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Immunologic Concepts in Kidney Transplantation

John R. Lee and Thangamani Muthukumar

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<http://dx.doi.org/10.5772/53798>

1. Introduction

On December 23, 1954, the first successful kidney transplantation was performed at the Peter Bent Brigham Hospital in Boston with the donor being an identical twin of the recipient [1]. This sentinel event marked one of the first in the history of modern medicine that a perfectly healthy individual underwent an invasive surgical procedure for the benefit of another. It also revealed that kidney donation and kidney transplantation were feasible and thus ushered the era of renal transplantation. Crossing immunological barriers between donor and recipient became the next major hurdle in the field. Over the past decades, advances in immunosuppression and crossmatch techniques have significantly improved the survival of renal transplants. Allograft survival has improved from 10% in the 1960's to over 90% in the modern era [2,3].

This chapter will introduce the basics of transplantation immunology with an emphasis on the HLA system and mechanisms for HLA typing. It will also provide an overview of different crossmatch techniques and also expound upon methods to determine sensitization to the donor. Lastly, it will introduce the basis for acute rejection, the diagnostic criteria that is currently employed, and noninvasive methods to diagnose acute rejection.

2. HLA system

The Human Leukocyte Antigen (HLA) is composed of proteins expressed on all nucleated cells encoded by a gene cluster called the major histocompatibility complex (MHC) and is the cornerstone of how mammals can differentiate between self and non-self. The work on MHC originated from Peter Gorer in the 1930's where he identified a set of four blood-group antigens [4]. Since these important findings, much research has been performed in investigating the MHC system and the equivalent H-2 system in mice. Understanding the

HLA system is critical to transplantation because differences in HLA between donor and recipient allow the immune system of a recipient to reject a donor's kidney. The HLA system is divided into two major classes of molecules: class I and class II.

2.1. HLA class I system

There are three major alleles of the HLA class I genes: HLA-A, HLA-B, and HLA-C. The HLA class I molecule is a membrane-bound glycoprotein that is expressed on the surface of all nucleated cells and presents peptides for recognition by the host immune cells. The structure of the HLA class I molecule can be divided into the following regions: a cytoplasmic tail, a transmembrane segment, and an extracellular alpha component that has three external domains: a1, a2, and a3. In order to be able to present antigen to lymphocytes for recognition, the HLA class I molecule must associate with B2-microglobulin. Between the a1 and a2 domains is a groove like structure that can bind peptides of 8 to 10 amino acids.

To understand the process of presentation in the HLA class I system, we will use an example of an intracellular bacteria that has invaded a nucleated cell of the host. Presentation of antigen by an HLA class I molecule requires an important step called loading of the antigen on the HLA class I molecule. Inside each cell is a protein-degrading machinery called the proteasome which constantly degrades proteins into amino acids. Following bacterial invasion, some of the bacterial proteins will be degraded by the cell's proteasome. The short peptide segments of the bacterial proteins are loaded onto the groove of the host HLA class I molecule. The HLA class I molecule is then brought to the surface of the cell and can now present the peptide to the host's circulating lymphocytes. If a lymphocyte recognizes the bacterial antigen in the groove of the host HLA class I molecule, it can become activated and can initiate a cascade of intracellular changes, resulting in the proliferation of lymphocytes that can specifically attack the infected cell. It is through the host HLA class I antigen processing and presentation that the immune system can eliminate cells infected with intracellular bacteria. If presented without the host HLA class I molecule, naked bacterial proteins are not recognized by the host immune system [4,5].

For transplantation, this process can be summarized in a similar way. Just as the proteasome processes proteins associated with intracellular bacteria, it also degrades all proteins in a foreign cell including the HLA class I molecules. As such, donor leukocytes that travel along the kidney at the time of transplantation undergo the fate of an intracellular bacteria. They present peptides of the donor HLA class I molecule to circulating host lymphocytes. Lymphocytes recognize the donor peptides and mount a response against the foreign HLA class I molecule.

2.2. HLA class II system

An important difference between HLA class I molecules and HLA class II molecules is that the HLA class II molecules are expressed in a limited number of cell lines such as dendritic cells, macrophages, and B lymphocytes. Certain other cells like vascular endothelial cells and various epithelial and mesenchymal cells can be induced to express class II molecules. There

are 3 major alleles of the HLA class II genes: HLA-DP, HLA-DQ, and HLA-DR. The function of the HLA class II molecule is to present extracellular foreign antigens to the immune system.

The structure of the HLA class II molecule can be divided into the following regions: a cytoplasmic tail, a transmembrane segment, and an extracellular alpha component that has two external domains. Unlike the HLA class I molecule, the HLA class II molecule is composed of a dimer. Once a dimer is formed, the extracellular units of the dimer create a groove like structure that can bind peptides for presentation. This peptide binding groove is the place where foreign antigens can be presented to lymphocytes.

To understand the process of presentation in the HLA class II system, we will use an example of extracellular bacteria. A macrophage, which is one of the cell types that express HLA class II molecules, can endocytose an extracellular bacteria. The endocytosed bacteria fuse with lysosomes, which contain enzymes that will degrade peptides of the bacteria. Peptides from this degradation event are loaded onto the HLA class II molecules which are brought to the surface for display to the immune system. The HLA class II molecules present the peptides to the host lymphocytes resulting in various immune responses that culminate in the killing of the bacteria.

For transplantation, the immune response can be summarized in a similar way. Specialized recipient cells that express the HLA class II molecule routinely endocytose contents of their environment. As such, these specialized cells can endocytose the contents of donor cells which may include donor HLA class I or class II molecules. These peptides are subsequently loaded onto the HLA class II molecule and can now be presented to the recipient's circulating lymphocytes.

A major functional difference between the two classes of HLA is that the class I molecules present peptides derived from intracellular proteins to cytotoxic CD8 T cells, whereas class II molecules present peptides derived from extracellular proteins to CD4 T cells. After a kidney transplant, donor antigen presenting cells migrate to the draining lymph nodes of the recipient. The recipient CD4+T cells respond to donor class II HLA-peptide complex and the recipient CD8+T cells respond to donor class I HLA-peptide complex. This is called the direct pathway and is fundamentally different from the way the immune system responds to a foreign antigen. Contrary to MHC-restriction by which the T cells that respond to foreign peptides do so only when the foreign peptides are presented by antigen presenting cells expressing the same MHC as the responding T cells, T lymphocytes of the recipient respond and proliferative to donor derived non-self HLA molecules. In the indirect pathway, the sequence of events is similar to the host response towards an extracellular pathogen; recipient's antigen presenting cells internalize donor derived peptides and present as recipient HLA-donor peptide complex to the recipient lymphocyte [6].

2.3. HLA typing

Determining the HLA make up of an individual is called HLA typing. The HLA system is extremely diverse with hundreds of known alleles for each HLA class molecule. We have two alleles, one derived from each parent, for each HLA molecule. Alleles derived from each

parent that are transmitted together is called a haplotype. Matching or mismatching between donor and recipient is reported based on haplotypes for living related transplants and on individual antigens for deceased donor transplants. Typically, there are three major HLA alleles that are routinely assessed: 2 alleles of HLA-A, 2 alleles of HLA-B, and 2 alleles of HLA-DR. It is important to remember that, immunologically, a 6-antigen matched deceased donation is not the same as a 2-haploidentical living-related donation. Similarly, due to differences in minor histocompatibility antigens, donation between 2-haploidentical siblings is not the same as donation between identical twins. In the following sections, we will review the methods for determining the composition of the HLA antigen system of an individual.

2.3.1. Serology based methods

Serology based typing was the first method used to identify the HLA of an individual. This technique is similar to blood group typing. It utilizes viable lymphocytes of the individual to be typed. The lymphocytes are mixed with antisera that contain antibodies to a wide spectrum of HLA. Although simple, there are several limitations with this method. The coverage of HLA antigen screening is not comprehensive as antisera for corresponding HLA have been largely developed in Caucasian population. Variability exists in the production of antisera resulting in differences among laboratories. Viable lymphocytes are necessary for serology typing. Given these limitations and the significant decrease in cost for DNA typing methods, serology based methods have been largely supplanted [7,8].

2.3.2. DNA typing methods

Currently, the major techniques in DNA typing include sequence-specific primers (SSPs), sequence-specific oligonucleotide probes (SSOPs), and sequencing-based typing (SBT). The SSP method takes advantage of the polymerase chain reaction (PCR) and the specificity of primers. A PCR will not undergo amplification unless the sequences of the primers are nearly perfect in binding. Primer sets have been designed in order to detect the variation in sequences for HLA typing. The SSP method can thus detect the HLA typing of an individual. Given the significant reduction in cost for PCR, the technique is inexpensive and can provide HLA typing in few hours. The SSOP method provides a complementary approach. The method utilizes 6 to 19 length nucleotide probes that will bind to DNA sequences specific to HLA alleles. The probes are labeled with a marker like digoxigenin that will allow for identification of HLA alleles.

Because of increasing number of HLA alleles being identified, higher resolution methods have been developed. Both the SSP and SSOP methods can only identify known HLA alleles. In contrast, SBT method does not require prior knowledge of the HLA alleles and can reveal new alleles. This method does provide the most comprehensive understanding of an HLA typing but is time consuming and expensive. Many centers utilize SSP method and SSOP method for routine identification of the typing of an individual [7,8,9].

2.4. HLA matching

It was recognized that allograft survival was superior in siblings who shared the same serologically typed HLA when compared to non-matched deceased donor transplants. Given advances in immunosuppressive therapy and improved crossmatching techniques, transplantation across HLA barriers is now routinely performed. In the early 1990s, it was shown that there was an increased rate of one-year graft survival and estimated half-life for matched grafts compared with mismatched grafts [10]. Long term graft survival rather than early rejection is affected by the degree of HLA mismatch. As per the 2010 annual report of the Scientific Registry of Transplant Recipients, the five-year allograft survival for deceased donor kidney transplants was 77% with zero HLA mismatch and 67% with six HLA mismatch (3). Two haplotype matched living transplants are estimated to have a half life of approximately 30 years while one haplotype matched living transplants have a half life of 18 years [11].

In addition to the major HLA described above, minor antigens may play a significant role in allograft survival. An important antigenic molecule that is well described is the MHC class I polypeptide-related sequence A encoded by the MICA gene. The MICA gene is located on human chromosome 6 but unlike class I molecule, does not associate with beta-2-microglobulin and thus does not present antigens like HLA class I molecules. However, it is highly polymorphic and over 50 antigens have been described. Transplant recipients who have antibodies to MICA have worse graft survival compared to those who do not [12,13,14].

3. Blood group matching

In order to transplant a kidney, the same rules of blood transfusion apply. Thus, ABO blood group matching is the fundamental first step. Rh matching, however, is not required as Rh proteins are predominantly expressed only on red blood cells. Aside from HLA matching, ABO incompatibility has been another successful barrier that has been crossed in renal transplantation. In Japan, over 1000 transplant patients have received ABO incompatible transplants with good allograft survival. One-year and 3-year graft survival rates in this cohort were 96% and 94%, respectively [15]. Desensitization protocols have been developed that include use of rituximab, splenectomy, or plasmapheresis to successfully achieve high rates of graft survival.

4. Complement Dependent Cytotoxicity (CDC) crossmatch test

The CDC crossmatch test was a landmark *in vitro* test that propelled transplantation into a new era. Developed by Paul Terasaki in the late 1960's, it is still used today and is a prerequisite for any renal transplantation. The CDC crossmatch test essentially screens for preformed antibodies in the recipient that may immediately react against the donor. In this test, T lymphocytes are isolated from the donor and mixed with serum from the recipient. When preformed antibodies from the recipient recognize the HLA class I molecule, these antibodies bind to them. Following the addition of complement, the cells undergo lysis. A dye that penetrates lysed cells is utilized to detect the strength of the cell death. In contrast, if no antibodies in the recipient's serum bind to the T-lymphocytes of the donor, complement

will not be activated and there will be no cell death. No dye will be taken up by the cells and the test is considered a negative reaction. The result of the crossmatch test is reported as the percentage of dead cells relative to live cells as determined by microscopy. The reading is on a semi-quantitative scale with 0 representing no dead cells, 2, 4, and 6 representing increasing severity of cell death, and 8 representing complete lysis of cells. When there is greater than 20% cell lysis, the test is reported as positive and is generally considered a contraindication for transplantation [6].

The importance of the CDC crossmatch test in transplantation cannot be underscored. In Terasaki's initial series, 24 out of 30 transplants that tested positive for the CDC crossmatch test had immediate allograft failure while only 4 out of 17 transplants that tested negative had immediate allograft failure [16]. Since the development of this test, hyperacute rejection with immediate allograft failure has largely disappeared.

4.1. Significance of B lymphocyte CDC test

At the present time, a positive CDC crossmatch test utilizing T lymphocytes of the donor is considered an absolute contraindication for kidney transplantation. T cells do not constitutively express HLA class II molecules. Hence, the result of a positive T lymphocyte crossmatch test generally reflects antibodies to HLA class I only. A positive CDC crossmatch test using B lymphocytes of the donor has different implications. B lymphocytes express both HLA class I and HLA class II molecules. A CDC crossmatch test that is positive against donor B lymphocytes but negative against donor T lymphocytes can be interpreted to represent a HLA class II antibody that reacts against the donor or to represent low levels of HLA class I antibodies against the donor. The expression of HLA class II molecule is not universal like HLA class I molecule and is limited to macrophages, dendritic cells, and B lymphocytes. A positive B lymphocyte CDC crossmatch test is not an absolute contraindication to proceeding with the transplantation. However, it has been associated with reduced long term graft survival [17]. A positive T lymphocyte CDC crossmatch in the presence of a negative B lymphocyte CDC crossmatch could possibly be a technical error related to B lymphocyte viability and is usually repeated [18].

4.2. Advances in CDC testing

In order to enhance the sensitivity of the CDC crossmatch test, anti-human globulin (AHG) has been utilized [6]. Efficient complement activation in the CDC crossmatch test depends not only on the antibody binding to the donor cells but also the concentration of antibodies on the surface of the cells. It is possible to have a false negative T lymphocyte CDC crossmatch test if the concentration of antibodies binding to the T lymphocytes is below the threshold for complement activation. Addition of AHG will enhance the concentration of antibodies if specific binding of antibodies are already present and thus increase the sensitivity of the CDC crossmatch test.

As both IgG and IgM can fix complement, the CDC crossmatch test cannot distinguish IgG from IgM antibodies. IgM antibodies are usually autoantibodies. IgM antibody exists as a

pentamer that is held by disulfide bonds. Such disulfide bonds can be broken down by the use of the reducing agents like 2-mercaptoethanol or dithiothreitol or by heating the serum to 63°C for 10 minutes. As such, a CDC crossmatch test that is positive against B lymphocytes but negative when the same serum is treated with heat at 63°C likely indicates an IgM antibody. However, reducing agents or heat can also inactivate low levels of IgG antibodies. The significance of the presence of IgM antibodies is not well understood.

4.3. Caveats to CDC crossmatch testing

While the standard CDC crossmatch test with B lymphocytes and T lymphocytes has helped the transplant community to avoid hyperacute rejection, the test does have some limitations. Although lymphocytes express class I HLA and class II HLA molecules, they do not provide the full representation of all antigens against which antibodies from a recipient can react. Examples to consider are antibodies to MICA or anti-endothelial antibodies. MICA, as described previously, are HLA like molecules expressed on the surface of cells with many different allelic variations. Importantly, MICA is expressed on many cell lines like endothelial cells, dendritic cells, fibroblasts, and epithelial cells but not on lymphocytes. Thus, the standard CDC crossmatch tests with B lymphocytes and T lymphocytes are unable to detect donor specific antibodies against MICA. In a similar fashion, antibodies against donor endothelial cells may also be missed on the standard CDC crossmatch test as the targeted antigen may be present on the endothelial cells but not on the lymphocytes. A CDC test with endothelial cells has recently been developed and employed. In reports of hyperacute rejection despite a negative CDC crossmatch test, investigation with the endothelial cell CDC crossmatch test has revealed the presence of antibodies against donor endothelial cells [19]. While it is possible that non-HLA antibodies can cause a hyperacute rejection, this is likely a rare event.

5. Flow cytometry crossmatch test

Advances in the field of transplantation have led to the development of a more sensitive test called the flow cytometry crossmatch test. The flow cytometer utilizes laser-based technology to evaluate the status of single cells one at a time. In a flow crossmatch test, cells from the potential donor are isolated and are labeled using a fluorescent marker. A fluorescent labeled antibody against CD3 or CD19 is used as a marker to distinguish T from B lymphocytes. The donor cells are incubated with the recipient's serum to allow for potential antibodies to bind. If there are donor specific anti-HLA antibodies, the Fab portion of the antibody binds to the HLA antigens on the cell surface. Fluorescein-labeled goat anti-human antibody is then used as the reporter fluorescent dye to detect the binding of this alloantibody. This secondary antibody can detect either IgG or IgM antibodies. Thus, if there is a positive reaction between the recipient's serum and donor lymphocytes, the flow cytometer will be able to detect this interaction as it will recognize the fluorescent-labeled anti-CD3 or CD19 antibody and the fluorescent labeled antibody against the Fc portion of the donor specific antibody. If there is a negative reaction between the recipient's serum and donor lymphocyte, the flow cytometer will recognize the fluorescent labeled anti-CD3 or

CD19 antibody but not detect any fluorescent labeled antibody against the Fc portion of donor specific antibody [6].

The flow crossmatch test has many advantages and is now routinely used prior to renal transplantation. It is considered to be more sensitive than the CDC crossmatch test. Complement fixation or high-titer antibodies are not required to obtain a positive result. During the early years of transplantation, patients continued to experience hyperacute rejections despite a negative CDC crossmatch test. Studies using flow crossmatch tests found that many acute rejections were associated with a positive flow crossmatch [20]. More recent studies have also suggested that transplant recipients with negative flow cytometry crossmatch test have better renal survival compared with those with positive flow cytometry crossmatch [21,22].

It is important to note a few caveats in the interpretation of the flow crossmatch test. Flow cytometry results are reported as positive or negative based upon the median channel shift caused by the binding of a specific antibody. The number of channel shifts required to call a test positive or negative varies among laboratories and has not been standardized. Although modifications can be made to detect IgM antibodies, typically the standard flow crossmatch test only detects IgG that is bound to donor cells. A possible scenario is that the CDC crossmatch test is positive against T lymphocytes but negative against T lymphocytes when heated to 63°C, thus suggesting the presence of an IgM antibody. The flow crossmatch test will be negative as it cannot detect donor specific IgM antibody. Unlike the CDC crossmatch test, the flow crossmatch test is also not dependent on complement. A possible scenario is that the CDC crossmatch test is negative against T lymphocytes but the flow crossmatch test is positive against T lymphocytes. A possible interpretation of these results is that the antibodies that are binding to the donor T lymphocytes are non-complement fixing antibodies like IgG2.

5.1. Significance of a positive flow crossmatch test

The significance of a positive result in the presence of a negative CDC crossmatch is not entirely clear. In the absence of prior sensitization, a positive T or B lymphocyte flow crossmatch is not associated with increased risk of acute rejection. In patients who are sensitized prior to transplantation, the graft survival is inferior [18]. The outcome of a positive B lymphocyte flow crossmatch is less clear [23]. Some studies have not found that a positive B lymphocyte flow crossmatch influences graft function. These studies evaluated deceased donor transplantations and did not find a difference in the one year and three year graft survival [24]. Other studies have found that a positive B lymphocyte flow crossmatch test is associated with worse survival. One study evaluated 145 patients and found that patients with a positive B lymphocyte flow crossmatch had significantly poorer graft survival than those with a negative one (68% vs. 90% at 1 year) [25].

6. Panel Reactive Antibody test

Instead of utilizing donor T lymphocytes and B lymphocytes as used in the standard CDC, the panel reactive antibody (PRA) test utilizes a panel of lymphocytes from approximately

100 blood donors that represent the local population of potential donors. Percentage of PRA is the number of reactions within that panel. This test allows for characterizing the sensitization of a recipient. For example, if the serum of a recipient causes lysis of cells and hence a positive reaction in 80 out of 100 samples, the PRA is 80%. Theoretically, if a donor is available from that donor pool, the recipient would experience acute rejection 80% of the time (6). The PRA test, however, is not comprehensive. The panel of individuals does not represent all HLA class I and class II molecules. Moreover, the antigen specificity is not known. Despite these drawbacks, the PRA test has been extremely useful in providing information about the sensitization of a recipient.

Now, with refinement in technology, it is possible to determine the antigen specificity against which an individual produces antibody. These antigens against which an individual has antibodies are called unacceptable antigens. Currently, several centers do not perform routine PRA and instead calculate the PRA (CPRA). By knowing the frequency of unacceptable antigens in the national pool of donors, it is possible to calculate the likelihood that a recipient and a donor will be incompatible. Patients with CPRA that is greater than 80% receive additional points for the allocation of a kidney.

7. Solid phase assays for donor specific antibodies

Development of solid phase assays in the past decades has advanced the ability to identify antibodies in the blood to specific HLA. Two methods, one based on ELISA and one based on fluorescent microspheres (Luminex®), are currently being used to determine the presence of HLA class I and HLA class II antibodies.

In the ELISA method, specific purified HLA molecules are immobilized on a plastic surface. The serum of the patient of interest is then incubated on the plastic surface. If there are antibodies directed against a specific HLA, these antibody binds to the antigen. A second anti-human IgG directed against the Fc portion of antibody is now added to detect the serum antibodies that have bound to the HLA. An enzyme is usually attached to this second antibody. If the second antibody binds to the Fc portion of the specific anti-HLA antibody, addition of a substrate for the attached enzyme will generate a colored product that can be quantified.

In the fluorescent microsphere method, specific synthetic HLA molecules are immobilized on fluorescent microspheres. The Luminex® system consists of 100 fluorescently dyed 5.6 micron-sized polystyrene microspheres. These are internally dyed with red and infrared fluorophores. When excited with laser, each microsphere generates a unique spectral signature allowing for powerful multiplexing. The serum of the patient of interest is incubated with the microspheres coated with HLA molecules. A second fluorescent-labeled anti-human IgG directed against the Fc portion of the antibodies is then added to the system. A flow cytometer will detect the amount of fluorescent labeled anti-human IgG that is bound to a particular HLA molecule. The strength of the antibody titer is quantified as the mean fluorescence intensity (MFI). Currently, Luminex® based anti-HLA detection is available as a screen to determine the presence of anti-HLA antibodies (LABScreen PRA) as well as to detect the specificity of the antibodies (LABScreen single antigen) [26].

It is important to note some limitations with the solid phase assays. The chosen panel of HLA in solid phase assay usually represents the most prevalent HLA in the population and so can miss some of the less common HLA. The solid phase assay is also more of “in vitro” test as the HLA in the ELISA method and fluorescent microsphere method are not expressed on cells but are rather synthetically generated and placed on plastic plate or beads, respectively. As such, the solid phase assays will be able to detect HLA in secondary structure but may miss detection of antibodies to HLA in quaternary structure as might be detected on an assay like the CDC crossmatch test. Commonly used solid phase assays are currently designed to detect IgG antibodies and so an IgM antibody can be missed. Finally, the solid phase assays does not distinguish complement fixing from non-complement fixing antibodies. Despite these limitations, solid phase assays is the most robust test that is currently available to detect donor specific antibodies and have provided a wealth of information about a recipient’s sensitization to a donor kidney.

7.1. Significance of donor specific antibodies

Due to sensitization from prior transplants, pregnancy, or transfusions, potential transplant recipients may have preformed donor specific antibody (DSA) against HLA. Several studies have examined the significance of having preformed antibodies and have found an association with worse graft survival and increased antibody mediated rejection. In one study that investigated DSA in over 400 transplant recipients, those with preformed anti-HLA DSA had inferior graft survival at 8 years as compared with those with no preformed DSA (93% vs. 61%) [27]. Another study investigated pre-transplant DSA in 334 patients and found a higher incidence of clinical/subclinical antibody mediated rejection in those with DSA (55% vs. 6%) [28]. The strength of the DSA as measured by MFI may also play a role in the development of antibody mediated rejection [27].

While many studies have found that the presence of pre-transplant anti-HLA DSA is a risk factor for inferior graft survival and increased AMR, not all anti-HLA DSA may be pathogenic. In one study, 30 out of the 67 patients who had anti-HLA DSA did not have a clinical/subclinical antibody mediated rejection [28]. Five-year death censored graft survival among this group was similar to the transplant patients without anti-HLA DSA. Clearly, further research is needed to elucidate the characteristics of anti-HLA DSA that are pathogenic.

Transplant recipients may not have anti HLA-DSA prior to the transplant but can develop them after transplantation. Similar to preexisting DSA, de novo anti-HLA DSA has been found to be a risk factor for graft failure. In an international cooperative study of transplant recipients who did not have anti-HLA antibodies prior to transplant, the one-year allograft survival after the detection of antibodies was worse among the post transplant recipients with de novo anti-HLA antibodies (9%) than among the post transplant recipients with no post transplant anti-HLA antibodies (3%) ($P < 0.001$) [29].

7.2. Monitoring and treatment

Transplantation in the presence of preformed anti-HLA DSA is associated with increased risk of antibody mediated rejection and graft failure. Transplant centers have utilized

desensitization protocols with agents like intravenous immunoglobulin or rituximab to reduce the antibody titers prior to transplant. Acceptable patient and graft survival have been reported [30]. Furthermore, transplanting highly sensitized patients after desensitization may be beneficial as compared to maintaining them on dialysis. One study that evaluated 211 HLA sensitized patients found a survival benefit of a desensitization protocol as compared with a matched control on dialysis awaiting a deceased donor transplant (81% vs. 31% survival at 8 years, $P < 0.001$, respectively) [31]. It is not clear how patients who are sensitized and transplanted following desensitization should be monitored. One study found that an increase in DSA by one week after transplant following desensitization therapy was significantly associated with antibody mediated rejection [32].

8. Transplant rejection

Acute rejection continues to remain a significant problem after transplantation. Categorization of rejection is based on the Banff classification schema. In the early 1960's, hyperacute rejection was described in cadaveric kidney transplantations. Shortly after the vascular anastomosis, the allograft became cyanotic. On microscopic examination, the major findings included neutrophil and platelet margination in glomerular and peritubular capillaries, red blood cell stasis, acute tubular necrosis, and variable degree of cortical necrosis. Immunofluorescence studies demonstrated the presence of IgG in peritubular capillaries [33].

Many of these hyperacute rejections have largely disappeared following the development of the CDC crossmatch test. Currently, acute rejection is classified into two major categories: T cell mediated acute rejection (ACR) and acute antibody mediated rejection (AMR) (Table 1). The following sections will provide an overview of the evolution of the criteria for both major types of acute rejection.

8.1. Renal transplant biopsy adequacy

The gold standard for diagnosis of transplant rejection is a renal biopsy. Diagnosis of transplant rejection depends on the availability of an adequate specimen for evaluation. In 1991, the Banff schema defined an allograft biopsy specimen as adequate if it contained seven or more glomeruli [34]. Further revisions in 1997 required two cores of tissues, 10 or more total glomeruli, and the presence of at least two arteries [35]. The presence of seven glomeruli and one artery is the threshold for a minimal sample. The diagnosis of acute rejection thus depends on an adequate specimen as defined by the Banff criteria.

8.2. Acute T cell-mediated rejection

In the 1980's and 1990's it was observed that interstitial inflammation found in transplant kidney biopsies, in many cases, had a negligible effect on graft survival and thus was not pathognomonic for acute rejection [34]. Tubulitis, the infiltration of lymphocytes into the tubules of kidney, was associated with allograft dysfunction and became the hallmark of acute rejection. The Banff 1991 consensus defined acute rejection as the involvement of tubulitis (>4 mononuclear cells/tubular cross section) and/or intimal arteritis (the infiltration of

lymphocytes in the arterial wall). Further revisions of the Banff criteria in 1997 provided a classification of acute T cell-mediated rejection that is currently used today [35]. Grade 1A rejection requires moderate to severe interstitial inflammation (>25% of parenchyma affected) (i2 or i3) and foci of moderate tubulitis (>4 mononuclear cells/tubular cross section) (t2). Grade 1B rejection requires moderate to severe interstitial inflammation (>25% of parenchyma affected) (i2 or i3) and severe tubulitis (>10 mononuclear cells/tubular cross section) (t3). Grade 2A rejection requires intimal arteritis (presence of lymphocytes within the intima) (v1). Grade 2B rejection requires severe arteritis (involving >25% of luminal area) (v2). Grade 3 rejection requires transmural arteritis and/or necrosis of medial smooth muscle cells (v3) (Table 1).

Banff 4: Acute T Cell Mediated Rejection	Banff 2: Acute Antibody Mediated Rejection
Grade IA: >25% interstitial inflammation (i2,i3) AND foci of moderate tubulitis (t2)	1. Morphological evidence of kidney injury A) Acute tubular necrosis or B) Neutrophils and/or mononuclear cells in peritubular capillaries and/or glomeruli, and/or capillary thrombosis or C) intimal arteritis/fibrinoid necrosis/intramural or transmural inflammation in arteries
Grade IB: >25% interstitial inflammation (i2,i3) AND foci of severe tubulitis (t3)	
Grade IIA: mild to moderate intimal arteritis (v1)	2. Immunopathological evidence of antibody action A) presence of C4d in peritubular capillaries or B) immunoglobulin and complement in arterial necrosis
Grade IIB: severe intimal arteritis involving >25% (v2) of luminal area	3. Serological evidence of circulating antibodies A) antibodies to donor HLA or B) antibodies to donor endothelial antigens
Grade III: transmural arteritis and/or arterial fibrinoid changes with accompanying lymphocyte infiltration (v3)	All 3 criteria are necessary to make a diagnosis of antibody mediated rejection; 2 out of 3 criteria makes a diagnosis of suspicious for antibody mediated rejection

I Scoring and V Scoring Definitions

I scoring: i0 (<10% parenchymal inflammation), i1 (10 to 25% parenchymal inflammation), i2 (25 to 50% parenchymal inflammation), i3 (>50% parenchymal inflammation)
T scoring: t0 (no mononuclear cells in tubules), t1 (foci of 1 - 4 mononuclear cells/tubular cross section), t2 (foci of 5-10 mononuclear cells/tubular cross section), t3 (foci of >10 mononuclear cells/tubular cross section)
V scoring: v0 (no arteritis), v1 (mild to moderate intimal arteritis in one arterial cross section), v2 (severe intimal arteritis with at least 25% luminal area lost), v3 (transmural arteritis and/or arterial fibrinoid change with lymphocytic infiltrate)

Table 1. Banff Classification of Acute Rejection (adapted from references 35,37,41)

8.3. Acute antibody mediated rejection

Feucht et al. reported the presence of complement-split products (C4d) in early biopsies of patients with high immunological risk [36]. The Banff 97 classification defined antibody mediated rejection as rejection demonstrated to be due, at least in part, to anti-donor

antibody. Two forms, immediate and delayed, were recognized. With the description of staining for C4d as a marker for antibody mediated rejection, an update to the Banff 97 classification was reported in 2003 that defined AMR with three characteristics: i) evidence of morphological injury in the form of either a) acute tubular necrosis, b) neutrophils and/or macrophages in glomeruli or peritubular capillaries or thrombi in glomeruli, or c) intimal arteritis/fibrinoid necrosis/intramural inflammation; ii) immunological evidence of antibody as either a) presence of C4d in peritubular capillaries or b) immunoglobulins in arterial fibrinoid necrosis; and iii) serological evidence of circulating antibodies to donor HLA or other endothelial antigens. If 2 out of 3 characteristics are present, the renal biopsy is considered suspicious for antibody mediated rejection [35, 37] (Table 1).

8.4. Borderline changes

When there is tubulitis and interstitial inflammation but the definition of ACR is not met, the biopsy findings are categorized as borderline changes: 'suspicious' for acute cellular rejection. The criteria for this diagnosis includes: i) no intimal arteritis; ii) mild tubulitis (1 to 4 mononuclear cells/tubular cross section); iii) at least 'i1' inflammation (10-25% of parenchyma involved). The significance of borderline biopsies on renal outcomes is not clearly defined. One study of 100 kidney allograft biopsies categorized as borderline changes found a progressive increase in serum creatinine over time [38]. Nevertheless, management of a borderline diagnosis has not been clearly defined. More recently, a study that compared 40 borderline changes, 35 T-cell mediated rejection, and 116 nonrejection biopsies observed that most cases designated borderline by histopathology were found to be nonrejection by molecular phenotyping [39].

8.5. Other types of acute rejection

ACR and AMR are not mutually exclusive and frequently coexist. In a study of 87 patients with C4d positive AMR as defined by Banff criteria, 32 (37%) had evidence of concurrent ACR. The presence of concurrent ACR was an independent risk factor of allograft failure in kidney transplant recipients with C4d positive acute AMR [40]. It is important to emphasize that Banff criteria are not all-inclusive. Plasma-cell rich acute rejection is an entity where the interstitial inflammation and tubulitis are predominantly composed of plasma cells in addition to lymphocytes. Allergic interstitial nephritis closely resembles ACR and are all not always associated with eosinophil infiltrates. Thus, morphologically, it may be also be useful to categorize acute rejection as (i) interstitial rejection characterized predominantly by interstitial inflammation and tubulitis, (ii) vascular rejection characterized predominantly by intimal arteritis and (iii) capillary rejection characterized predominantly by glomerulitis and peritubular capillary inflammation usually in the presence of circulating DSA.

8.6. Chronic rejection

The Banff classification also defined three forms of chronic rejection: (i) chronic active T cell-mediated rejection characterized by arterial intimal fibrosis with mononuclear cell

infiltration in fibrosis and formation of neo-intima, and (ii) chronic active antibody-mediated rejection characterized by C4d+, presence of circulating antidonor antibodies, and morphologic evidence of chronic tissue injury, such as glomerular double contours and/or peritubular capillary basement membrane multilayering and/or interstitial fibrosis/tubular atrophy and/or fibrous intimal thickening in arteries, and (iii) interstitial fibrosis and tubular atrophy without evidence of any specific etiology that may also include nonspecific vascular and glomerular sclerosis [41]. Morphological evidence of chronic active antibody mediated tissue injury but with negative C4d is being increasingly recognized.

9. Non-invasive molecular techniques for assessing acute rejection

The platforms for molecular based biomarker discovery and validation are: (i) Real-time polymerase chain reaction (RT-PCR), Microarray and RNA sequencing (detection of expression of single or multiple genes), (ii) Elisa and protein microarray (detection of single or multiple proteins), (iii) ELISPOT (detection of cytokine producing cells), (iv) Immuknow (detection of adenosine triphosphate [ATP] levels in activated T-lymphocytes), and (v) Luminex® (detection of cytokines or alloantibodies) [42].

Urinary cell and peripheral blood cell messenger RNA (mRNA) profiling of transplant recipients has been studied extensively as a tool for the noninvasive diagnosis and prognosis of kidney transplant rejection. This technique involves quantification of mRNA levels of mechanistically informative genes using RT-PCR assay from urinary cells or peripheral blood cells of kidney transplant recipients. As an example, measurement of granzyme B and perforin (m) mRNA activity in urinary cells has been reported to be a sensitive and specific marker for the detection of acute cellular rejection [43]. In a study of 83 kidney transplant recipients; 36 with acute rejection, 18 with chronic allograft nephropathy, and 29 with normal biopsy results, urinary cell mRNA levels of regulatory T-lymphocyte marker, FoxP3, quantified at the time of biopsy diagnosis predicted reversal of acute rejection with 90 percent sensitivity and 73 percent specificity. Urinary cell mRNA levels of FoxP3 also identified subjects at risk for graft failure within six months after the incident episode of acute rejection. Urinary cell mRNA levels of CD3 (marker of T lymphocytes), CD25 (marker of activated T-lymphocytes), and perforin did not predict rejection reversal or graft failure [44]. Recently, a large multi-center trial sponsored by the National Institutes of Health trial validated the utility of urinary cell mRNA levels in the diagnosis of ACR [45]. The role of urinary cell mRNA level as a noninvasive tool is not limited to the diagnosis of acute rejection. A recent study identified a urinary cell mRNA signature for the diagnosis of fibrosis in human kidney allografts [46].

The Cylex Immuknow assay is an FDA approved blood test for the detection of cell mediated immune response in populations undergoing immunosuppressive therapy for organ transplants. The test quantifies the amount of ATP produced by lymphocytes of the transplant recipient upon activation. Based on the ATP levels there are two cut-off values: ≤ 225 ng/ml represents a low immune cell response and ≥ 525 ng/ml represents a strong immune cell response. In between values represent moderate immune cell response. Some studies have found a relation between the Cylex Immuknow assay and acute rejection [47].

However, more recently, a study that evaluated 1330 ImmuKnow assay values in 583 renal transplant recipients at a single center from 2004 to 2009 failed to show an association between single time point ImmuKnow assay values and the subsequent development of an adverse event (acute rejection or opportunistic infections) in the subsequent 90 days [48].

In a recent study of 64 kidney transplant recipients with graft dysfunction, a panel of 21 cytokines secreted by peripheral blood mononuclear cells was assayed using the Luminex® platform. In the initial training cohort of 32 patients, IL-6 was the best predictor of acute rejection. In the validation cohort of 32 patients, IL-6 predicted acute rejection, using a training set derived cut-point, with 92% sensitivity and 63% specificity [49].

Rapid advancements in our understanding of the role of microRNAs in transplantation [50] and in molecular techniques such as RNA sequencing have opened up new avenues for biomarker discovery and has resulted in better insight on the mechanistic basis of allograft dysfunction. In the future, we anticipate personalized management of transplant recipients with a combination of traditional pathology and the 'omics' based approach (genomics, proteomics, and metabolomics).

Author details

John R. Lee and Thangamani Muthukumar

Division of Nephrology & Hypertension, Department of Transplantation Medicine, New York-Presbyterian Hospital/Weill Cornell Medical College, New York, NY, USA

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