization should allow allograft to function normally with normal histology [44], an ideal situation called prope tolerance, near tolerance [45,46], partial tolerance [41] or minimal IS tolerance [47]. Most studies have concentrated on corticosteroids and calcineurin inhibitor minimization [48]. These two groups of medications that are well known to cause direct side effects are yet the backbone of all IS therapies. Careful patient selection and close monitoring of graft function are mandatory steps for a successful conduct of a drug minimization attempt to avoid rejection and graft loss [49]. Besides, the use of various lymphocyte-depleting agents for 'induction therapy' could help to create a milieu in which the graft is well tolerated under an 'umbrella' of low dosage IS [41].

New agents

The past decade has witnessed unprecedented advances in renal transplantation propelled by novel and effective IS drugs. The introduction of mycophenolate mofetil (MMF), tacrolimus, cyclosporine microemulsion, sirolimus, a new generation of monoclonal antibodies (the anti-interleukin-2 receptor blockers, daclizumab and

basiliximab) and the depleting polyclonal biological thymoglobulin has provided transplant physicians with a wide choice in selecting effective IS regimens [50–53]. More recently, new agents targeting B-lymphocytes and other mechanisms involved in the alloimmune response (including complement and other mechanisms) have also been introduced [54]. These include antibodies and fusion proteins interfering with T-cell-mediated activation via LFA-1/ICAM-1, CD2/LFA-3, CD40/CD154, and CD28/B7.1 and B7.2 interactions [55]. Intracellular targets involved in T- and B-cell activation pathways are also being evaluated, including protein kinase C inhibitors, Janus-associated kinase (JAK) inhibitors and proteasome inhibitors. Other new medications demonstrate promise in inhibiting donor-directed humoral immunity by targeting B-cell-activating factor (BAFF) and complement activation pathways. Finally, other drugs aim also at targeting the 'memory' component of the T-cell repertoire [56] or the regulatory component [57]. Some of these new drugs are being evaluated in clinical trials in attempt to reduce the burden of side effects and complications of agents currently available [58,59].

Tolerance induction

Inducing tolerance is becoming a main goal in transplantation [31] as the risk for IS-related side effects would be nullified, and consequently, we could expect an improvement in patient and graft survivals and overall outcomes, as well as in quality of life. This concept was first experimentally demonstrated in 1953 [60] when *in utero* injection of bone marrow cells to mice resulted in acceptance of skin graft from the same inbred donor while maintaining the ability to reject grafts from other breeds. Since then, a large number of cell-based and noncell-based strategies have been developed for tolerance induction [61–64]. Many of these techniques are closely linked to the development of potent pharmacologic and biological agents – as the ones used to block costimulation and cytokines [65–68] – or the utilization of available IS drugs – as the ones used for induction treatment to deplete the lymphocyte pool [69–71]. If induction of tolerance has largely been an achievable feat in animal, particularly murine models [72–74], nonhuman primate also identified some successful preclinical tolerogenic approaches, from T-cell depletion and mixed chimerism to costimulation blockade and cellular therapies [75,76]. Some criteria (applicability, robustness, stability over time, compatibility and measurability) are pointed as important features of a clinically useful tolerance-inducing strategy [77,78]. In the light of this, some ethical issues in tolerance induction trials also have to be considered [79]. The fact that IS morbidity is now clearly less than the morbidity of a failed organ [80] challenges the assumption that IS weaning would be worth the risk of graft loss. The success of a tolerance-inducing trial must therefore be better or equivalent to standard IS care and the patients participating in such trials must be chosen carefully. Up to now, successful induction of clinical tolerance in renal transplantation has been rarely achieved and most promising intents relied on concurrent stem cell transplantation and the achievement of mixed bone marrow chimerism [81,82] as attested by three independent interim reports [69–71] showing that bone marrow transplantation from the donor enables some kidney recipients to be totally weaned off IS 1 year after transplantation.

Pitfalls and limits of current strategies

The prospect for approval by the Food and Drug Administration of newer and more specific IS drugs that lack toxicity is several years away [53] so that it is thus unlikely that novel therapy for renal transplantation will be soon approved. In the interim, drug minimization regimens are being explored [48]. Nevertheless, in spite of impressive efforts for minimizing adverse effects by close drug monitoring and multiple drug combination [83], transplant specialists have to face the reality that for unknown reasons this strategy has not had the expected impact on long-term outcomes of renal allograft. The changing demographics of donors and recipients as well as latent subclinical rejection not adequately controlled by low-dose IS may be implicated in this failure [84]. More than a decade later, no consensus has emerged yet [85]. We cannot fully inhibit IS side effects yet [86]. Minimization remains a risky procedure and an active field of research. It becomes evident that for success, IS minimization should be carefully individualized [87].

In spite of slow but steady progress in tolerance induction [88], translation of experimental data from rodents into the clinics has proven difficult [89,90]. While the size of the memory cell pool in inbred rodents is very limited (almost naïve), heterologous immunity resulting from previous immunological exposure could acts as a barrier to tolerance induction in humans [91]. More broadly, immunity in human is amazingly complex and regulatory mechanisms involved in the development of tolerance are not solved yet [92–94]. In addition, there is also evidence that some commonly used IS drugs inhibit some mechanisms of tolerance induction so that it is difficult to know how to introduce a new tolerance-inducing protocol in the setting of standard IS therapy. Although occasionally achieved [69–71], these protocols are currently limited to very small cohorts of patients meeting very specific criteria hampering their generalization [95]. Thus, these strategies are not yet available for daily clinical practice [64,96]. The efficacy, safety and impact on the long term of these protocols [77] also still remain to be evaluated.

An alternative to solution these limits

Improving long-term transplant outcome by IS minimization or withdrawal remains a major goal in kidney transplant medicine and may eventually be achieved in patients who have developed tolerance through active induction therapy. At present, according to the limits of these two strategies, robust parameters to define transplant immunological unresponsiveness and tolerance and monitor its persistence in clinical transplantation need to be established [97,98] to define transplant recipients amenable to drug minimization or withdrawal. Needless to say, it is not an easy task as it implies to find tolerant patients and define biomarkers that could identify them [99].

Biomarkers, as defined by the 'Biomarkers Definition Working group', 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 intervention [100]. Ideally, a biomarker is accurate and reproducible, with high sensitivity and specificity as well as high positive and negative predictive values. A biomarker should also be widely available, rapid, easy to use and inexpensive if it is to find a place in routine clinical practice [100]. At present, the allograft biopsy remains the 'gold standard' of assessing the status of the graft [101], but this invasive procedure is not suitable for monitoring the graft on a regular, sometimes daily basis. Evaluation of graft function is thus based on creatinine and proteinuria levels. They give essential information on kidney function and allow the medical staff to adapt the patient's treatment. Therefore, these parameters lack specificity as they can vary under normal physiological conditions as well as with disease [102]. For instance, the rate of production of creatinine is dependent on muscle mass, which is subject to the major modifying effects of age, gender and ethnicity. Although widely used in clinical practice of kidney injury, they correlate poorly with actual kidney function and offer little useful prognostic information regarding the likelihood of organ failure or recovery. Moreover, they provide no information about the immune status of the organ recipient. Thus there is still an urgent need to develop biomarkers of tolerance [103]. Biomarker discovery is an active domain of research in kidney transplantation [104] to establish molecular diagnostics [105] personalized medicine [106,107], especially by improving post-transplant monitoring [108,109] and prediction of long-term outcome [110]. For defining a state of operational tolerance, biomarkers should be assessable noninvasively using, for example, peripheral blood or urine [111], the latter being minimally invasive. Establishing biomarkers of operational tolerance may provide tools to select patients who are eligible for enrolment in IS drug weaning or withdrawal, as well as surrogate endpoints for tolerance induction trials.

The characterization of the tolerance status also needs the availability of tolerant patients. Amazingly, 'Spontaneous operational tolerance (SOT)' has been observed in humans [112] and refers to rare noncompliant recipients and others deliberately removed from IS who did not develop rejection even long after the event [113]. These cases provide the proof that tolerance can be achieved [114] and represent a unique opportunity to dissect the mechanism implicating in the development and/or maintenance of this status and identify relevant biomarkers [115]. Elucidation of the related mechanisms is a prerequisite to induce this state and the identification of biomarkers of tolerance, the individualization of IS and real-time monitoring of post-transplant immune responses may help to achieve this goal [116].

Operational tolerance as a unique model of research

Definition of the clinical status

The original definition of Medawar in the 1950s referred to nonresponsiveness to antigens [117]. In animal studies, tolerance may be defined as good long-standing graft function in the presence of a competent immune system, with no signs of graft immune injury. The latter definition is obviously not useful in human transplantation, and therefore, 'operational tolerance' is the term most widely used. Given that no biopsy can be performed, kidney transplant recipients who have been successfully weaned from IS and have maintained stable graft function for 1 year or more are referred to as functionally or operationally tolerant [118–120]. These cases are usually observed by chance when transplanted recipients no longer taking their IS drugs do not reject their graft. Spontaneously tolerant patients stop their immunosuppressive treatment for two major reasons: noncompliance and the occurrence of deleterious side effects of the IS drugs (drug toxicity or malignancy) [118–120]. The precise prevalence of tolerance among kidney recipients is currently unknown. These cases are rare (<1‰ of kidney-transplanted recipients) [121], and current estimates report 100 cases over the world [112]. This number is certainly higher as a substantial part of the compliant kidney recipients could be in fact tolerant [113]. The rarity of these patients renders difficult their study.

These patients can maintain for decades with excellent graft function [118,122,123]. The majority of them had received IS treatment in the past involving azathioprine (AZA) and corticosteroids. This observation is particularly compelling as AZA, one of the oldest pharmacologic IS agents in use today [124], was shown to prolong renal

allograft survival [125]. Accordingly, this agent is associated with very long stable renal transplant recipients [126–128]. The good functional results of these patients should be interpreted taking into account the fact that most of them were transplanted before the beginning of the CNI era. The absence of CNI use probably translated into a prevention of chronic nephrotoxicity together with a high-incidence acute rejection as observed before the introduction of CNIs as a modern IS therapy at the beginning of the 1980s [129]. This finding is of importance because the concept of CNIs leading to chronic nephrotoxicity has actually been challenged by studies demonstrating that immune injury is the leading cause of graft damage and loss and that CNI nephrotoxicity has a minor, if any, impact on chronic allograft nephropathy [130–134]. One can thus speculate that avoiding CNI use could contribute to very long-term graft survival in the absence of tubulointerstitial fibrosis.

Accordingly, AZA was used in 1962–1963 at the University of Colorado [135] for the generation of a bellwether series of long-surviving kidney allograft recipients that established renal transplantation as a clinical service. The recipients of kidneys from 46 live related donors were pretreated with AZA for 1–2 weeks before transplantation and then given AZA monotherapy afterwards. Prednisone was added only for the indication of overt rejection. This protocol was based on the assumption that if the mechanisms of tolerance can be subverted by the customary heavy immunosuppression [136], this undesired consequence could be prevented by observance of two therapeutic principles: recipient pretreatment and the use of minimal post-transplant IS [136]. From this study, nine of the kidneys subsequently functioned for the next four decades and are among the longest surviving organ allografts in the world [137]. Importantly, seven from the nine patients became drug-free tolerant for a period of 3–38 years [137]. Despite the fact, this strategy makes kidney recipients more tolerant and thereby less IS dependent; no similar cluster of tolerant kidney recipients was produced subsequently, anywhere in the world. The explanation for the failure to duplicate these results was evident. In the end of 1963, pretreatment was de-emphasized because a significant number of IS-related infectious complications had occurred prior to transplantation. A second modification was prompted by losses of kidney allografts whose rejections could not be reversed or controlled once they had begun. In contrast to minimal post-transplant IS, high doses of prednisone were now instituted from the time of operation, rather than as specifically indicated.

Tolerant patients do not differ from other transplant recipients as to whether they received a kidney from a deceased or living donor, and the number of HLA incompatibilities is at the same level as in other transplant recipients [72,118,121,138–141]. If the individual parameters and history of these patients are extremely variable [118–120], several interesting features could emerge from their careful follow-up. First, operational tolerance can develop even in the presence of either HLA mismatches at baseline or anti-HLA antibodies during follow-up, as well as in patients having experienced acute rejection [118]. Second, tolerant cases had not been nonspecifically immunosuppressed, as they did not present any significantly increased risk for either opportunistic/severe infections or cancers, but showed responses to vaccination comparable to the general population [142]. Third, operational tolerance process has been shown to be metastable over time, as demonstrated by a non-negligible proportion of patients who lose their graft for immunological reasons or simply due to physiological age defects [120]. Finally, operational tolerance corresponds to an immunocompetent situation [142] associated with immune regulation as shown by a decrease of the donor-reactive delayed-type hypersensitivity (DTH) response specific to the donor [140,143].

Technical considerations on the biodetection of tolerance

The research on these patients is intense [115]. Many studies have tried to dissect the phenotype of tolerance in kidney transplantation to understand its mechanisms but also to be able to identify patients under conventional IS who could have developed tolerance to their transplant. However, these studies on operationally tolerant kidney patients are very heterogeneous, either due to the techniques, controls used or to the various clinical profiles of the tolerant recipients [118–120,140,143–159]. Moreover, the cohorts studied are small, which prevent a robust statistical approach. These considerations markedly contribute to the difficulty for generalization and standardization of the results [157]. Finally, the lack of biopsies is a problem in these patients, as some indications of graft deterioration may not be detected [120]. In the search for new biomarkers and more specifically tolerance biomarkers, there are three major dilemmas: the first concerns the technology to be used, the second is the origin of the samples, and the third is the control population.

Choice of the technology: A major barrier to clinical tolerance is the absence of a method to detect it prospectively. In animal models, donor and third-party skin grafting has been used as a robust test, but this is not a practical clinical approach for many reasons. In humans, many immunological assays have been used as surrogate tests to monitor the immune response after transplantation [97,98]. Tests on antigen-specific T-cell responses (mixed lymphocyte reaction, limiting dilution) have not been shown to predict the development of tolerance or been helpful guides for IS withdrawal. More recent tests of precursor frequency

(enzyme-linked immunosorbent spot, tetramer analysis and others [160,161]), although promising during IS, have not yet been applied in tolerant patients. Benefiting from advances in genomic science [105,162], very sensitive molecular techniques have become available to quantify relevant gene expression patterns and protein signatures in biological samples [163,164]. They have been of added value [165] to characterize spontaneously tolerant transplant recipients [166] and establish a tolerance gene signature [167,168].

Choice of the compartment: Regarding the origin of the samples, peripheral blood, graft biopsy [145] and urine have all been used for analysis [157]. In kidney transplantation, the graft biopsy is recognized as the 'gold standard' for rejection diagnosis [101]. However, in most cases, operationally tolerant patients refuse biopsy and it is ethically questionable to perform a biopsy of a fully functional graft. Cellular infiltrates have been reported to be very low in tolerant kidney graft [145] and would therefore probably not yield a great deal of information [169]. Analysing the peripheral blood [111,170] has the advantage of being less invasive and less expensive than biopsy, which makes it the main method used to analyse gene profiles [111]. Blood is a promising source of diagnostic markers and therapeutic molecules [171,172], but it probably does not always reflect what is happening in the graft [173]. In kidney transplantation, analysing urine may have several advantages [111,170]: its collection is noninvasive and inexpensive and urine is in direct contact with the grafted organ, which could give relevant information on kidney function [174,175]. Unfortunately, high variability in concentration and volume makes the quantification of biomarkers often difficult and occasionally unreliable.

Choice of the control: The other significant dilemma is choosing the right control population, which is not easy for tolerant patients [104,176]. On one hand, these patients have been grafted and have good graft function, so we might suppose that stable patients under immunosuppressant treatment would be the best control, but the absence of immunosuppressive drugs in the tolerant patients could influence the results. On the other hand, the absence of treatment makes tolerant patients similar to healthy individuals, but we cannot ignore the absence of transplantation in the latter group. Comparison of tolerant patients with patients undergoing chronic transplant rejection has also been used, but these patients are clinically very different. Moreover, it cannot be excluded that some tolerant patients could present functionally undetectable subclinical graft lesions, and the absence of systematic kidney biopsies for these tolerant patients prevents any conclusions from being drawn. Faced with the lack of the perfect control population, the use of multiple controls may be the best alternative.

In the last 10 years, a number of studies have been conducted to identify new, robust, tolerance biomarkers, and two major consortia have been involved in the discovery of such biomarkers: the Immune Tolerance Network (ITN) in USA (http://www.immunetolerance.org/In) and the Indices of Tolerance (IOT) in Europe (http://www.rise-tfp6.org/). The existence of such consortia is essential, as operationally tolerant patients are rare (as previously stated, less than 100 known in the world), and so, it is the only way to develop multicentre studies and to have access to larger cohorts of patients.

Tolerant recipient display a b-cell gene signature

Our group and others have analysed the transcriptome of peripheral blood mononuclear cells. These five analyses revealed an increased expression of B-cell-related genes in tolerant patients compared with stable patients [146,154,157,159,177]. In a *princeps* study [177], tolerance was characterized by a footprint of genes signing immune quiescence and implicating the overexpression of B-cell markers such as such as CD79a, CD79b, CD19 or CD20 [177]. Such enrichment of B-cell markers was also reported in other following studies: 23 genes (77%) [178], six genes (60%) [159] and 24 genes (69%) [154].These genes were especially involved in the proliferation, activation and maturation of B cells [158] in accordance with the higher number of B cells in these patients [153]. Also many genes expressed by naive and transitional/immature B cells were overrepresented in tolerant patients confirming the enrichment of these subsets in this group [157]. Some of these genes could be commonly identified between the different studies [154] such as the CD20 protein, which was reported to be strongly expressed in blood from tolerant recipients in three of the five transcriptomic studies [154,157,159] and also detected in their urines [157]. As the number of samples in each of the studies was relatively low, the significance of these results was assessed through a meta-analysis [179]. The high number of samples analysed (96 samples from 50 tolerant recipients from three independent multicentric cohort: French, UK and USA) led to the identification, for the first time, of a specific gene signature. This signature could unequivocally distinguish tolerant from other recipients (>90% accuracy) through cross-validation and was remarkably enriched in B-cell-related genes. Of interest, these markers linked to B cells were among the best discriminative ones between tolerant and stable recipients. A minimal selection of the top 20 genes (see Fig. 1), mostly enriched in B-cell markers, yielded similar performances of discrimination and could be validated in an independent set of 18 samples including new tolerant cases. These data provide proof of principle that tolerance can be identified among transplanted recipients by the use of a

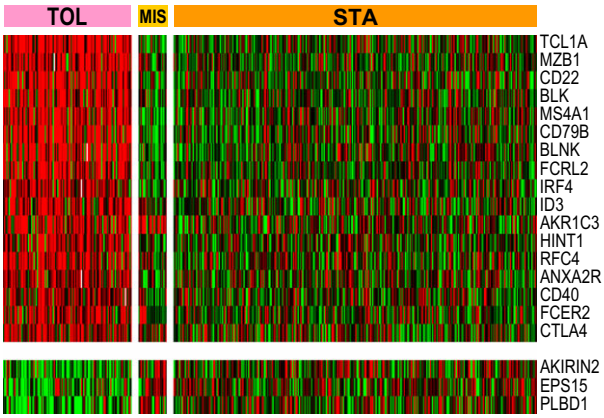

Figure 1 Expression of the 20 genes related to tolerance in peripheral blood samples. Results from microarrays are depicted for tolerant (TOL; $n = 114$), recipients with stable function under minimal immunosuppression (MIS, $n = 25$) or standard immunosuppression (STA, $n = 322$). Gene expression values are visualized by a heat map using green for gene under expression, black for gene expression close to the median and red for gene overexpression.

20-gene predictor, mostly centred on B cells [179]. Hence, these biomarkers could be used to detect tolerance and stratify kidney recipients in clinics. First, they may help for a better follow-up of the tolerant recipients. Several lines of evidences indicate that tolerance is likely not a stable situation for 'entire life' [177]. In such situation, these biomarkers could predict future graft loss and immunotherapy could be reinstated before the first clinical symptoms appear. Second, these biomarkers may help to monitor recipients under IS regimens. Among stable cases, those detected as having a low risk of rejection would be highly eligible for progressive IS weaning.

Tolerant recipient display expanded b-cell subsets

Accordingly, cellular analysis by flow cytometry reported an increase in absolute number of B cells in tolerant patients compared with immunosuppressed recipients [153]. This finding has been further replicated and validated by three studies [157–159]. This increase was associated with an enrichment in naive and transitional B-cell subsets in the peripheral blood mononuclear cells of tolerant patients [157,159], and a lack of plasma cells attributed to a default in B-cell differentiation and a higher sensibility to apoptosis in the late stages of differentiation [180]. Phenotypic analysis identifies a global inhibitory profile with a diminution of CD32a/CD32b ratio, increased expression of BANK-1 (which negatively modulates CD40-mediated AKT activation) and augmentation of CD1d CD5-expressing B cells [158], which are considered to be regulatory phenotypes [181].

These studies thus support the fact that operationally tolerant recipients display a strong B-cell signature. This feature is unique to kidney tolerant recipients as not observed in liver tolerant patients [154]. At present, the exact reasons for the expansion of the different B-cell subsets in the blood of tolerant recipients are unknown. Therefore, it is interesting to note that first recipients with end-stage renal disease have a significant reduction in the peripheral total B-cell count [182–184]. Second, after transplantation, patients are treated with a strong IS therapy which guarantees a stable function of the graft but strongly alters the immune system. But some patients who stopped their treatments managed to tolerate their kidney allograft and are subject to a strong immune reconstitution with an increase of naïve or immature B cells in their blood compared with stable recipients. This phenomenon is observed in kidney transplantation tolerance mediated by mixed chimerism with a repopulation of transitional B cells in tolerant recipients [185]. Accordingly, a recent study suggest that B cells could play a role in the maintenance of tolerance but not in its induction and that their development may be due to the progressive weaning off IS, which may allow regulatory populations to emerge [140]. These data suggest that the repopulation by immature and naïve B cells could be a feature facilitating tolerance in kidney transplantation [186] and reinforce the essential role of the B-cell compartment [187].

Therefore, whether these findings are truly linked to the development and/or maintenance of tolerance or the unique reflect of an absence of treatment is still hotly debated in the transplant community. This is an important question because noncompliance prevalence has been proven elevated among transplanted recipients [188] so that based on biomarker expression, cases identified as low-risk transplanted recipients under IS [150] could be only reflective of nonobservance. Assuming this hypothesis would not alter the main conclusions and even reinforce the utility of these findings in clinic. In such situation, the signature would allow to detect uncompliant but highly stable patients who are thus *de facto* true 'operationally tolerant'. Nevertheless, several elements strongly suggest that this signature is not the unique result of IS cessation. First, this signature was not shared by most of kidney recipients on weaning (minimal immunosuppression with corticosteroids) [159,177,179]. Second, neither kidney recipients with chronic rejection under dialysis and totally off IS [177,179] nor tolerant recipients with liver allograft [154] displayed this signature. Third, patients with atopic dermatitis treated with doses of cyclosporine A equivalent to those of transplanted recipients did not display the changes characteristic of B cells from kidney transplant recipients under IS [180]. By contrast, tolerant recipients and healthy volunteers display roughly the same profile [179] both at the

transcriptional and B-cell phenotype levels [157,189]. This suggests that, as previously mentioned [190], tolerant patients may harbour a global preservation of their 'phenotype', especially the B-cell compartment, that may contribute to maintain a physiological cell homoeostasis counteracting inflammation and preserving a 'healthy profile' in these patients. Indeed, while deep depletion of this compartment is associated with higher incidence rejection [191], its preservation would be a prerequisite to favour the development of tolerance [189]. Accordingly, as operational tolerance in kidney transplants is more often detected in patients who have carried the graft and thus IS for a long time [118,119], one can hypothesize that long IS may create a immune restart [140,156] towards a homoeostatic equilibrium, as the one observed in healthy volunteers [190].

An unsuspected role for b cell in operational tolerance

The implication of the B-cell compartment in the development and/or maintenance was further evidenced by the tolerogenic property of these cells. In a rat model of long-term cardiac allograft [192], tolerant rodents were shown to display an increased B-cell number in their blood, a blockade in the IgM to IgG switch recombination process and overexpressed BANK-1 and CD32b. Most important, B cells from tolerant rats were able to transfer tolerance [192]. Thus, as observed in humans [158], tolerant rats have a accumulation of B cells exhibiting an inhibited and regulatory profile [37] strengthening their role in the maintenance of transplantation tolerance. These observations strongly support the fact that B cells may exert regulatory functions in tolerance.

Indeed, B cells with immune-regulating function (Bregs) largely contribute to immune regulation [193,194] and although limited at present, such regulatory B cells could also play a crucial role in the development and/or maintenance of tolerance to allograft [178,187,195–198]. The term 'regulatory B cells' was first introduced following the identification of Bregs as an IL-10-producing B-cell subset [199]. Therefore, a unique marker defining a Breg phenotype has not yet been described, and consistent differences have been reported between murine and human Bregs [181,199,200]. The pathways whereby regulatory Bregs exert immunosuppressive functions essentially include the secretion of two cytokines, IL-10 and transforming growth factor-beta (TGF-B) [181,199–202] or the production of the serine protease granzyme B (GrB) [203].

In the setting of operational tolerance, the identification of Bregs subsets producing TGF-B [159], IL-10 [157,196] or GrB [204] bring mechanistic considerations linking B cells to suppressed immunity in tolerance to allograft. An increase of TGF-B-producing B cells was observed in tolerant recipients [159]. This suggests that B cells of tolerant patients had a skewed cytokine response, with a higher propensity for TGF-B production than B cells from other study groups [159]. These results are corroborated by a regulatory profile with marked increase of TGF-B expression [156] and the implication of the TGF-B pathway in operationally tolerant recipients [150]. This production of TGF-B in B cells could be regulated by increase of miR142-3p through a negative feedback loop [152]. Although IL-10 was detected for any study group (tolerant and stable recipients), no different production was observed [159]. Indeed, polyclonal activation of total B cells revealed no difference in cytokines secretion in all groups of transplanted patients [158,159]. By contrast, stimulation of transitional B cells showed an enrichment in IL-10-secreting B cells in tolerant and healthy donors compared with stable patients [157,180]. These results could be explained by the fact that transitional B cells constituted only 0–5% of total B cells [205]. Consequently, this response could be undetectable in total B cells. While these transitional B cells could represent a regulatory B-cell population based on their increased IL-10 production [187], no difference in B-cell subsets (total, naïve and transitional cells) or inhibitory cytokines (IL-10 and TGF-B) was detected when compared with healthy controls [157]. This is corroborated by the observation that a functional B-cell regulatory compartment is preserved in blood from operationally tolerant and healthy volunteers, with normal capacity to phosphorylate signal transducer and activator of transcription 3 (STAT3) after activation [189]. Compared with stable recipients and healthy volunteers, as chronic rejection patients display B cells with impaired suppressive function (inability to efficiently inhibit autologous T-cell proliferation) [206] and a quantitative decrease of Bregs with a skew in their cytokine polarization (decreased IL-10/TNF-a ratio) [207], it is likely that B-cell-inducing tolerance could be explained by a preserved B-cell compartment. Recently, the regulatory function of Breg was evidenced by GrB rather than IL-10 production [204]. In this study, tolerant recipients harboured a higher number of B cells expressing GrB and displaying a CD19+ CD5+ CD27+ CD138+ phenotype. These Bregs were able to actively inhibit effector T cells through a contact and granzyme B-dependent pathway [204].

These data on B cells, with a an increase of B-cell populations with regulatory properties and a decrease in plasma cells producing deleterious antibodies, in tolerant patients, are very encouraging. They have been reproduced in varying studies in different cohorts of tolerant patients and strongly suggest that a critical balance of the B-cell compartment is essential in tolerance (see Fig. 2). Reconciling the function of these B cells with other regulatory cells as Tregs [208] and with a concomitant role for T cells will not be difficult due to their well-documented interaction and

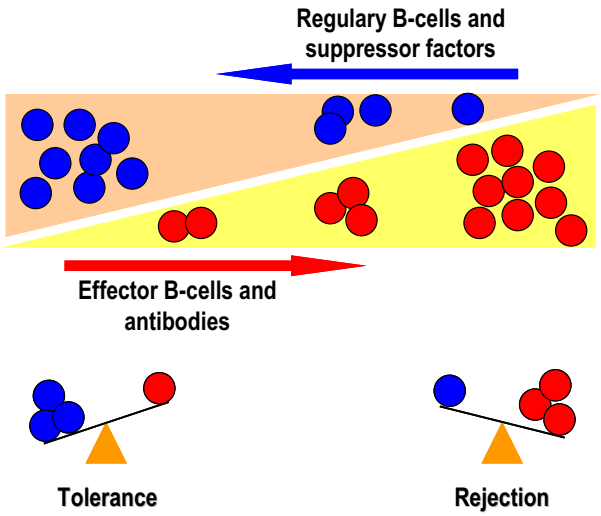

Figure 2 The balance between regulatory (Breg) and effector (Beff) functions of the B-cell compartment. This balance emphasizes the role of the number of Bregs in the development and/or maintenance of tolerance to kidney allograft. In the presence of too large numbers of Beffs (red circles), the regulatory mechanisms, consisting of Bregs (blue circles) circles) and suppressive factors (cytokines, Gzb), are unable to attenuate the immune response, which therefore leads to graft rejection. However, in the presence of a sufficient number of Bregs, the mechanisms of regulation can suppress the immune response, which leads to graft tolerance.

the strong link between cellular and humoral immunity [209,210]. However, it is difficult in the present data to determine whether the 'B-cell signature of tolerance' preceded the development of tolerance and thus possibly contributed to its development or whether it arose after tolerance had developed and could reflect a tolerant state *per se*. These considerations should have important implications for the design of protocols of IS minimization as well as tolerance-inducing regimens and assays for detecting tolerance.

The future for tolerance

Identification of kidney recipients eligible for is minimization

One of the major goal of nephrologists is thus to define patient eligibility for immunosuppressive drug weaning, which could improve graft survival and patient quality of life in the long term. Although histological examination of graft biopsies is the 'gold standard' for assessing recipient status [101], this procedure is invasive and cannot be easily repeated in stable recipients. Given that some patients continue to have well-functioning grafts after IS withdrawal, it is reasonable to assume that a certain percentage of renal transplant patients under IS are susceptible to becoming spontaneously tolerant after IS weaning. The development of a 'B-cell' biomarker signature as diagnostic test of tolerance [179] opens up the possibility of rationally designed IS weaning protocols by improving individual monitoring [85] and clinical decision aids [211]. These biomarkers would help to define patient eligibility for IS interruption or minimization procedures [177]. The principle is based on the assumption that stable transplanted recipients under IS and harbouring these markers [177] are potentially tolerant and present a low risk of rejection [85]. This is strongly supported by a loss of peripheral tolerance-related markers in some patients that correlated with a change in clinical phenotype from operational tolerance to rejection [177].

Based on previous analyses, it is expected the tolerance signature to be present in a small percentage (5–10%) of patients with stable kidney function [150,159,177]. Recent analyses confirmed these previsions as 7% of stable recipients under IS accurately displayed the tolerance gene signature [179] (see Fig. 3). Remarkably, among these stable patients, some cases on weaning (corticosteroid monotherapy) harbour this gene signature [159,177,179] and also an indirect T-cell response close to that of tolerant patients [140], demonstrating that *prope* tolerance [45] is achievable through IS minimization. Therefore, these results also highlight the rarity of candidates eligible for IS minimization. They are supported by the large survey of 6000 kidney-transplanted recipients in the United States performed over 20 years showing that from the 48 patients who stopped IS therapy, only six conserved stable renal function for more than 3 years [121]. This is very low compared with transplanted liver recipients as operational tolerance is most commonly in as much as 20% of patients [212,213]. The main reason given for this success is the so-called hepatic tolerogenicity [214,215]: all allografts are not created equal and liver is more tolerogenic than other as an immune-privileged site. Conversely, kidney graft should be more susceptible to rejection as attested by stronger T-cell proliferation and differentiation than in other organs [216]. Liver is also an organ with regenerative capacity such as it can silently endure an immune attack that would lead to loss of a more sensitive allograft. Regardless of severity, the vast majority of acute cellular rejection episodes in hepatic transplantation are not are not life- or allograft-threatening, do not produce significant morbidity, and current IS can easily reverse [217,218] most often totally [217,219,220]. Liver allografts are also more resistant to antibody-mediated rejection [221] and even the early phases of chronic rejection are reversible [222]. Therefore, if a liver allograft recipient develops acute or the early chronic rejection during or after weaning, the process is likely to be completely reversible without significant damage [217,222]. At present, IS minimization in kidney recipients remains a risky procedure (irreversible graft damage)

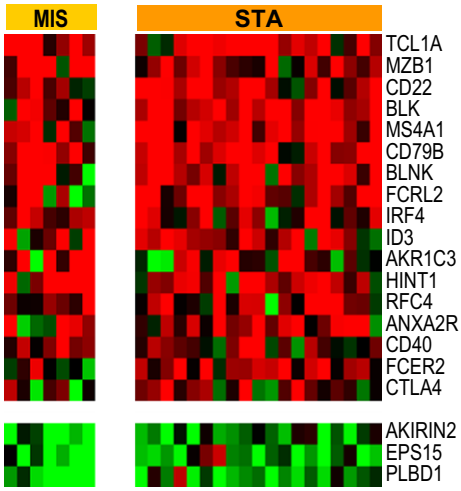

Figure 3 Expression of the tolerance gene signature in blood samples from a subset of stable recipients. The expression of the 20 genes related to tolerance is depicted for seven minimally immunosuppressed recipients (MIS) and 19 patients under standard immunosuppressive regimen (STA). These kidney recipients under IS have a good graft function and display a tolerant profile in their blood. Results from microarrays are visualized by a heat map using green for gene under expression, black for gene expression close to the median and red for gene overexpression.

and appropriate selection of the patients is a necessity for the safety of the strategy [85]. In spite of the low number of potentially tolerant among stable recipients, this strategy remains advantageous regarding drug costs and quality of life (IS side effects).

Nevertheless, the number of eligible patients should be greater as there is an increase body of evidence that tolerance could develop with time. Operational tolerance in kidney transplants is more often detected in patients that have carried the graft for a long time [118,119]. Likewise, in liver transplants, the probability of acute rejection during IS weaning protocols is higher in patients with a short follow-up [2], and recent data suggest that tolerance markedly increases with time (6% at 3 years, 33% at 6 years and >60% at 10 years post-transplantation) [223]. Among kidney recipients with stable function, potential tolerance is more often detected among 'highly stable' recipients who keep a good graft function on the long term [150]. Accordingly, increased numbers of B cells, and particularly high numbers of transitional and naïve B cells are occasionally observed in very long-term survivors with a single renal transplant [126]. Although limited at present, recent data on the expression of B-cell tolerance-related markers in stable kidney transplant patients who are still on IS strengthen these observations. Compared to patients with rejection, immature and naïve B-cell-related and operational tolerance-associated transcripts (CD20, TCL1A, CD79B, TOAG-1) were upregulated in the peripheral blood in rejection-free kidney transplant recipients within the first year post-transplantation [224]. Accordingly, the expression of two other B-cell-related genes (IGKV1D-13 and IGKV1-4), expressed specifically in operational tolerant [157], showed a time-dependent increase in blood of stable recipients (see Fig. 4) associated with a specific increase of naïve and transitional B cells [128]. This increase was only observed for patients under calcineurin inhibitor (CNI), not azathioprine (AZA), an effect linked to intrinsic differences between the two drugs on the immune system as demonstrated in experimental settings [225]. The markers were expressed by 0% at 1, 7% at 5 and 25% of CNI cases at 10-years [128]. At that time, only 4 of the 15 CNI cases displayed levels of markers similar to the ones from operationally recipients [128].

Nevertheless, it has been proposed that long-term surviving grafts in patients on AZA constitute just a selection of cases with a favourable immune adaptation to the graft regardless the IS protocols and may possess some unique immunological characteristics [126,127]. In such patients, in contrast to tolerant recipients, AZA was shown to decrease the B-cell compartment [126,128]. This effect on B-cell homoeostasis could be attributed to the suppression of T cells as shown for many IS agents [226]. Indeed, AZA exerts its immunosuppressive effect by halting DNA replication [124] and turning the costimulatory signal CD28 into an apoptotic signal, resulting in lymphocyte depletion [227–229]. Accordingly, antigen-specific tolerance has been achieved in experimental systems using AZA, which led to T-cell anergy or apoptosis [230]. Unfortunately, the tolerizing effect of AZA has been less robust in human solid organ transplants, resulting in movement towards newer, more potent IS drugs such as CNIs (cyclosporine A and tacrolimus) and antiproliferative agents (mycophenolate mofetil and rapamycin). Therefore, if AZA has lost its place as first-line therapy in kidney transplantation, it holds promise for the development of new drugs that could induce allograft-specific tolerance [229]. With the knowledge that an AZA metabolite can block CD28 signalling via Rac1 [229], one could envision that chemical modifications may result in a more specific compound that alone, or in combination with others, could induce long-lived antigen-specific tolerance. In this way, the first costimulation blocker belatacept (an anti-B7 compound designed to block CD28) has recently been approved and provides a promising alternative to calcineurin inhibitors in renal transplant recipient [231,232]. The bulk of data argues that belatacept's targets intersect with pathways relevant to Treg generation and function [66,233]. Very interestingly, AZA but not CNI treated stable patients had a higher number of regulatory peripheral T-lymphocytes (Tregs) than patients with chronic rejection [128]. Several studies, including ours, have suggested a potential role for regulatory T cells (Tregs)

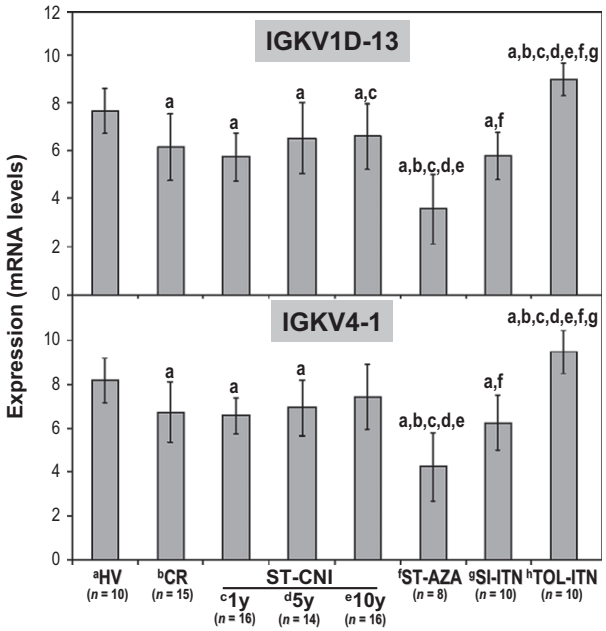

Figure 4 Expression of IGKV1D-13 and IGKV4-1 genes in peripheral blood samples. Results from RT-PCR are presented as Log2(2(−ΔΔCq)) values and displayed for groups of patients from local cohorts and from the Immune Tolerance Network (ITN). These groups include healthy volunteers (aHV); patients with chronic rejection (bCR); patients with a stable renal function under calcineurin inhibitor treatment (ST-CNI) at 1 year (c1y), 5 years (d5y) or 10 years (e10y) post-transplantation; patients with a stable renal function under azathioprine (fST-AZA); patients from the ITN registry with a stable renal function under standard immunosuppressive regimen (gSI-ITN); and tolerant recipients from the ITN registry (hTOL-ITN). Significant differences ($P < 0.05$) are represented by the letters corresponding to the groups in comparison (e.g. 'a' indicates significant difference with HV).

in tolerant recipients as suggested with overexpression of several genes associated with regulatory functions [147,155,156,159,177]. Even more, stable patients and recipients with ongoing chronic rejection display lower levels and proportions of CD4+ CD25+ Foxp3+ T cells compared with operationally tolerant patients and healthy volunteers [147,155]. More recently, a specific expansion of $CD4^+$ $CD45RA^-$ $Foxp3^{hi}$ memory Tregs was exclusively found in tolerant patients [208]. These memory Tregs exhibited a specific increased demethylation of FOXP3 TSDR (Treg-specific demethylated region) and stronger suppressive properties in tolerant patients [208].

Altogether, these findings suggest that multiple cell subsets, including B- and T-cell subsets, are potentially involved in the development and/or maintenance of tolerance in humans. Also, they suggest that different IS schedules modulate the immune cell populations that participate in the graft acceptance. This observation deserves further investigation on the use of 'tolerance permissive' IS regimens and is discussed in next section. These data also suggest that under IS, the critical threshold to achieve operational tolerance depends on a long period (a decade) after transplantation. This is of major interest, as weaning protocols for stable kidney transplant should be aimed at patients with a long follow-up. To date most of the attempts to minimize IS have been performed soon after transplantation, after 1 year or less [234–236], and were associated with a significant increase of rejection.

Of course, such studies do not provide formal proof that operational tolerance can be achieved in these patients and the fact that some stable patients are really tolerant is a hypothesis that only a weaning/minimization of IS could confirm. Only prospective studies on very large cohorts and at later time (at least 5 years post-transplantation) will satisfy the 'proof of concept' of this assumption.

Induction of tolerance by b-cell therapies

According to the potential pivotal role of the B-cell compartment, B-cell-directed therapy is thus emerging as a key component in achieving transplantation tolerance and long-term graft survival [237]. Presently, a B-cell approach for tolerance induction is promising, but further investigation on how these cell populations regulate alloimmune response is necessary [238]. Moreover, this strategy may be limited due to the prohibitive costs, availability (with only a few centres capable of amplifying cell populations to sufficient numbers), and issues of standardization and biological regulation [239]. As an alternative, the existence of some specific 'tolerance permissive' IS regimens may help to expand specific B-cell subsets [185,186,240].

In this context, the application of the tolerance gene signature for the immune monitoring of the patients should be a great advantage to identify potentially tolerant recipients and could also help to develop new permissive regimens.

Kidney transplant recipients undergoing B-cell depletion (use of the monoclonal antibody to CD20 rituximab) for desensitization experienced reconstitution with transitional B cell, while the donor HLA-specific memory B-cell repopulation was significantly delayed [241]. Alemtuzumab induction therapy effectively depletes B cells and is followed by rapid repopulation up to levels exceeding baseline [186]. The reconstitution of the lymphocyte compartment was characterized by a marked transient increase in transitional B cells and cells with phenotypic characteristics of regulatory B cells, as well as a long-term dominance in naive B cells [186]. Finally, in a model of tolerance induction based on a combined kidney and bone marrow transplantation, B-cell reconstitution with a high frequency of peripheral transitional B cells was observed in tolerant recipients (three of the four patients) [185] showing that the involvement of B cells in the mechanisms of tolerance is not

limited to operational tolerance but also concerns patients with therapeutic-induced tolerance.

Similar observations came from non-human primate (cynomolgus macaque) studies as long-term allograft acceptance has been achieved by augmenting traditional immunotherapy with B-cell-depleting antibodies. An induction immunotherapy regimen, consisting of rabbit antithymocyte globulin (thymoglobulin) and rituximab, promoted long-term islet allograft survival in macaques maintained on rapamycin monotherapy [240]. The B-cell reconstitution began 100 days after transplantation and long-term survivors exhibited immature and transitional B cells in contrast with early rejectors [240]. Rituximab (plus cyclosporine) also prolonged cardiac graft survival, inhibited DSA production and attenuated chronic rejection [242].

These studies support that selective use or pairing of B-cell-depleting agents can generate tolerance-promoting B-cell phenotypes and eliminate factors leading to chronic rejection. As B-cell depletion is inadequate for preventing xeno-specific antibodies [243] and has mixed results in desensitization [244–246], further evaluation is needed to optimize its use in transplantation. In the light of this, several new promising molecules could be considered.

For instance, recent data in rodents demonstrated that depletion of the B-cell compartment, at the time of transplantation, can induce tolerance by the deletion of alloreactive clones and so remodelling of the BCR repertoire [247,248]. In clinical setting B-cell tolerance has also been observed in the developing immune system of heart-transplanted children under ABO-incompatible conditions [249]. The overexpression of genes involved in class switch and receptor editing strongly support that remodelling of BCR repertoire in tolerant recipients to kidney allografts [157]. Reinforcing this observation is the fact that B-cell reconstitution in combined bone marrow-/kidney-transplanted tolerant recipients was preceded by elevated serum BAFF level (a member of the TNF family involved in proliferation, survival and maturation of B cells) and coincided with the development of alloantibodies and auto-antibodies [185]. Of major interest, selective targeting of B-cell activation through inhibition of BAFF was shown to promote tolerance in murine allograft models by depleting follicular and alloreactive B cells, promoting an immature/transitional B-cell phenotype, abrogating the alloantibody response and sustaining a regulatory cytokine environment [237,250]. Together, these data suggest that the presence of alloantigens could remodel the humoral repertoire of tolerant recipients [251] and help the emergence of Breg subsets.

Conclusion

The results summarized within this review are encouraging. Through impressive conjoint efforts from important transplantation networks (Indices of Tolerance – IOT and Reprogramming the Immune System for the Establishment of Tolerance – RISET in Europe; Immune Tolerance Network – ITN in the United States), a big step forward towards our understanding of tolerance and its detection has been realized in the last decade. The constitution of multicentric cohorts of transplant recipients, gathering the rare cases of spontaneous operational tolerant observed in the clinics, has offered the possibility to identify and validate the expansion of B-cell subsets and related gene markers. The fact that some of these subsets present proven regulatory functions adds to the crucial role for this compartment in the development and/or maintenance of the tolerance status.

The described biomarkers and functional assay will help to develop new strategies and to identify tolerant patients and can be used to shape the drug weaning protocols of transplanted patients. Large-scale clinical studies are now warranted to validate the utility of this tolerance signature as a mean to identify spontaneous clinical operational tolerance in long-term kidney recipients with stable graft function, to determine the timing of appearance of the observed tolerant footprint post-transplantation and to test the stability of this profile over time. These analyses will especially help to identify an optimal window for future protocols. At present, IS minimization and induction trials have unfortunately concentrated on the early post-transplantation period, which could explain the discouraging results in most attempts. Not proven but very convincing so far, observations from operational tolerant or recipients under IS regimen suggest that tolerance develops later (from 5 to 10 years). Whether it depends solely on time or long IS exposure remains to be ascertained. Therefore, future protocols should consider later favourable periods, when spontaneous tolerance is to be the most probably achieved. In such a favourable window, minimizing IS or inducing tolerance is to be easier.

Altogether, these results may be helpful to establish individualized therapy for kidney transplant recipients and may enable to improve long-term graft outcomes. Of course, B cell is not the unique feature operating in tolerance and there are several emerging reports evidencing the role of other regulatory subsets. Immunological tolerance is a multifaceted situation involving a large array of participants and regulations. It is certain that adopting an integrated view of multiple data sources (cellular, immunological, phenotypic, genetic, epigenetic proteomic, metabolomic among all) by a system biology approach will help to model tolerance and answer why a recipient accepts his graft while the others do not. This approach will be facilitated by the accelerated improvement of immunological and molecular techniques but also of analysis. Regarding what have been achieved to date on the road to the 'Holy Grail', personalized medicine for kidney transplant recipients might underpin clinical practice for the coming decade.

To conclude, improvements in the efficiency of the donation process, public awareness and live donation, development of standardized operation and postoperation protocols have made donation and organ transplantation more widespread. With increasing the number of transplantations and improvement in both surgical technologies and tolerance induction regimens, transplantation is entering a new promising era.

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