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REVIEW

Biopsy diagnostics in renal allograft rejection: from histomorphology to biological function

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Summary

Histological assessment of allograft biopsies is still the gold standard for typing and grading renal allograft rejection episodes**.** The technology employed for biopsy assessment and the resulting diagnostic classification did however not always keep pace with the rapidly evolving knowledge about the immunological mechanisms of rejection. As accurate recognition of these mechanisms is crucial for specific therapy and reliable risk assessment, it is mandatory to constantly adjust our diagnostic standards to current immunological knowledge. The introduction of antibody-mediated rejection as a diagnostic category a few years ago exemplifies the importance of defining renal allograft rejection according to the prevailing immunological mechanism. Current challenges are the diagnostic implementation of novel concepts like sub-clinical rejection or accommodation of grafts. This requires a reassessment of current diagnostic standards and likely also the development of new diagnostic tools. This article reviews novel concepts arising from studies on protocol biopsies and experimental models with specific focus on the potential and limitations of current diagnostic procedures for the detection and classification of recently appreciated conditions like sub-clinical rejection, accommodation and C4d-negative antibody-mediated rejection.

Introduction

Histological assessment of allograft biopsies is still the only way to prove rejection of renal transplants and remains the gold standard for typing and grading allograft rejection episodes. This is somewhat surprising for several reasons: (i) Obtaining a renal allograft biopsy is a painful procedure, is not without risk of severely damaging the graft, and yields a randomly sampled tiny piece of tissue. (ii) The technology applied for investigating the biopsy has not significantly changed over the last 50 years and still mainly relies on conventional histomorphology. (iii) There is a considerable discrepancy between a quite sophisticated knowledge about immunological mechanisms of rejection and the rather simplistic concepts of diagnostic transplant pathology.

Despite these obvious shortcomings of biopsy diagnostics, less invasive strategies for monitoring graft function (analysis of urine, blood/serum or clinical assessment of graft function) even in combination with sophisticated methods like flow cytometry, gene-expression profiling or proteomic analysis [1–4] (though yielding promising results in some aspects) were not yet able to replace allograft biopsies in post-transplant patient-care.

Allograft biopsies will therefore likely continue to be the primary tool in rejection diagnostics. Constant adjustment of the classification scheme to current concepts of transplantation immunology is however required to further enhance its clinical utility.

This article will focus on recent efforts to achieve a biologically adequate and thus clinically relevant diagnostic depiction of rejection processes.

Histomorphological features of renal allograft rejection

The historical evolution of diagnostic criteria

Immune cells infiltrating the graft are the obvious histological hallmark of allograft rejection. In transplanted kidneys, for decades, classification of rejection episodes was almost exclusively based on extent and location of infiltrating immune cells (interstitial versus vascular versus glomerular) [5–7] and on the putative clinical course (acute versus chronic) [6,8]. A uniform, widely accepted terminology did however not exist until 1991, when the first Banff Conference on Allograft Pathology was held in Canada in order to reach consensus on a standardized classification system for renal allograft pathology. The resulting Banff Classification largely adopted the approach outlined above, by categorizing rejection episodes according to the extent and location of immune cell infiltrates. It however also introduced several additions to previous diagnostic standards [9].

Despite considerable criticism especially regarding the arbitrary definition of some diagnostic thresholds and the rather complex scoring system, which furthermore suffers from high inter-observer variability [10], the classification system gained rapid international acceptance. Biannual follow-up conferences introduced several adjustments and minor changes to the classification scheme [11,12] and also adopted features of the similarly designed Cooperative Clinical Trials in Transplantation (CCTT) classification system published in 1997 [13]. The classification however still retained the concept of defining rejection almost exclusively based on cell-mediated immune mechanisms.

Towards a pathogenesis based classification of allograft rejection

Increasing clinical suspicion [14,15] and the availability of a novel diagnostic marker (deposition of the complement fragment C4d within vessels of the graft) [16–18] however indicated that donor-specific alloantibodies (DSA) might play a more important role in rejection than previously appreciated. In 2001, the Banff Classification underwent its first major modification by defining renal allograft rejection according to the prevailing immunological mechanism. In the revised classification scheme, acute rejection was subdivided into a T-cell-mediated and an antibody-mediated type [19] thereby paving the way for therapeutic strategies selectively targeting specific immunological mechanisms. Seminal work from the Boston group [16] had a major impact on this important step towards a pathogenetically oriented rather than morphologically descriptive structure of the classification system. The principle of defining diagnostic categories based on the underlying pathogenic mechanism was however not yet applied to chronic fibrosing lesions of the graft, which still were collectively termed CAN in the 2001 update of the Banff Classification [19]. The indiscriminate use of the term CAN however obscured the theoretically well-accepted fact that a variety of quite different pathogenic mechanisms (both immune- and nonimmune-mediated) may lead to progressive tissue fibrosis and loss of graft function. Perception of CAN as an entity (rather than a purely descriptive term) was not only biologically inadequate but also impeded the development of therapeutic strategies, as efficiency of treatment is mainly determined by our ability to first identify and then specifically target the causative pathological mechanism. It was thus mandatory to move the focus of the classification system away from assessing the severity of fibrosing lesions towards the identification of underlying causative mechanisms [20,21]. The 2005 revision of the Banff Classification indeed abandoned the term CAN in favour of a terminology aimed at specifically addressing the underlying pathogenesis and also defined diagnostic criteria for 'chronic active T-cell-mediated' and 'chronic active antibody-mediated' rejection [22].

Current challenges in renal transplant pathology

Currently, we are facing a huge gap between sophisticated immunological concepts of rejection mechanisms and the quite simplistic way of assessing and categorizing allograft rejection by conventional histology. Discrimination between cellular and humoral mechanisms of graft rejection is an important step into the right direction, but has to be complemented by further elaboration of the diagnostic recognition of immunological mechanisms in both acute (early) and chronic (late) rejection.

Moreover, we need to validate the utility of diagnostic criteria (that had been developed for biopsies from dysfunctional kidneys) for assessing protocol biopsies from patients without clinically obvious graft dysfunction.

Accurate and timely diagnosis of chronic rejection

The specification of diagnostic criteria for chronic rejection was formally very important. The current definition however requires already established chronic lesions like arterial intimal fibrosis or chronic transplant glomerulopathy (CTG). These lesions represent an advanced stage of the rejection process, are likely irreversible, and are markers of unfavourable outcome [23]. It therefore is crucial to identify chronic rejection at the earliest stage possible in order to prevent irreversible damage to the graft by timely application of specific treatment. Early and reliable

detection of possibly minor, clinically unapparent, alloimmune reactions is however still a complex diagnostic challenge.

Studies on protocol biopsies, performed at predefined time-intervals after transplantation, irrespective of the status of graft function at the time of biopsy, fundamentally contributed to current concepts about mechanisms and the clinical course of chronic rejection.

Sub-clinical cellular rejection as a precursor of chronic graft fibrosis

David Rush *et al.* were among the first to employ protocol biopsies for specifically investigating the predictive value of histomorphological lesions in clinically unsuspicious renal allografts for long-term graft function. They observed morphological signs of rejection (at 1-, 2- or 3-month post-transplantation) in 30% of grafts with normal function [24] and concluded that this might represent sub-clinical rejection (SCR) [25]. In a subsequent prospective study, they found that signs of rejection and chronic lesions in protocol biopsies within the first 6 months after transplantation were indeed associated with impaired renal function at 2 years [26]. Nankivell *et al.* confirmed and extended the concept of SCR as being causative for chronic allograft injury [27,28]. In a cohort of 119 patients, monitored by protocol biopsies for up to 10 years after transplantation, they observed that SCR (including Banff borderline lesions) was a frequent finding early after transplantation (in 61% and 46% of biopsies at 1- and 3-month post-transplantation). If SCR persisted, it was not only associated with a higher degree of interstitial fibrosis and tubular atrophy (IF/TA) but also resulted in significantly decreased renal function at 2 years after transplantation [28]. Their quite stringent definition of true cell-mediated chronic rejection, which occurred in 5.8% of patients, was SCR persisting for at least 2 years [28]. A more recent study from Hannover also seems to support the view that persistence of inflammation might be crucial for the subsequent development of graft dysfunction. Mengel *et al.* calculated the cumulative inflammatory burden by summing up the number of inflammatory infiltrates in sequential biopsies (at 1.5-, 3- and 6-month post-transplantation). Persistence of inflammation in sequential biopsies regardless of its severity, localization or cellular composition-predicted creatinine clearance at 1 and 2 years [29]. Whether persistent inflammation resulted in progressive IF/TA could not be investigated because of lack of subsequent follow-up protocol biopsies in this study.

The concept of persisting inflammation (even of minor severity) causing progressively accumulating tissue injury that only becomes clinically apparent at advanced stages of tissue fibrosis, is biologically plausible, and is also in line with clinical experience from other fields of clinical medicine (i.e. long-standing hypertension or diabetes mellitus causing chronic vascular injury).

Alloantibodies and complement activation promoting chronic graft injury

It is tempting to extend the concept of sub-clinical immune injury causing cumulative organ damage to antibody-mediated rejection (AMR). Numerous studies linking the presence of circulating anti-HLA antibodies to impaired long-term outcome seem to indeed support this view [30–35]. In a recent review article, Terasaki *et al.* strongly advocated the causative role of alloantibodies in chronic rejection [36]. Serological studies on the impact of alloantibodies on late allograft loss however have some important limitations:

(i) Evidence for a causal role of antibodies for graft loss is circumstantial. (ii) Methods for detecting circulating alloantibodies are far from being standardized, making it difficult to directly compare results from different studies. (iii) Definition of positive results is arbitrary and depends on the method employed.

Moreover, in almost all of the studies cited above, a certain proportion of recipients with circulating antibodies (sometimes even the majority of patients) did not experience clinical problems that were likely to have been related to alloantibodies.

Other studies however, searching for direct evidence of chronic AMR in renal allograft biopsies, also supported an association and even causal relationship between alloantibody (as evidenced by serology and/or C4d deposition within the graft) and CTG, the morphological hallmark of chronic rejection [37–39]. It thus appears that in analogy to sub-clinical cellular rejection, antibody-mediated mechanisms might also lead to progressively accumulating chronic tissue damage (mainly affecting the vasculature) that results in clinically overt graft dysfunction only at advanced stages. Therefore, indication biopsies, because likely being performed too late, might be inadequate for guiding successful treatment. The diagnostic challenge is again (like in cell-mediated SCR) detection of clinically quiescent immune mechanisms as early as possible, before likely irreversible chronic tissue injury occurs. It seems however questionable whether serological testing alone, given the limitations mentioned above, is sufficient for immunological monitoring and accurate risk assessment in individual patients.

In a recent study, we therefore tested sequential serum samples from 164 patients with functioning grafts for circulating anti-HLA-antibodies (by flow-cytomeric cross-match testing and highly sensitive solid-phase assays). In

order to specifically investigate the predictive value of circulating alloantibodies, we identified a subgroup of 34 patients with uneventful 1-year post-transplant course and excellent graft function. Nine of these patients (27%) had circulating anti-HLA-antibodies (DSA in five cases). Frequencies of positive test results were not significantly lower than those documented for the other 130 patients. Remarkably, in patients with excellent 1-year graft function, anti-HLA reactivity was not associated with reduced GFR or proteinuria at a later time (median follow up 65 months) [40].

As in patients without graft dysfunction, circulating antibodies (even if donor-specific) are not necessarily associated with subsequent accelerated graft loss, they are of only limited predictive value and thus inadequate for individualized risk assessment. A potential strategy for a more accurate risk assessment in those patients might be staining of protocol biopsies for C4d, which is of proven utility as marker of AMR in patients with graft dysfunction [19].

The diagnostic value of C4d staining in protocol biopsies

Mengel *et al.* investigated the diagnostic relevance of C4d in 551 nonselected protocol biopsies. C4d deposition was detected in 4.4% of the cases but was not associated with inferior outcome (median follow-up 43 months) [41]. A more recent study by Yoon *et al.* [42] detected C4d in 4/79 (5.1%) protocol biopsies and also did not observe graft dysfunction during a median follow-up of 30 months. Even more remarkable, studies on protocol biopsies from ABO-incompatible transplants revealed that C4d was present in 80% [43] to 94% [44] of biopsies but was not associated with morphological signs of AMR [43,44] or adverse outcome [44].

C4d, a highly reliable indicator of AMR in patients preselected for graft dysfunction, seems to be of only limited diagnostic value in grafts with stable function.

One possible explanation for the apparent insensitivity of many ABO-incompatible and some ABO-compatible transplants to antibody- and complement deposition is accommodation, an acquired state of resistance to antibody-mediated injury [45]. The mechanisms of accommodation are however not well understood, making it difficult to reliably confirm its presence in individual grafts and to assess its frequency and role in recipients with anti-HLA antibodies.

Another explanation for C4d positivity without clinical or morphological implications might be that humoral reactivity is only transient. The studies by Mengel and Yoon cited above did not systematically analyse whether C4d deposition persisted in sequential biopsies. Yoon *et al.* however reported a loss of C4d staining in the only two follow-up biopsies performed in four C4d positive recipients [42].

A study by Haas *et al.* on the course of sub-clinical AMR detected in 10/83 highly sensitized cross-match-positive patients successfully transplanted after desensitization, showed that the presence of sub-clinical AMR (C4d positivity and capillaritis/glomerulitis) in a first biopsy predisposed the patients to significantly more intense chronic lesions in follow-up biopsies, as compared with recipients without AMR [46]. Interestingly, in this study a persistence of at least focal C4d staining was observed in 9/10 follow-up biopsies. Persistence of C4d positivity in sequential protocol biopsies might thus be of practical diagnostic relevance for discriminating potentially harmful sustained alloantibody activity, from innocent transient responses.

An additional feature indicating prognostically relevant sub-clinical rejection might be the presence of capillaritis/glomerulitis, which was only rarely (4–18%) found in ABO-incompatible protocol biopsies [44] but almost always present (86–100%) in sub-clinical and clinical cases of AMR reported by Haas *et al.* [43]. Lerut *et al.* [47] observed in a small series of protocol biopsies that peritubular capillaritis at 3 months was significantly associated with signs of chronic antibody-mediated rejection at 1 year. The findings outlined above might be translated into the following diagnostic approach: The isolated occurrence of C4d in a protocol biopsy seems to be of limited value for therapeutic decisions. This finding might however trigger, especially if accompanied by peritubular or glomerular capillaritis, a follow-up biopsy. Persistence or even increase of the lesions in the follow-up biopsy might then be more seriously considered as indication for therapy, their disappearance on the other hand would likely rule out AMR.

Chronic AMR without C4d deposits?

In acute allograft dysfunction, C4d is a reliable marker of AMR suggesting that complement activation within the graft might also crucially contribute to the pathogenesis of the condition [48]. Reports on the prevalence of C4d in biopsies with CTG, the morphological hallmark of chronic AMR, are more controversial. Although statistically associated with CTG [38], C4d is far from being universally present even in cases with serologically detectable anti-HLA antibodies. Issa *et al.* [23] found CTG being positively correlated with increasing levels of (pre-transplant) anti-HLA-class II antibodies but could demonstrate C4d in only 24% of the biopsies investigated. Previous studies from the same group and others detected anti-HLA in 82% [49], 77% [50] and 70% [51] of CTG cases, but C4d deposits were present in only 25%, 9%

and 36% respectively of antibody-positive cases. Akalin *et al.* reported a prevalence of only 36% for DSA and 14% for C4d staining in a cohort of 28 CTG cases [52]. These results might be explained in several different ways:
1 Chronic transplant glomerulopathy is not specific for AMR and might also be caused by antibody-independent mechanisms.
2 A previously active AMR might already have resolved at the time of biopsy, leaving behind capillary lesions but no more complement deposits.
3 Low-level but still biologically active complement deposits might escape detection by immunohistochemistry. Moreover, divergent results could also be caused by unequal sensitivity of staining methods [immunofluorescence on frozen sections (IF) is likely more sensitive than immunoperoxidase on paraffin sections] [53].
4 It is conceivable that anti-HLA antibodies without the ability to activate complement could still mediate injury to endothelial cells.

The latter two concepts, despite representing potential mechanisms of alloantibody-mediated tissue damage, are not recognized by current diagnostic standards that require C4d deposition for the diagnosis of AMR [54]. Recent experimental data from animal models and *in vitro* research however support the concept of anti-HLA antibody-mediated, complement-independent injury to endothelial cells that still might result in progressively accumulating vascular damage. Uehara *et al.* demonstrated that chronic transplant arteriopathy (CTA) could be induced in hearts transplanted to RAG1 KO mice (devoid of T and B cells) by passive transfer of anti-MHC antibodies. Repeated administration of antibody over 4 weeks induced CTA. Endothelial C4d deposits were present upon antibody treatment but disappeared during further follow up (56 days) while CTA did not diminish [55]. A series of *in vitro* studies by the group of Elaine Reed demonstrated that (monoclonal) antibodies against HLA class I antigens exert effects on human endothelial cells by complement-independent activation of signalling pathways involved in cell survival and proliferation. Phosphorylation of focal adhesion kinase (FAK) resulted in downstream activation of ERK or the phosphoinositol-3-kinase (PI3K)/Akt signalling pathway. They further reported that ERK phosphorylation was mammalian target of rapamycin (mTOR)-dependent and could be blocked by siRNA or pretreament with rapamycin [56]. It is of particular interest that signal transduction pathways are activated in a time- and dose-dependent manner, with low doses of antibody-inducing protective mechanisms antibody-inducing protective mechanisms (Bcl-2, Bcl-xL up-regulation) while higher titres of the same antibody might induce expression of fibroblast growth factor receptor (FGFR) thereby enhancing cell proliferation and possibly contributing to vascular damage [57]. Yamakuchi *et al.* demonstrated that anti-HLA antibodies triggered exocytosis of von Willebrand factor and P-selectin from endothelial cells in a complement free *in-vitro* system. This finding was confirmed in human skin transplants to nude mice perfused with the anti-HLA antibody *in vivo* [58].

Although none of the studies cited above was done in kidneys and we are not aware of similar investigations in renal transplant models, the findings are likely to be of universal importance as they highlight the complexity of antibody-mediated responses that go far beyond complement-mediated cell activation/damage. Duration and dosage (titre) of antibody exposure are obviously crucial for the nature of molecular events triggered by antibodies bound to cells surfaces. These findings from studies in transplanted hearts and endothelial cell culture should stimulate similar investigations in renal allografts and the search for novel diagnostic markers of AMR and/or accommodation.

Does treatment of SCR prevent chronic graft injury?

If SCR is a major driving force of chronic rejection, one would predict that early treatment of SCR must be a highly efficient option in preventing graft fibrosis and graft loss. Treatment of SCR is indeed a common strategy in centres performing protocol biopsies as part of standard patient care. Unfortunately only few controlled studies evaluated the benefit of early therapy of cellular SCR [59] and a systematic evaluation of treatment in sub-clinical AMR is not yet available. Treatment of SCR however, does not seem to be as beneficial for long-term allograft function and survival as could be anticipated in view of the evidence outlined above [59]. One reason for the limited success of therapy might be that SCR and/or chronic rejection is not simply the persistence of acute rejection, as we know it from indication biopsies early after transplantation, but also involves different mechanisms. It might therefore be inappropriate to apply the same diagnostic rules that were developed for classifying clinically overt acute rejection occurring early after transplantation, to clinically quiescent SCR detected months or years after transplantation. One strategy to refine the assessment of immunological events within the graft might be a more detailed analysis of immune cells infiltrating the graft.

Immunophenotyping of graft infiltrating cells

From a biological point of view, it seems overly simplistic to assess allograft rejection by just estimating (or at best counting) the amount of infiltrating immune cells without considering the variable function of different cell

types. Although it was observed that not only T cells but also monocytes/macrophages (MO), B cells and other immune cells infiltrate rejected grafts, T cells are assumed to be the most deleterious immune cell type. They are therefore still the main diagnostic and therapeutic target in clinical transplantation [54]. Several immunohistochemical studies however noticed that MO could account for the majority of graft-infiltrating cells [60,61] and some studies also indicated that they actively contribute to rejection [62–64]. A recent publication even suggested that an interaction of T cells with MO is even crucial for the development of acute rejection and the associated renal dysfunction [63].

Graft-infiltrating B-cells are less numerous than MO or T cells [65,66] and received only little attention until Sarwal *et al.* [67] observed an association between B-cells infiltrates and steroid-resistant acute rejection and poorer outcome in one of the first studies utilizing DNA-arrays for gene-expression profiling of allograft biopsies. This observation was confirmed in a subsequent immunohistochemical study by Tsai *et al.* [68]. Nonetheless, other studies did not observe any association of B-cell-rich infiltrates with resistance to conventional anti-rejection treatment, impaired renal function or reduced graft survival [69–71].

Studies on immunotyping of graft infiltrating cells commonly reported cells counts but not always paid attention to the microanatomical location and structure of infiltrates.

Kerjaschki *et al.* [72] took a different approach and specifically examined nodular immune cell aggregates that are not uncommon in late allograft biopsies. They observed that these cellular aggregates frequently represented clusters of $CD4^+$, $CD8^+$ and $CD20^+$ lymphocytes and dendritic cells, which were arranged around newly formed lymphatic vessels. Numerous chemokine receptor (CCR7)-positive immune cells within the nodular infiltrates suggested an active role of chemokine (SLC/CCL21)-secreting lymphatic endothelial cells in the formation of organized immune cell aggregates. The authors hypothesized that this type of nodular infiltrates reminiscent of 'tertiary' lymphatic organs (TLO) [73] might play a role in launching and perpetuating alloreactivity.

The design of this study unfortunately did not allow any conclusions on the clinical impact of this type of nodular infiltrates. A potential role of TLO in chronic rejection was however suggested by animal models and by findings in explanted human allografts [74].

Hippen *et al.* reported a higher rate of steroid-resistant rejection and reduced graft survival in patients with B-cell clusters [75], a finding that was not confirmed by others [69–71]. It is however important to note that definition of B-cell clusters was not uniform among these studies and never as sophisticated as in the initial report from Kerjaschki *et al.* It thus remains unclear as to what degree B-cell clusters investigated by Hippen [75], Bagnasco [69], Kayler [70] and Scheepstra [71] indeed represented TLO.

Another reasonable strategy for gaining more detailed insights into the role and function of immune cells infiltrates within the graft is to specifically search for subsets of immune cells with well-defined functional properties such as the regulatory T-cells (Tregs). The capacity of Tregs, usually defined by co-expression of CD4, CD25 and forkhead box P3 (Foxp3) [76], to counteract T-cell mediated injury and induce tolerance was originally demonstrated in animal models [77–79]. Because of their ability to limit immune responses, Tregs gained increasing interest in human transplant immunology. The fact that they might represent graft-infiltrating lymphocytes that are beneficial rather than detrimental is also of major diagnostic importance as this concept would challenge the assumption that T cells within the graft invariably are equivalent to rejection. Staining for Tregs might be an attractive option for further characterization of otherwise prognostically doubtful inflammatory infiltrates in protocol biopsies or borderline lesions in indication biopsies. Bestard *et al.* investigated 37 protocol biopsies with SCR for Tregs and found that the number of FoxP3-positive T-cells positively correlated with graft function after 2 and 3 years [80]. Mansour *et al.* determined the mRNA levels for FoxP3 in 46 cases of untreated Banff Borderline lesions. FoxP3 expression was significantly higher in patients who did not experience further increase of serum creatinine values after biopsy [81]. Remarkably, other studies analysing the role of Tregs in indication biopsies did not report a beneficial effect of FoxP3-positive cells on outcome but rather reported an association of Tregs with rejection and reduced graft survival [82,83]. This discrepancy again highlights the fact that diagnostic findings in biopsies for indication do not necessarily have the same pathological significance if observed in protocol biopsies.

Immunotyping of immune cells within the graft is not yet part of the standard work up of renal allograft biopsies and is also not specifically recommended by the Banff classification. It however remains a valid tool for research and might also help in guiding specific therapy in individual patients. For instance, there are some reports on successful therapy of B-cell rich rejection with rituximab (anti-CD20 antibody) [84,85], and staining for Tregs in protocol biopsies might be a useful tool for refined risk assessment in cases of SCR.

Conclusion

The diagnostic potential of renal allograft biopsies is by far not yet fully exploited. Data from studies on protocol

biopsies indicate that clinical signs of dysfunction are not very sensitive indicators of graft injury and protocol biopsies might thus help in detecting early stages of progressively accumulating organ damage. We must however consider that diagnostic standards derived from early indication biopsies might not be fully appropriate for the assessment of clinically inconspicuous conditions in protocol biopsies like sub-clinical rejection, accommodation and C4d-negative antibody-mediated rejection. The challenge is to translate the vast knowledge about immunological mechanisms of rejection into practically applicable diagnostic criteria and novel diagnostic tools.

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