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The mechanisms of acute transplant rejection revisited

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Abstract

For many years, acute rejection has been considered as a typical response of the adaptive immunity system. However, recent investigations have revealed a critical role for innate immunity as a pivotal trigger in adaptive immune responses. Danger signals released by cells damaged or killed by injury or disease may be intercepted by Toll-like receptors (TLRs) that alarm the dendritic cells (DCs) through the activation of transcription factors. In the presence of an inflammatory milieu created by other components of the innate immunity, DCs become mature and present the antigen to naïve T cells. The activation of T cells requires both a signal engendered by the presentation of the antigen to the T cell receptor and costimulatory signals generated by the contact between molecules displayed by antigen-presenting cells (APCs) and by T cells. Once activated, T cells encode and synthesize interleukin 2 (IL-2) and other cytokines that provide the signals for cell differentiation and proliferation. Until recently, little attention was paid to the role of antibodies in renal transplantation. However, there is mounting evidence that a number of kidney allografts fail as a consequence of a rejection caused by antibodies specifically directed against major histocompatibility complex antigens, class I or II, of the recipient. A critical role in antibody-mediated rejection (AMR) is played by complement. A number of therapeutic attempts have been tried to prevent or treat AMR. The still open question is whether the antibodies we detect are those responsible for tissue damage or not.

Key words: *Acute rejection, Antibody mediated rejection, Antigen recognition, Dendritic cells, Innate immunity*

Introduction

Until recently, the complex machinery of transplant rejection was considered to be orchestrated by T cell adaptive alloimmune responses. However, there is now evidence that the innate immune system of the recipient modulates adaptive immune responses through activation of a number of cells, ligands, receptors, transcription factors, chemokines and cytokines. Moreover, also B cells, plasma cells and their associated antibodies may play an important role in the alloimmune response. In this paper, the different steps eventually leading to acute cellular and humoral rejection of a renal transplant will be reviewed.

Alloantigen-dependent cellular rejection

The typical acute rejection usually occurs a few days after a transplant – the time necessary for the activation, proliferation and differentiation of T cells. This immune response requires 3 signals: the alloantigen recognition, the activation of T cells and the signal for T cell proliferation.

Alloantigen recognition (signal 1)

The human major histocompatibility complex (MHC), also called the human leukocyte antigen (HLA) system, is a polypeptide chain that resides on chromosome 6 and contains a large number of genes that encode cell-surface antigen presenting proteins. There are 2 main classes of HLAs (1). Class I proteins form a functional receptor on most nucleated cells of the body. There are 3 major (A, B and C) and 3 minor class I genes (E, F and G). The major molecules of class I present peptides derived from internal proteins that are degraded by

the proteasomes. These small peptides are presented to cytotoxic $CD8^+$ T cells. The binding groove of class I molecules is essential for antigen binding and presentation on T cells. It has been suggested that the structural features of class I molecules determine the recognition of different ligands and light chains, which are responsible for their corresponding functions through an inherent mechanism (2).The minor molecules may interact with $CD8^+$ cells and natural killer (NK) cells. Class II molecules (DP, DM, DOA, DOB, DQ and DR) present antigens from outside the cell to $CD4^+$ T cells (3). These particular antigens stimulate the multiplication of T-helper cells, which in turn stimulate B cells to produce antibodies to that specific antigen.

The alloantigens presented by the donor are recognized by a specialized cell type of dendritic cells (DCs) that are critical for triggering the immune response. These specialized DCs are called antigen-presenting cells (APCs). DCs are derived from the differentiation of hematopoietic stem cells and are recruited to peripheral tissues (4). Immature DCs are tolerogenic, since they present innocuous self- and nonself-antigens in a fashion that promotes tolerance, at least in part, through the control of regulatory T cells (5). However, when DCs come into contact with an antigen in the presence of an inflammatory milieu created by the response to pathogens or tissue damage, they become mature. Mature DCs capture and process the antigen by degrading its proteins into small pieces, and through lymphatic vessels, migrate out of the graft to lymph nodes (6). Here, DCs up-regulate cell-surface coreceptors such as CD80 (B7.1), CD86 (B7.2) and CD40 that enhance the activation of T and B cells (7, 8) and act as APCs by presenting epitopes to T cells.

There are 3 possible pathways for alloantigen recognition: (i) the direct pathway, (ii) the indirect pathway and (iii) the semidirect pathway. In the **direct pathway**, alloreactive T cells directly recognize intact allogeneic MHC molecules expressed on foreign cells (9), namely donor professional APCs, which are mainly immature DCs. As reported above, DCs migrate out of the graft toward secondary lymphoid organs, where they mature and present the antigen to naïve T cells. Of note, the trafficking and maturation of DCs require proinflammatory signals. In the second, **indirect pathway**, it is the recipient APC that captures and processes the allogenic HLA molecules of the donor that have been shed through apoptosis or necrosis. In the third, **semidirect pathway**, the alloantigens are transferred from donor APCs to recipient APCs, through cell–cell contact or through transfer of donor exosomes (10, 11). The direct pathway is generally used in early rejections. However, the number of donor DCs tends to decline over time, so that the indirect or semidirect pathways are frequently used in late rejection.

A critical role in creating the inflammatory environment necessary for the maturation of DCs is played by innate immunity. **Innate immunity** is an ancestral form of defense against invading microorganisms. The response is initiated by a limited number of germline-encoded receptors that recognize ligands displayed by pathogens (12). These receptors are expressed by a number of cells, including monocyte macrophages, lymphocytes, DCs, NK cells, polymorphonuclear cells, epithelial and endothelial cells. The main family of danger signal receptors is composed of the **Toll-like receptors** (TLRs). These are small proteins that are on cell surface membranes or on endosomal membranes. During an infection the pathogen-associated molecular patterns released by bacteria or viruses are recognized by TLRs that recruit adapter molecules within the cytoplasm. These adapters activate several kinases that amplify the signal, leading to activation of nuclear factor kB (NFkB) and interferon regulator 3 (13). These transcription factors can induce or suppress genes that orchestrate the immune and inflammatory response (14). TLR2 in combination with TLR1 and TLR6 recognize many ligands and functions. TLR4 together with the accessory proteins CD14 and MD-2 recognize bacterial proteins and lipopolysaccharides; TLR3 recognizes double-stranded RNA. TLR5 is specific for bacterial flagellin. TLR9 is a receptor of ingredients of DNA of gram-positive and gram-negative bacteria, viruses and damaged or dying cells (15, 16). Mitochondria are an important component of innate immunity and antibacterial responses. Indeed, a subset of TLRs (TLR1, TLR2 and TLR4) can recruit mitochondria to macrophage phagosomes and induce the generation of mitochondrial reactive oxygen species (17). In the setting of organ transplantation, there is accumulating experimental and clinical evidence that TLR signaling is involved in the immune recognition of allografts. In fact, TLRs can be engaged not only by microbial-associated molecular patterns but also by molecules that are released by cells damaged or killed by injury or disease (18-20). These endogenous molecules that represent an early signal of danger for the organism are called danger-associated molecular patterns (DAMPs). A number of DAMPs coming from kidney injuries have been identified. They include advanced glycation end products and their multiligand receptors, high-mobility group box 1 protein, the S100 protein family, heat shock proteins, genomic double-strand DNA, uric acid, neutrophil-derived alarmins, extracellular matrix proteins (fibronectin, proteoglycans, fibrinogen etc), adaptor proteins and caspase-1 (21). When released into extracellular space, DAMPs are recognized as danger signals and initiate inflammation by activating TLRs (22). The major initiator of alloimmune response has been identified in the damage-induced molecule high-mobility group box 1 protein and its binding to TLR4 (23).

A central facet of innate immunity is the **complement system**. Three biochemical pathways can activate the complement cascade: the classical, alternative and mannose lectin pathways. Each of these pathways may be activated by different mechanisms and can modulate the alloresponse in different directions. DAMPs may activate the classical pathway by binding to C1q (classical pathway), the alternative pathway by binding to C3, or by binding to mannose-lectin/ficolins (24). It has recently been demonstrated that also several subsets of human DCs can express many of the components of the classical, alternative and terminal pathways of complement (25). Complement may also be up-regulated and activated in the kidney as a direct result of brain death in the donor (26, 27), or of the inflammatory environment created by ischemia reperfusion injury (28). Only 30 minutes of renal ischemia are sufficient to activate C4d/C1q (29). The complement component C5a acts directly on C5a receptors expressed on DCs, resulting in cell activation, and subsequently enhances its capacity for allospecific T cell stimulation (30). Polymorphonuclear cells and macrophages, recruited by C5a, create an inflammatory milieu leading to maturation of DCs, which further activate the complement cascade. In turn, the terminal components of complement, C5b-C9, bind to APC receptors and this binding favors direct recognition with activation of Th1 and adaptive immunity (31).

NK cells, another critical component of innate immunity, can play different roles in transplant rejection. NK cells can become activated by microbial products or endogenous proinflammatory ligands released during mechanical, microbial or ischemic injury. Experimental evidence suggests that specific subsets of NK cells can develop long-lived and highly specific memory for a variety of antigens (32). NK cells are equipped with an array of receptors that can either stimulate or dampen their reactivity. Activating receptors include receptors that interact with soluble ligands such as cytokines and receptors that interact with cell surface molecules. Inhibitory receptors include the MHC class I receptors which mediate NK cell functions so that they selectively kill target cells (33).

Activation of T cells (signal 2)

Naïve T cells require 2 signal for their activation: The first signal is provided by the contact between the alloantigen presented by the APC and the specific receptor located on the surface of T cells. However, this is an anergic/apoptotic signal. A second signal (**costimulation**) is needed to rescue T cells from apoptosis and to activate them. Costimulation requires the contact between adhesion molecules located on the surface of the APC (CD40, and CD80 and CD86 of the B7 family) and T lymphocyte (CD28, CD40L and CTLA-4). Belatacept, a fusion protein composed of the Fc fragment of human IgG1 immunoglobulin linked to the extracellular domain of CTLA-4, binds to CD80 and CD86 and provides potent immunosuppression in clinical renal transplantation (34). However, the efficacy of costimulation blockade can be reduced by environmental perturbations such as infection or inflammation, which activate TLRs and the innate immunity system (35, 36).

Once a T lymphocyte is activated, there is a large influx of Ca^{++} ions into the cytoplasm, leading to activation of calcineurin, a protein phosphatase which dephosphorylates a family of proteins called nuclear factor of activated T cells (NFAT). Once dephosphorylated, NFAT can enter the nucleus where it participates in the codification and synthesis of interleukin-2 (IL-2) and other cytokines. The contact with APCs also activates the mitogen activated protein kinase pathway, and the protein kinase C/NFkB pathway. The result is an activation of IL-2, IL-2 receptor, NFkB and a further activation of APCs (37).

Proliferation of T cells (signal 3)

The ligand of IL-2 to its receptor (CD25), together with IL-15 and probably other cytokines, activate a kinase called Janus kinase 3, and delivers growth signals through the family of phosphatidylinositol 3 kinase (PI3-k) which together with a protein-kinase B (Akt) govern several signal pathways by activating a cascade of other kinases, which provide the signals for cell proliferation. The downstream effector of PI3-k is a serine-threonine kinase called **mammalian target of rapamycin** (mTOR), which regulates cell growth and cell proliferation (38). mTOR is the subunit of 2 complexes: mTORC1 or raptor complex and mTORC2 or rictor complex (39). While the mTORC2 signaling pathway is poorly defined, the mTORC1 signaling pathway phosphorylates the 40S ribosomal 6 kinase and cyclin dependent kinases, providing the transduction of proliferative signals to T cells (40, 41). After receiving the signal for proliferation, activated T lymphocytes require the synthesis of nucleotides to proliferate and differentiate into T cell effectors (42).

The effector arm

Activated T cells may differentiate into Th1, Th2 and Th17 effector subsets, as well as regulatory T cells (Tregs). The local cytokine environment established by innate immunity favors the differentiation into Th1 and Th17 cells. These **alloreactive T cells** mediate allograft injury through direct

contact with tubular epithelial cells and endothelial cells, as well as through the release of cytokines and chemokines that cause necrosis of the transplanted tissue. Th1 cells, which release interferon-γ and IL-2, mediate both the cellular arm of the immune system and B cell class switching to complement immunoglobulin G (IgG) fixing antibodies. Th17 differentiation, which is stimulated by transforming growth factor β and by a number of cytokines, produces the proinflammatory IL-17. This interleukin plays a key role in the immune response since it mediates inflammation and stimulates production of inflammatory cytokines and inflammatory chemokines that promote the recruitment of neutrophils and macrophages (43).

In contrast to Th1 and Th17, Th2 cells release antiinflammatory cytokines such as IL-10 and IL-4. Tregs can prevent the production of IgG antibodies and have suppressor functions that may favor tolerance. However, a number of barriers can impede the development of tolerance. The balance between graft-protective Tregs and graft-destructive T effectors is disproportionally against Tregs, which represent only 5% to 10% of T cells. Moreover, graft inflammation fosters the generation of donor-reactive Th1 or Th17 effector subsets while preventing the production of Tregs and can also alter the inhibitory activity of Tregs by converting them into inflammatory, effector-like phenotypes (44-46).

Although T cells play the major role in cell-mediated rejection, a number of non–T cells may have a broad impact on graft rejection. **B cell** receptors can act as APCs by taking up the antigen and presenting it in the form of peptide to T cells (47). B cells may also contribute to tissue damage by producing alloantibodies against MHC antigens, and by helping T cells differentiate into memory T cells (48). Once again, the contribution of innate immunity is of paramount importance. It is now accepted that **NK cells** may represent a bridge between innate and adaptive immunity, since they exert biologic functions that may be attributed to both these types of immunity. NK cells may be involved in allogeneic graft rejection by promoting the cytotoxic lysis of target cells and the release of proinflammatory cytokines, such as interferon-γ and tumor necrosis factor-α, which amplify the immune response (49, 50). On the other hand, NK cells may also play an unexpected role in the induction of transplant tolerance since they can kill donor DCs, thus limiting the priming of alloreactive T cells by the direct pathway (51). Polymorphonuclear cells may produce chemokines (52), which can activate effector function and T cell proliferation (53).

The innate immune system comprises also a humoral arm. Components of the humoral arm include members of the complement cascade and pentraxins. The late components of the **complement cascade** mediate chemotaxis and activation of neutrophils and macrophages and further contribute to the rejection process either by killing allogeneic targets in a complement-dependent fashion or by opsonizing donor cells and forming immune complexes (54). It has been demonstrated that the local synthesis of complement component C3 is capable of modulating the rejection of renal allografts in vivo and regulating T cell responses in vivo and in vitro (55, 56). **Pentraxins** are a family of multimeric pattern-recognition proteins that can be divided into short pentraxins and long pentraxins (57). The short pentraxins, C-reactive protein and serum amyloid P component, are opsonins produced in the liver in response to proinflammatory cytokines such as IL-6, and contribute to innate immunity responses. The prototypic long pentraxin is pentraxin 3 (PTX3) which is produced by DCs and macrophages, in response to TLRs and inflammatory cytokines. PTX3 acts as a functional ancestor of antibodies, recognizing microbes, activating complement and facilitating molecular pattern recognition by phagocytes (58). PTX3 is expressed by human proximal renal tubular epithelial cells and may play a role in the innate immune response and inflammatory reactions in the kidney (59). Thus, the prototypic long pentraxin PTX3 is a multifunctional soluble pattern recognition receptor at the crossroads between innate immunity, inflammation and matrix deposition. These opsonins may participate in removing the inflammatory and immunogenic material produced by ischemia-reperfusion injury or rejection (60). On the other hand, PTX3 correlates with acute rejection in renal transplant recipients, and the beneficial effects of thymoglobulin were shown to be related to its capacity to down-regulate PTX3 synthesis (61). Besides TLRs, recently a new family of cytoplasmatic receptors has been identified, the nucleotide-binding oligomerization domain (NOD) receptors. The **NOD-like receptors** are cytoplasmic proteins that may have a variety of functions in regulation of inflammatory and apoptotic responses. NOD-like receptors can recognize pathogen or endogenous signals of danger and may be involved in the formation of inflammasomes – i.e., intracellular macromolecular complexes that may be activated by danger signals (62). The inflammasomes are required for the activation of caspase-1, which promotes the maturation of inflammatory cytokines, IL-1β and IL-18 (63). There is experimental evidence that inflammasomes may regulate B cell activation (64) and direct the T cell response toward Th-17 and proinflammatory IL-17 (65), thus suggesting their potential role in enhancing the alloimmune response against the transplanted kidney.

Antibody-mediated rejection

Antibody-mediated rejection (AMR) is a rare form of acute rejection caused by antidonor antibodies which is usually associated with graft loss. AMR most frequently occurs in sensitized patients or in those with a history of previous failed allograft and usually carries a poor prognosis (66). There is the clinical impression of an increasing incidence of AMR, probably related not only to a better recognition of this form of rejection, but also to the increasing numbers of renal transplants and of recipients with preformed circulating antibodies and/or a high HLA mismatch.

Donor-specific antibody-mediated rejection

The typical AMR is caused by antibodies directed against HLA class I-II antigens. Antibodies against donor alloantigens target the capillary endothelium but not the arterial endothelium, with fixation of complement, resulting in tissue injury and coagulation. Antibodies can activate complement through the classical pathway by binding C1 and/or by binding to the mannose-binding lectin pathway. Once activated, C3 splits into C3a and C3b. C3b amplifies the alternative pathway, while the chemoattractant C3a and C5a recruit macrophages and neutrophils, causing additional endothelial injury. The final result is that arteries and basement membranes are remodeled, leading to fixed and irreversible anatomical lesions that permanently compromise graft function (67). The role of complement activation in antibody-mediated rejection is demonstrated by the abundant presence in peritubular capillaries of C4d, a terminal component of the complement cascade which persists in graft tissue (68). This finding is today considered a reliable marker of humoral rejection (69-71) and has been incorporated in the Banff classification (72). The diagnosis of donor-specific AMR is based on 3 main parameters: (i) morphologic evidence of acute tissue injury such as (a) acute tubular injury, (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; (ii) immunopathologic evidence for antibody action such as (a) C4d and/or (rarely) immunoglobulin in peritubular capillaries or (b) immunoglobulin and complement in arterial fibrinoid necrosis; and (iii) serologic evidence of circulating antibodies to donor HLA or other antidonor endothelial antigens (73).

Both the pretransplant presence of donor-specific alloantibodies and the development of de novo donor-specific antibodies (DSAs) after a renal transplant may be associated with an increased risk of AMR and chronic rejection (74-78). On the other hand, not all of the circulating antibodies are responsible of antibody-mediated rejection, since some anti-HLA antibodies may even favor graft acceptance (79-80), and it is difficult to distinguish between harmful and protective antibodies or to distinguish patients in whom antibodies will precipitate AMR (81). Nevertheless, though only scarce data exist connecting DSAb to AMR, 2 recent papers find such a relationship and have stated that preformed DSAb, assessed by the luminex single-antigen assay, is a high risk factor for an adverse outcome (82, 83). A further contribution may be provided by the quantitative measurement of DSAs, but the existing platforms for DSA detection provide only qualitative assays (84).

Although the significance of circulating anti-HLA antibodies remains obscure, the current practice is to try to eliminate preexisting antibodies to reach a negative crossmatch and to prevent AMR or chronic rejection. Attempts with plasmapheresis, intravenous immunoglobulins and/or rituximab showed the possibility of removing circulating antibodies and successfully performing transplantation in sensitized patients (85-87) or in AB0 incompatible subjects (88-90).

Less exciting are the results with treatment of AMR. Therapies based on apheresis, rituximab or intravenous immunoglobulins only rarely obtained reversal of rejection. Better results have been reported in a limited number of cases, with eculizumab, a monoclonal antibody directed against the C5 component of the complement cascade, and with the proteasome inhibitor bortezomib (91-93). However, it should be kept in mind that although antibodies produced by B cells modify the outcome of allografts, recent studies pointed out a potential role of a peculiar blood B cell phenotype in maintaining graft tolerance (94, 95). Future attempts to target B cells will need to address the problem of how to inhibit effector B cells, while enhancing those with regulatory capacity.

Non anti-HLA antibodies

The allograft endothelium may be the target also of non-HLA antibodies. Dragun et al (96) reported the absence of anti-HLA antibodies in 16 of 33 transplant patients with vascular rejection and malignant hypertension. Activating IgG antibodies targeting the angiotensin II type 1 receptor (AT1) were detected in serum of these patients. The authors suggested that these antibodies may attack the endothelial cells which have 1 AT1 receptor and activate a downstream signaling cascade, mimicking the action of angiotensin II and inducing damage to the allograft.

The role of non-HLA antiendothelial cell antibodies is still uncertain. These antibodies may cause apoptosis of endo-

thelial cells in vitro (97). Some investigators reported that the presence of these antibodies was associated with a higher number of acute rejection episodes and lower long-term graft survival in kidney transplants (98, 99) probably by stimulating proinflammatory and proproliferation signals. However, the lack of standardized screening assays for detecting antiendothelial cell antibodies makes it difficult to reach definite conclusions (100).

Other non-HLA antibodies include various minor histocompatibility antigens, vascular receptors, adhesion molecules and intermediate filaments. Non-HLA antibodies may induce a wide variety of allograft injuries (101).

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