MONITORING ALLOIMMUNE RESPONSE IN KIDNEY TRANSPLANTATION

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Abstract

Currently, immunosuppressive therapy in kidney transplant recipients is generally performed by protocols and adjusted according to functional or histological evaluation of the allograft and/or signs of drug toxicity or infection. As a result, a large fraction of patients are likely to receive too much or too little immunosuppression, exposing them to higher rates of infection, malignancy and drug toxicity, or increased risk of acute and chronic graft injury from rejection, respectively. Developing reliable biomarkers is crucial for individualizing therapy aimed at extending allograft survival. Emerging data indicate that many assays, likely used in panels rather than single assays, have potential to be diagnostic and predictive of short and also long-term outcome.

While numerous cross-sectional studies have found associations between the results of these assays and the presence of clinically relevant post-transplantation outcomes, data from prospective studies are still scanty, thereby preventing widespread implementation in the clinic. Of note, some prospective, randomized, multicenter biomarker-driven studies are currently on-going aiming at confirming such preliminary data. These works as well as other future studies are highly warranted to test the hypothesis that tailoring immunosuppression on the basis of results offered by these biomarkers leads to better outcomes than current standard clinical practice.

Keywords

Acute rejection; Biomarker; Adaptive immunity; T and B-cell ELISPOT; MLR; Kidney transplantation

Introduction

Measuring immunosuppressive drug levels in blood is the most frequently used tool to monitor transplant immunosuppression. However, even stringent obedience to the recommended drug levels (frequently chosen on empiric bases) does not prevent either

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overimmunosuppression resulting in infections and malignancies or underimmunosuppression associated with an increased risk of acute rejection or chronic immune injury of the graft. Indeed, different genetic background may result into different immune reactivity across patients despite similar levels of immunosuppressive drugs in the blood [1, 2]. Acute rejection is suspected when serum creatinine increases without signs of dehydration, drug toxicity, or urological complication and, most importantly, it usually increases when overt allograft injury has already occurred. Knowing blood levels of immunosuppressive drugs is helpful, but is usually insufficient *per se* to differentiate rejection from toxicity, thus necessitating biopsy. Hence, identification of biomarkers of immune reactivity is a true priority in transplantation research and development of strategies for immune monitoring will be crucial for distinguishing transplant recipients who will benefit from a reduction in, or even the withdrawal of, immunosuppression from those who require more intense, lifelong immunosuppression. Finally, some of these assays may be useful biomarkers not only of acute rejection but of long term outcomes which are increasingly dissociated from the excellent results achieved in the first three years after transplantation. This review provides an overview of the most promising biomarkers to quantify alloreactivity.

T cell alloreactivity

Development of T cell alloreactivity biomarkers starts with the understanding of anti-donor T-cell immunity. There are two main pathways of HLA alloantigen recognition: the direct and the indirect pathway. The '*direct*' pathway requires the recognition of intact donor HLA alloantigens on the surface of donor cells, whereas the '*indirect*' pathway of HLA allorecognition involves the internalization, processing, and presentation of alloantigens as peptides bound to recipient HLA molecules [3, 4]. Since professional donor antigen presenting cells (APCs) disappear after the first weeks or months after transplantation, priming by the direct pathway has classically been thought to be important for the pathogenesis of acute rejection in the early post-transplantation period [5-7]. Priming via the indirect pathway has long been considered to play a pathogenic role in late graft failure [8-10], but recent studies suggest that both pathways persist during the entire life of the graft and can contribute to the pathogenesis of chronic injury, as direct recognition of donor alloantigens in recipient professional APCs may also occur *via* the so called third or semi-direct pathway [11, 12].

A set of assays has been developed to quantify T cell alloreactivity. Measurement of the primary *in vitro* response to direct recognition of allogeneic molecules occurs in the mixed lymphocyte reaction (MLR), in which recipient T cells are tested for reactivity to donor cells. In an MLR, peripheral blood lymphocytes from two individuals are mixed together in tissue culture for several days; donor lymphocytes are inactivated, thereby allowing only the recipient lymphocytes to proliferate in response to foreign histocompatibility antigens (one-way MLR) (**Table 1** and **Figure 1**) [13]. This reaction was first described in the 1960s using 3Hthy incorporation as a read-out, and has been extensively used to study anti-donor T cell responses. Low levels of T cell expansion are considered a sign of over-immunosuppression, whereas high T cell expansion in response to donor antigens is read as increased risk of allograft rejection. In 19 recipients of cadaveric renal allografts, donor-specific hypo-

responsiveness assessed by MLR at 3 and 6 months after transplantation was associated with a better graft outcome at 1 year [14]. However, in this conventional form, MLR is poorly reproducible and has a limited predictive value in clinical transplantation [15].

Different assays and alternative readouts have been developed to obtain information regarding immunological responses that are of further clinical utility. Most assays test the direct alloreactivity pathway, but modifications in culture conditions can be made for almost all assays to measure indirect pathway responses (**Figure 1**).

Alloreactive T-cell Enzyme-linked immunosorbent Spot (ELISPOT) assay

Among notable advances in transplantation immunology research is the recognition that a great proportion of the alloreactive T cell repertoire derives from the memory pool [16], as the hallmark of adaptive immunity, whose unique properties indicate that they may be detrimental to transplant outcome [17, 18], and consequently, measurements of memory alloimmunity could be used as biomarkers for post-transplantation outcomes. Frequencies of donor-reactive memory T cells are detectable by cytokine enzyme-linked immunosorbent Spot (ELISPOT). This assay combines the features of the MLR with the concept of an ELISA assay (**Table 1** and **Figure 1**). Recipient peripheral lymphocytes are cultured in the presence of inactivated donor or third-party cells in tissue culture wells coated with a capture antibody against the cytokine of interest. The ELISPOT plates are then incubated for 18–24 h to allow responding cells to recognize alloantigens and to release cytokines, which are captured directly at their source of secretion, before they are diluted, degraded, or absorbed by receptors on nearby cell surfaces. The spot that develops represents a cell that had been primed to the stimulating antigen(s) *in vivo.* To increase reproducibility of the assay and reduce the time associated with visual counting, computerized plate readers using digital cameras have been developed. Thus, ELISPOT provides a measure of the frequency of previously activated or memory T cells that respond to specific antigens by producing a selected cytokine [19].

ELISPOT has been found to be predictive of poor graft function. In 55 kidney transplant recipients, IFN-γ ELISPOT levels during the first 6 months after surgery correlated significantly with graft function at 6 and 12 months following transplantation [20]. Multiple-regression analyses indicated that the correlations between the early ELISPOT measurements of IFN-γ and serum creatinine were independent of acute rejection, delayed graft function or the presence of panel-reactive antibodies before transplantation [20]. Similar results were reported by a subsequent study in 23 kidney transplant recipients, showing that pre-transplantation anti-donor IFN-γ ELISPOT had a significant inverse correlation with allograft function at 6 and 12 months [21].

Pre-transplantation measurements of recipient T-cell alloreactivity to donor antigen via the IFN-γ ELISPOT assay are consistently correlated with acute rejection after kidney transplantation. The first study to evaluate this assay reported that 7 of 9 kidney transplant recipients with pre-transplantation donor-specific IFN-γ spots ≥ 25/300,000 cells had acute rejection after transplantation, while none of 10 recipients with < 25 spots per 300,000 cells developed acute rejection [22]. In a larger cohort of 37 African-American recipients of

deceased donor transplants with pre-transplantation testing, 50% of IFN-γ ELISPOT positive recipients had a biopsy-proven acute rejection versus only 17% of IFN-γ ELISPOT negative recipients [23]. Expanding on this experience, the same group reported the association between a positive pre-transplantation IFN-γ ELISPOT response and acute rejection in a separate, larger cohort of 100 kidney transplant recipients [24]. This association was independent of HLA matching, delayed graft function, donor source, ethnicity, and dialysis vintage [24]. In studies outside of the United States, Nickel *et al.* described a correlation between acute rejection and pretransplantation IFN-γ ELISPOTs in 42 German patients [25], and others have described similar findings in Spanish, Korean and Polish kidney transplant recipients [26-28]. ELISPOT has also potential utility in the identification of patients who may benefit the most from antibody induction therapy. In a retrospective analysis of 130 kidney transplant patients, individuals with higher pre-transplantation ELISPOT levels were at increased risk of acute rejection. However, patients with high ELISPOT levels who received antibody induction therapy (anti-CD25 or thymoglobulin) had a significantly lower risk of acute rejection than their counterparts who did not received induction. Post-transplant conversion to a negative ELISPOT assay occurred in 86% of patients who received induction therapy vs. 35% of patients who did not [29]. A subsequent study from the same group showed that thymoglobulin induces a prolonged reduction in anti-donor IFN- ELISPOT response, whereas anti-CD25 depleting antibodies do not affect it [30]. In a similar retrospective analysis in 90 consecutive kidney transplant individuals, pre-transplant T-cell alloreactive patients showed a significantly higher risk of T-cell mediated rejection during the early period of time after transplantation. Likewise, this effect was particularly evident in patients not receiving T-cell depletion induction therapy [31].

Contrary to previous reports, the large Clinical Trials in Organ Transplantation-01 observational study failed to identify an association between pretransplant IFN-γ ELISPOT positivity and the incidence of acute rejection or estimated glomerular filtration rate (eGFR) at 6- or 12-month in 176 primary kidney transplant recipients treated with heterogeneous immunosuppression [32]. Secondary analyses showed that, in patients not induced with thymoglobulin, positive pre-transplant-ELISPOT was associated with lower 6- and 12-month eGFRs than negative IFN-γ ELISPOT. In contrast, IFN-γ ELISPOT status did not correlate with posttransplant eGFR in subjects given thymoglobulin, which suggests that thymoglobulin may reduce the immunogical risk of patients with high pre-transplant IFN-γ ELISPOT.

Given evidence suggesting that ELISPOT may quantify recipient risk for rejection, it has been used to tailor immunosuppression in 60 kidney transplant patients in a non-randomized pilot study, who were allocated to a calcineurin inhibitor-based or a calcineurin inhibitor-free immunosuppressive regimen on the basis of pre-transplantation "high" vs. "low" anti-donor IFN-γ ELISPOT assays, respectively [33]. High pre-transplant ELISPOT levels were frequent even in the absence of anti-HLA antibodies and represented a major risk factor for acute rejection and graft dysfunction, despite the use of more potent immunosuppression with calcineurin inhibitors. Though further investigations are needed, this study suggests that evaluating antidonor T-cell sensitization before transplantation may help define immunosuppressive regimens on a single patient basis.

The ELISPOT procedure takes 24-36 hours, which makes it less suitable to assess the pre-transplant immunological risk in deceased donor recipients. To address this issue and that of patients with no donor cell availability as well as to provide information on the overall T cell immunoreactivity of transplant patients, a T-cell reactivity index (panel of reactive T-cells, or PRT) based on the frequency of positive ELISPOT responses using a pool of donor antigens prior to transplantation that may be reflective of potential organ donors was reported simultaneously by 2 groups [34, 35]. Similar to the panel-reactive antibody (PRA) test for identifying individuals with elevated levels of anti-HLA antibodies, the PRT may identify patients at risk for post-transplantation cellular immune-mediated graft injury. In a small study of 30 kidney transplant patients, six of the seven (86%) patients with acute rejection were PRT-positive whereas only one had low PRT before transplantation [36].

A recent study by the Fairchild group showed that a significant subpopulation of alloreactive memory $CD28^-CD8^+T$ cells are not detected by the above-noted approaches [37-40]. Addition of IL-15 to the cultures rescues the proliferating capacity of these cells, making them detectable [41]. Since renal epithelial cells are able to produce IL-15 during inflammation, quantification of these cells might be relevant in quantifying the immune risk of kidney transplant recipients. Future investigations using MLR and ELISPOT should therefore consider the addition of IL-15 to cultures to assess this population of memory cells and their contribution to graft injury.

Monitoring indirect alloreactivity

Most of the aforementioned assays are designed to evaluate the direct alloimmune response. However, evidence that indirect alloreactive $CD4^+$ T cells are the only cells that can provide help to alloreactive B cells in animal models, leading to the formation of anti-HLA antibodies, prompted an increased interest in the development of novel assays capable of quantifying this pathway [42, 43]. Costs and complexity of the procedures, however, make the assessment of indirect alloresponses more challenging. Three approaches to address the indirect alloimmune response include: peptides from polymorphic regions of HLA, lysates of donor cells, and non-polymorphic peptides.

By using peptides from polymorphic regions of HLA, a cross-sectional study of 45 kidney transplant patients showed that increased IFN-γ ELISPOTs in both the direct and indirect pathways were associated with abnormal graft function [44]. This method has higher reproducibility, and other retrospective and cross-sectional studies have shown similar results [45-47], but no prospective study has been done yet. Moreover, this approach is challenged by the difficulties of creating large libraries of peptides to encompass the wide number of HLA polymorphisms.

An alternative approach is the use of donor cell lysates or fragments, which in theory should allow the use of for the testing of the full repertoire of alloantigen available. A study in mice showed that donor cell lysates generated through cell freezing and thawing were able to induce a T cell response in skin transplanted animals through the indirect pathway [48]. A similar approach was tested in humans by measuring the IFN-γ production of PBMC from kidney transplant patients with or without chronic rejection in response to donor cells

undergone through multiple cycles of freezing and thawing [8]. Indirect response alloreactivity was significantly lower in stable patients as compared to those with chronic rejection, a difference that was not captured by evaluating only the direct pathway. In another cross-sectional study of 34 longstanding living donor renal transplant recipients, indirect response IFN-γ ELISPOT against donor-cell fragments correlated with proteinuria, a major predictor of graft failure [11]. However, to date, no prospective studies have been done to validate this approach.

Alloimmune responses are frequently associated with intramolecular epitope spreading, and in experimental models, this includes responses to cryptic self-epitopes [46, 49]. In support of this concept, a cross-sectional study in 110 kidney transplant recipients showed that IFN-γ production by PBMC stimulated with nonpolymorphic HLA-derived peptides was associated with chronic rejection [50]. The assessment of responses to such peptides might, therefore, be used to provide evidence of alloimmunization without the need to account for donor or recipient HLA type, provided they reflect an immune response initiated by alloantigen that drives allograft injury. This does not even require that the peptide tested are allogeneic, because the response to "cryptic self-epitopes" could equally meet these criteria [51, 52]. As with the other approaches above, however, prospective studies are lacking, and will be required to validate the utility of these assays in the prediction of allograft outcomes.

Characterizing Regulatory T cells

Regulatory T cells (Treg) consist of a heterogeneous population of T cells with the ability to suppress immune responses. It has long been established that Foxp3 is the major transcription factor that determines the fate, identity, and function of Treg, and Treg regulate immune functions by producing cytokines such as TGF-β, IL-10, and IL-35 [53, 54]. However, there are subsets of Treg that do not express Foxp3. For example, TGF-β–producing Th3 and IL-10–secreting Tr1 Treg also can be potent suppressors in some experimental systems. Given the vast evidence demonstrating the contribution of Tregs regulating immune responses in different animal models, great hopes have flourished on the potential use of Tregs as markers of tolerance, transplant rejection, or prediction of graft outcomes.

However, while murine studies consistently showed a relationship between increased numbers of FoxP3$^+$ Tregs and tolerance [55], the induction of CD25 and FoxP3 upon activation of human Teffs has led to far more variable clinical data [54]. Findings of reduced numbers of Tregs in patients with acute or chronic rejection have not been consistent [50, 56-61], nor have findings of increased numbers of Tregs in tolerant recipients [56, 62-64]. Despite the growing list of markers useful for identifying human Tregs, none is able to clearly identify T cells with suppressive capacity. Thus, pure enumeration of immunophenotypic Tregs needs to be accompanied by an assessment of Treg suppressive function. Using autologous cells as responders in Treg assays of kidney transplant patients can be misleading, since immunosuppression can decrease their proliferative capacity, which may explain why Tregs from patients with rejection had impaired suppressive function in some studies but not others [58, 60, 61, 65, 66]. To address this issue, Akimova *et al.* developed a standardized suppression assay where CD25$^+$CD127^{low}CTLA4$_+$ FoxP3$^+$ Treg

function was tested against a Teff cell from healthy individuals, so that Tregs are the only variable. By using this strategy, they found that patients with tacrolimus levels >3.6 ng/mL had weaker Treg function than those with levels <3.6 ng/mL, whereas rapamycin therapy positively correlated with Treg numbers and their expression of CTLA4 [67]. Whether this new strategy to quantify Treg activity correlates with the risk of developing acute rejection is still unknown. Following a similar approach to an MLR, Canavan *et al.* developed a 7-hour flow-cytometric assay to assess regulatory T cell (Treg) suppressive function, by measuring CD154 and CD69 expression on T effector cells (Teff) in a suppression assay (**Figure 1**) [68]. Expression levels of these markers correlated excellently with gold-standard assays involving inhibition of CFSE dilution and cytokine production. This test could allow rapid evaluation of Treg suppressive function in clinical trials of cell therapy, enabling the translation of the large body of preclinical data from bench to bedside. Analysis of the epigenetic status (e.g degree of methylation) of FoxP3 can also provide helpful information on the suppressive function of Tregs. An additional way to characterize the suppressive function of Tregs is to measure the ratio between Teff/Treg. Lower values have been associated with better graft outcomes [69]. Together, these assays provide a new approach to monitor the immune status of transplant patients. However, no existing evidence suggests that measuring Treg activity can predict the risk of future acute/chronic rejection or infections. Similarly, conflicting evidence exists regarding an increased Treg presence in transplant patients with cancer, but the cross-sectional nature of the data prevents any analysis on the predictive power of such a parameter [70]. The entire picture may be complicated by evidence that increased Tregs associated with rapamycin are associated with higher risk of acute and chronic rejection and lower risk of tumors compared to cyclosporine, possibly secondary to differential effects of the two drugs on effector T and actively proliferating tumor cells [71, 72]. *Ad hoc* prospective studies are therefore needed to validate the utility of Treg characterization in the management of transplant patients.

B cell alloreactivity

The effector role of alloantibodies was already shown in the 1960s with the observation of the occurrence of hyperacute rejection in many sensitized transplant patients [73]. Even though the issue of hyperacute allograft rejection has been almost overcome by the introduction of novel crossmatch techniques [74], and more sensitive assays measuring the presence of circulating anti-HLA antibodies both before and also after transplantation [75], the persistence of humoral immunity against donor antigens still remains as the main cause leading to allograft rejection in the mid/long term.

Even though the assessment of circulating anti-HLA antibodies has significantly improved with the advent of novel highly sensitive assays, which have allowed a better understanding of the risk and impact of humoral immunity in kidney transplantation [75], the solely focus on circulating anti-HLA antibodies to determine the allosensitization state of a given transplant patient may underestimate the magnitude of the complete humoral immune response as it excludes the detection of the entire mBC pool. There are two major B-cell populations that contribute to the maintenance of immunological memory: long-lived plasma cells (LLPC) and memory B cells (mBCs); whereas the former reside primarily in the bone marrow where they continuously secrete antibodies that act rapidly on invading microbes

and are responsible of the maintenance of serum antibody levels, the latter are mainly located in peripheral lymphoid tissues and can, upon re-encounter with the priming antigen, differentiate into antibody-secreting cells (ASCs) and thus amplify the antibody response while also replenishing the pool of long-lived plasma cells to maintain long-term antibody levels in the absence of pathogen [76, 77]. A key element that distinguishes antibody-producing LLPC and mBC from activated naïve B cells is that they have both undergone class switching and somatic hypermutation in germinal centers [78], although in some settings mBC may lack these differential features [79]. Of note, mBCs may exist in the absence of detectable serum antibody levels and their rapid differentiation and antibody production may be of high relevance for a protective humoral response [80, 81].

Indeed, in the transplant setting, HLA-sp antibodies may appear and disappear in several situations such as after undergoing transplantectomy [82], re-transplant patients display worse allograft outcomes regardless of preformed DSAs [83] and highly suggestive histological lesions of humoral rejection are frequently observed despite no DSAs [84]. Therefore, the detection of functionally active anti-donor HLA-sp mBCs at in the context of transplantation could provide additional relevant biological information regarding the sensitization state of transplant candidates besides the accurate evaluation of circulating alloantibodies.

Recent relevant technical approaches have focused in tracking mBC using novel *in vitro* assays in the context of solid organ transplantation (**table 2**).

Quantification of HLA-specific mBC using Flow cytometry

Taking advantage of the introduction of the HLA tetramer technology to quantify antigen-specific T cells, Mulder and co-workers using either human B cell hybridomas [85] and HLA-specific B cells from pregnancy-immunized individuals [86], were the first to show the feasibility of tracking HLA-specific B cells binding epitopes of foreign HLA molecules in their original conformation through their B-cell receptor (BCR). This technology uses streptavidin-biotin complexes of HLA molecules conjugated to a fluorescent protein. Then, the tetramer-binding B cells can be accurately enumerated by flow cytometry and allow a rapid quantization of the B-cell response to the given HLA antigen. Zachary et al. [87, 88] added CD27 and CD38 markers to discriminate the tetramer-binding mBc and plasma cells, respectively. Interestingly, a high frequency of HLA-tetramer mBc was found in negative DSA patients at the time of transplantation that subsequently developed DSA after transplantation suggesting the presence of donor-specific mBc already prior to transplant surgery. This feature was particularly evident among patients that did not receive B-cell depletion therapy with rituximab [89]. A main drawback of such technique that should not be obviated is that a significant number of B cells, which can be up to 6% of total CD19+ B cells, may recognize non-HLA fractions of the tetramer, such as the streptavidin-phycoerythrin, leading to nonspecific staining [90]. Hence, more controls must be done such as using immunomagnetic depletion with streptavidine beads before staining or use similar tetramers conjugated to two different fluorochromes [86]. Importantly, while this technique allows for easy quantification HLA-specific B cells harboring a HLA-specific BCR using flow cytometry readouts, it does not really enumerate frequencies of HLA-specific mBC

capable of releasing antibodies. Furthermore, it bears low sensitivity for the detection of low level of responses, which is most likely the case when measuring mBC responses. In a similar new approach but using HLA-coated multiplex beads, circulating HLA bead-B-cell frequency and specificity might be also analyzed using flow cytometry (Ahmed Akl, Roitberg-Tambur, M Javeed Ansari, Abstract OR07 ASHI 2014). In a small cohort of kidney transplant patients, this group showed that transplant patients with poor graft outcome and circulating alloantibodies showed significantly higher frequencies and polyreactivity against both class I and II HLA antigens as compared to patients with good graft outcomes.

Assessment of anti-HLA antibodies in supernatants of ex vivo expanded mBC cultures

Since antigen-specific ASCs do not generally circulate in the periphery, but only transiently early after active or accidental immunization, and antigen-specific mBc do not secrete antibodies, *in vitro* differentiation of circulating mBc onto an ASC-like phenotype capable of secreting antibodies can be achieved by using a number of different antigen-independent polyclonal activation methods [91]. Most activator protocols generally include different stimuli. Most common antigen-independent activators used are CpG (a Toll-like receptor [TLR] 9 agonist), pokeweed mitogen (PWM) and Staphylococcus aureus Cowan (SAC) often combined with CD40-ligand (CD40L) and/or cytokines such as interleukin (IL-) 2 and IL-10 [92]. More recently, the use of the TLR7/TLR8 agonist R848 plus IL-2 was also shown to efficiently activate and differentiate mBc onto ASCs [81, 91]. Of note, to efficiently obtain sufficient ASC numbers, 5 to 7 days of *in vitro* stimulation either purified B cells or PBMCs are required.

A first attempt to evaluate the presence of HLA-specific mBc was carried out by Han and colleagues [93] after assessing alloreactive IgG antibodies in the supernatant of *ex vivo* expanded mBc cultures using solid-phase assays . By analysing the presence of anti-HLA antibodies of expanded mBc culture supernatants in a small group of transplant patients, transfusion-derived sensitized individuals as well as multiparous women with serum HLA antibodies, they observed that HLA-specific antibodies were detected in sensitized individuals, but not in the non-sensitized controls and furthermore, most transplant patients did also show antibodies against mismatched donor HLA antigens. Most interestingly, DSAs were found in some B-cell cultures but not in concomitant serum of the patients, illustrating that such DSAs were released by circulating mBc rather than by LLPC. In a recent interesting report using the same method for tracking HLA-specific mBc, Snanoudj et al. [94] depicted the presence of HLA-sp antibodies in expanded mBC culture supernatants in a high proportion of highly sensitized patients, showing a high burden of previous allogeneic sensitization, even long time after first allogeneic contact. In addition, the authors described a more restricted epitope reactivity of mBC antibodies as compared with circulating HLA-sp antibodies, suggesting that particular antigens eliciting strong immune responses are most likely responsible to induce and maintain circulating alloreactive mBCs over time. While this method seems a feasible approach to potentially translate to the clinic practice for questioning the mBc compartment, it has two main caveats: 1) it does not quantify the frequency of HLA-specific IgG-producing mBc in a funcitonal manner and 2) the relatively low frequencies of HLA-specific antibodies found in some expanded mBc cultures might lead to false negative results, regardless the use of highly sensitive solid-phase assay

platforms. Indeed, in a recent study done by our group (Bestard O et al., manuscript in preparation), the comparison between MFI values of HLA-specific antibodies assessed in either the serum or supernatants of expanded mBc cultures of the same individual as compared to circulating HLA-specific mBc frequencies against the same HLA antigen using a highly sensitive B-cell ELISPOT assay, revealed that while some alloantibodies and mBc frequencies could be simultaneously detected using all 3 approaches, some others were not found either in the serum or in the supernatants, whereas could be clearly enumerated when using a B-cell ELISPOT assay (**Figure 2**) thus, highlighting the higher sensitivity of the latter method to detect HLA-specific mBc responses. Furthermore, although alloantibody titers of these supernatants can be extrapolated to the total numbers of circulating mBC, this quantification differs from reality as it assumes that all ASCs secrete a constant amount of alloantibodies.

HLA-specific B-cell Enzyme-linked Immunosorbent Spot (ELISPOT) Assays

To accurately quantify the frequency of HLA-specific mBc, the most sensitive technical approach is to use a B-cell enzyme-linked immunosorbent spot (ELISPOT) assay platform. This assay was first described in 1983 by Czerkinsky et al [95] and has proven to be an important method for the detection of IgG-producing B cells. The assay has also been further developed for the detection of antigen-specific plasma blasts and mBc [77, 96, 97]. Recently, our group showed the capacity of the B-cell ELISPOT to accurately enumerate cytomegalovirus (CMV)-specific mBC frequencies within kidney transplant patients [98]. We demonstrated the high sensitivity of the assay to measure low frequencies of CMV-specific mBC in peripheral blood, even in transplant patients without anti-CMV IgG antibodies in the sera, and showing higher protection against viral infection. A first report in the context of pediatric heart transplantation using this assay was done by Fan et al, evaluating the frequency of ABO-specific antibody-producing B cells [99], demonstrating the immune B-cell tolerance state of this setting. Next, Perry et al. studied the presence of HLA-specific IgG-producing bone marrow–residing plasma cells obtained from bone marrow aspirates as well as from peripheral blood [100] and confirmed that most ASC reside exclusively in the bone marrow. A first attempt aiming at quantifying circulating HLA-specific mBC frequencies in the context of kidney transplantation using a B-cell ELISPOT was done by Heidt and colleagues [101]. For this purpose, they polyclonally activated during 7 days, isolated peripheral blood B cells in a CD40-driven culture system using either a complex L-CD40L cells or a more accessible anti-CD40 mAb in combination with a cytokine conceptail consisting of interleukin (IL)-2, IL-10, IL-21, and CpG DNA [102]. After such an activation protocol, B cells are transferred to streptavidin precoated ELISPOT plates, followed by biotinylated synthetic HLA class I or II molecules. In these initial studies they showed the feasibility of the assay to accurately detect low frequencies of HLA-specific mBc frequencies both in some pregnancy-immunized women as well as in a small group of kidney transplant patients against previously exposed HLA antigens, which were harbored in previous kidney allografts. More recently, our group developed a novel B-cell ELISPOT assay approach based on a cellular stimulation culture method based on the TLR 7/8 agonist R848 and IL-2 [103] and tested it in a large group of 70 highly HLA sensitized patients as well as in kidney transplant individuals. Differently from previously reported HLA B-cell ELISPOT methods, our approach allowed for using PBMCs rather than

sorted B cells. In addition, as frequencies of HLA-sp mBCs are relatively low, multimerized class I and class II HLA molecules were used, enabling clear detection of very low IgG-producing mBC frequencies. In this study, we confirmed that mBC frequencies against both class I and class II HLA antigens may be detected in peripheral blood in patients with obvious allogeneic sensitization background, even in the absence of detectable circulating anti-HLA antibodies. Remarkably, we described that high frequencies of d-s alloreactive mBCs are present in kidney transplant patients during ABMR and also before transplantation, reflecting an active baseline anti-donor sensitization state not always depicted by circulating DSA. Differently from a previous report published by Lynch et al [104] using a complex B-cell ELISPOT assay procedure based on cultured isolated donor fibroblasts to obtain a broader donor antigen repertoire, we did not find a universal detection of alloantigen mBC responses in all patients after transplantation. Currently, two large European observational studies (Leiden, Holland and Barcelona, Spain) are currently ongoing to assess the value of quantifying HLA-specific mBC in kidney transplantation. However, the 6-day cell culture and the use of cells as the biological specimen to analyze are major hurdles that may limit its implementation in the clinical practice. Furthermore, the need of the whole HLA antigen repertoire to be tested in these assays is also highly needed in order to cover all potential donor-recipient HLA mismatches of all transplant candidates around the globe. Similarly to the T-cell ELISPOT assay, these assays should undergo interlab validation within experienced laboratories and prospective large, observational trials are also warranted in order to obtain consistent data. These studies should be developed within international research networks in close collaboration with creative biotech industry, aiming at bringing these tests to the clinic.

Monitoring free DNA

With 10-100 billion fragments per milliliter of plasma, circulating cell-free DNA is an information-rich window into human physiology, for rapidly expanding applications in cancer diagnosis and therapy and prenatal diagnosis. More recently, cell-free donor-derived DNA (cfdDNA) has been proposed as a candidate marker for noninvasive diagnosis of graft injury. For female recipients of a graft from a male donor, donor-specific DNA can be identified using molecular assays targeting the Y chromosome. Single-nucleotide-polymorphisms (SNPs) distributed across the entire genome do allow discriminating donor and recipient DNA molecules also regardless from their gender. A retrospective study on 43 samples from 7 heart transplant patients showed a significant correlation between the fraction of cfdDNA and acute rejection episodes determined by endomyocardial biopsy [105]. A subsequent larger prospective-cohort study evaluated the performance of cfdDNA to measure allograft rejection in 565 plasma samples collected longitudinally from 65 adult and pediatric heart transplant recipients. Comparison to endomyocardial biopsy results (356 samples) indicated that this approach can be used for the discrimination of rejecting and nonrejecting grafts and demonstrated the utility of the technique for the detection of acute cellular and antibody-mediated rejection in adult and pediatric heart transplant recipients, as well as in patients requiring a second heart transplant. These findings indicate that cfdDNA measurements have the potential to replace the endomyocardial biopsy and that these measurements can possibly be used also to predict acute rejection events and managing

immunosuppressant dosing. More recently, quantification of cfdDNA has been shown to identify acute rejection in lung transplant recipients [106], indicating that cfdDNA is a broadly applicable marker of graft injury that could be used also to monitor kidney graft status.

Conclusions

The development of noninvasive, accurate, and reliable assays to monitor alloreactivity in kidney transplant recipients could potentially lead to a new treatment paradigm in transplantation, with a conceivably safer, more tolerable, and cost-effective approach to immunosuppression. Over the last two decades, many assays have been developed to noninvasively quantify the level of alloreactivity in transplant patients. While some of these assays have been standardized across different laboratories [107, 108] and validated prospectively, no studies have formally tested whether or not these assays can be used as an alternative to, or in combination with an allograft biopsy to improve kidney transplant outcomes and to reduce the morbidity and mortality associated with over- and under-immunosuppression. Similar to the ongoing European BIO-DRIM (BIOmarker-Driven personalized IMmunosuppression) consortium aiming at stratifying patients to high or low burden of immunosuppression according to pre-transplant donor-specific IFN-γ ELISPOT (CELLIMIN study; NCT02540395 ClinicalTrials.gov), future randomized clinical trials should assess whether titrating immunosuppressive therapy according to the results of one or some combination of these assays provides comparable results to the conventional approach based on serum creatinine levels, allograft biopsies and immunosuppressive drug levels. It is unlikely that a single immune-monitoring assay will provide adequate information representative of the complex pathophysiology of an individual's alloresponse. To overcome this major limitation, multiple tools could be combined to measure the immune response from different perspectives, including not only T and B cell alloreactivity, but also antibody profile and gene-expression pattern. A systems approach, where the focus is moved from analysis of individual cell types towards more integrated studies of the entire immune system, has been also proposed as a way to better measure alloreactivity and to personalize immunosuppressive therapy [109].

The task of translating immune-monitoring assays into day-to-day clinical practice is challenging, but will be the key to individualized immunosuppression in the future. Notably, the relevance of these assays extends beyond transplantation, since they could provide vital information also pertinent for the management of patients with autoimmune diseases.

Acknowledgements

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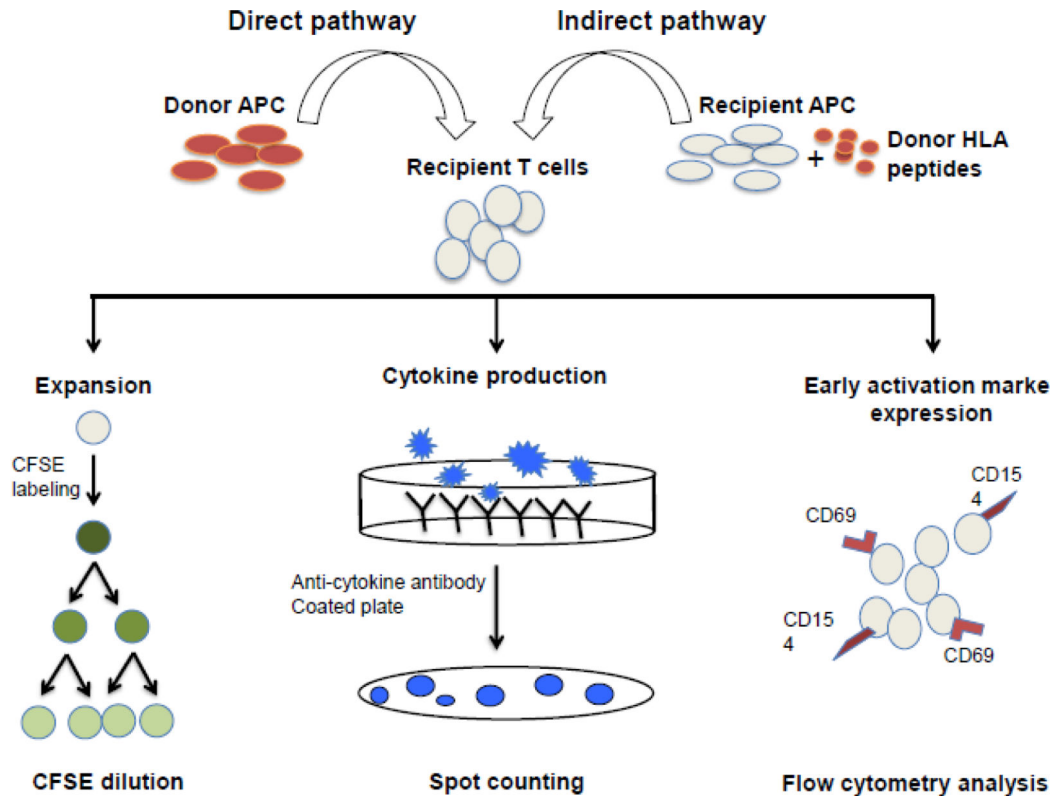
Figure 1.
Biomarkers of T cell alloreactivity: common assays measuring the magnitude of direct or indirect activation of recipient T cells include the mixed lymphocyte reaction (left), cytokine ELISPOT (center), and detection of activation markers (right). In a mixed lymphocyte reaction (MLR) stimulated T cells are labeled with a fluorescent dye (left). The degree of fluorescence dilution is directly related to the extent of T cell proliferation. ELISPOT assays (center) use plate-bound antibody to detect cytokines (*e.g.* IFN-γ) secreted by an individual cell, manifest as a "spot" after use of a secondary antibody and enzymatic developer. Activation markers on alloreactive T cells (right) are expressed within 24–36 h after incubation with alloantigen, allowing for their identification. From: Transplant Reviews (2015) 29:53-59
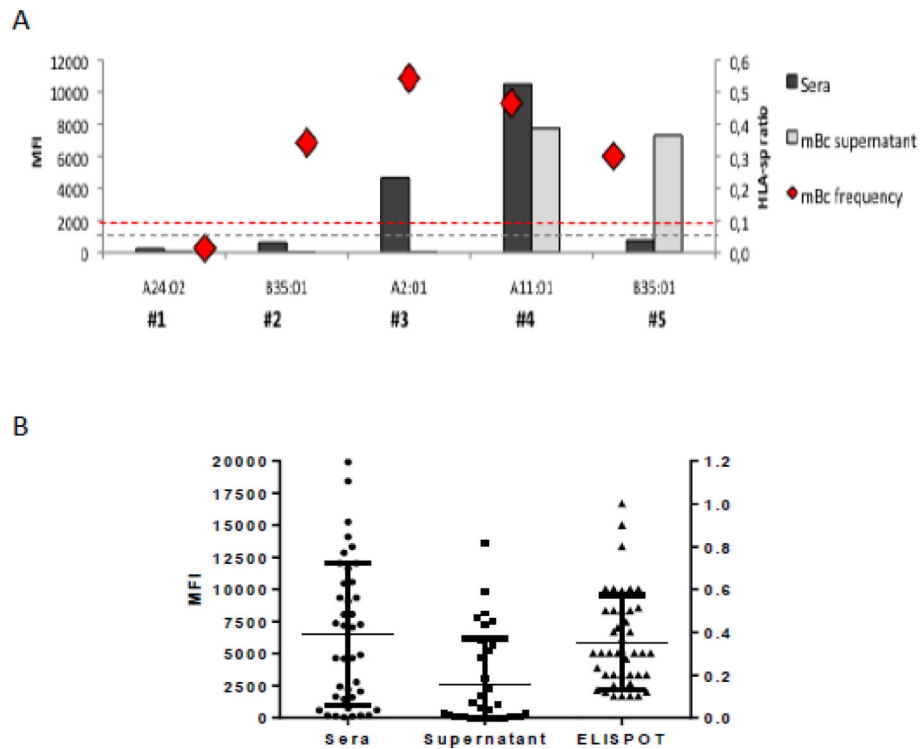
Figure 2.
Comparison of HLA-specific Ab detection in serum, supernatant of ex-vivo expanded mBC cultures and HLA-sp mBC frequencies using a HLA-sp B-cell ELISPOT. **A.** Illustrative examples of 5 transplant candidates on the waiting list for kidney transplantation. Different possible information may be obtained when assessing the presence of HLA-sp B-cell sensitization when evaluating either the serum, the supernatants of ex-vivo expanded mBC cultures or mBC frequencies using a B-cell ELISPOT assay. While patient #1 did not show any evidence of anti-HLA sensitization in any compartment, patient 4 displayed anti-HLA sensitization by looking at either circulating anti-HLA Ab in the serum, HLA-sp mBC frequencies or the respective Ab in the supernatant of the expanded mBC culture. Of note, patients #2 and #5 showed circulating HLA-sp mBC using the B-cell Elispot assay but the respective Ab was not found neither in the supernatant of patient 5 nor in the serum of both patients. Alternatively, patient #3 showed a high HLA-sp mBC frequency as well as the respective Ab in the serum, but was not detected in the supernatant of the expanded mBC culture. B. In general, while sensitized transplant patients show a broad range of detectable HLA-sp mBC frequencies, which fit with the respective circulating anti-HLA Ab in the serum, an important proportion of patients the respective Ab assessed in the supernatant of the exvivo expanded mBC cultures might not be detectable, even using the highly sensitive solid-phase assays.

Table 1

Options for monitoring T cell alloimmunity

Assay	Description	Pros/Cons
Mixed lymphocyte reaction (MLR)	Lymphocytes from donor and recipient are combined and co-cultured; dye-dilution assays with CFSE are used to measure T cell proliferation in the recipient in response to donor antigen presentation	Pros: inexpensive, relatively easy to perform Cons: poorly reproducible and time-consuming; older technique, so limited data in tacrolimus era
ImmunKnow®	Measures early response to stimulation by detecting intracellular ATP synthesis in CD4 cells selected from blood by monoclonal antibody coated magnetic beads; the amount of ATP present in stimulated blood specimens is a measure of lymphocyte activity	Pros: highly reproducible, rapid turnaround time, and relatively low cost Cons: poor sensitivity/specificity, no large prospective studies to support its predictive value
Enzyme-linked immunospot (ELISPOT)	Recipient lymphocytes are cultured in the presence of inactivated donor or third-party cells in tissue culture wells coated with a capture antibody against the cytokine of interest; plates are incubated for 18–24h; spots that develop represent a cells that had been primed to the stimulating antigen(s) *in vivo*, and are quantified using computerized plate readers	Pros: high throughput and functional readout; faster than MLR and amenable to standardization; multiple prospective studies to support its predictive value Cons: expensive (compared to MLR) and labor-intensive

Abbreviations: CFSE – carboxyfluorescein diacetate succinimidyl ester; ATP – adenosine triphosphate

Table 2

Assays for measuring alloreactive B cells.

Assay	Description	Pros/Cons
Flow cytometry HLA-specific Tetramer-binding B cells	Assessment of HLA-specific B cells using fluorochrome-conjugated HLA tetramers using flow cytometry. The HLA Tetramers are streptavidin-biotin complexes of 4 peptide-loaded HLA molecules conjugated to a fluorescent protein. The tetramer-binding B cells can then be enumerated by flow cytometry	Pros: Easy detection of B cells harboring a HLA-sp B-cell receptor. Cons: Non-sp B-cell binding to fluorochomes; Low sensitivity to detect low frequencies (does not quantify mBC capable of secreting alloAb
Anti-HLA antibodies of *ex-vivo* expanded mBC culture supernatants	Indirect assessment of HLA-sp mBC by measuring anti-HLA antibodies in supernatants of *ex-vivo* expanded mBC cultures. Anti-HLA Ab are obtained from the supernatant of *ex-vivo* activated PBMC containing mBC during 6 days. Thereafter, Ab may be detected using a Luminex platform	Pros: Simple detection of anti-HLA Ab released by mBC. Cons: Indirect quantification (assumes all ASC secrete a constant amount of IgG); Low sensitivity for low Ab titers (effect of dilution); needs 6-day polyclonal stimulation
HLA-specific B-cell ELISPOT assay	Enumerates the frequency of HLA-specific mBC capable of secreting anti-HLA Ab. Uses PBMC that are polyclonally activated either through a cognate stimulation with anti-CD40 mAB or CD40L, or using a TLR-derived stimuli together with a cytokine cocktail for 6 days. Afterwards, cells are seeded in IgG pre-coated ELISPOT wells and then multimerized biothynilated HLA class I or II molecules are added and IgG spots are visualized using an ELISPOT reader	Pros: Precise enumeration of HLA-sp mBC capable of secreting Ab (also low frequencies). Provides the frequency(strength) of alloresponse as it may be extrapolated to the total clonable mBC of a given individual. Cons: Needs 6-day polyclonal stimulation; need of all synthetic HLA molecules to asses the whole HLA repertoire; expensive and labor-intensive

Abbreviations: mBC_ memory B cells; HLA-sp: Human Leukocyte Antigens-specific; Ab: antibody