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Editorial

Compelling scientific and clinical evidence that non-HLA specific antibodies impact graft outcome independently and in concert with donor HLA specific antibodies

The vascular endothelium, expressing both alloantigens and autoantigens, is the first immune barrier encountered during the immune response following organ transplantation. Historically, the investigation of this initial response has been focused on alloreactivity against non-self HLA antigens mismatched between the donor and recipient resulting in antibody mediated injury [1]. With the implementation of reliable solid phase antibody testing reagents, the exquisite specificity, along with the breadth and strength of this response, could be more accurately determined [2]. These advances allowed for a more precise definition of the role of HLA specific antibodies in antibody mediated injury and graft loss. However, antibody mediated injury in the absence of donor HLA specific antibodies has also been described [3,4]. For over a decade, investigation of non-HLA specific antibodies and their impact on antibody injury and graft outcome has focused on antigens expressed by the vascular endothelium. Included in these antigens are the G protein coupled receptors (GPCRs) angiotensin type 1 receptor (AT_1R) and endothelin type A receptor (ET_AR) [3–13], as well as MHC class I chain-like gene A (MICA) [14–16], endothelial progenitor cell antigens [17,18], and a variety of proteins expressed by stressed endothelial cells including myosin [19,20,21], vimentin [22,23,24], Kα1 tubulin and collagen-V [25,26], and also perlecan [27,28,29].

This special issue of Human Immunology, which includes a variety of studies, will focus on presenting new concepts and the clinical impact of non-HLA specific antibodies on graft outcome. These studies provide evidence that the presence of non-HLA antibodies, whether alone or in conjunction with donor HLA specific antibodies, appears to provide a more comprehensive view of the immune status of the recipients. By assessing both HLA and non-HLA specific antibodies, a more accurate analysis of immune risk assessment can be made. Further, the pretransplant status of non-HLA antibody levels, donor HLA specific antibody strength and function, and HLA mismatch appears to identify a higher risk for early and long-term graft dysfunction.

Two reports describing the detrimental impact of non-HLA antibodies piqued the interest of the kidney transplant community. One report, focused on the outcome of HLA identical sibling transplants, showed the effect of PRA that became apparent after the first year posttransplant and that non-HLA immunity was associated with chronic graft loss [30]. A second report focused on the presence of AT_1R antibodies in kidney recipients with severe vascular rejection and malignant hypertension but no donor HLA specific antibodies [3]. This sentinel work by Dragun showed that a recipient of an HLA zero mismatched graft developed accelerated vascular rejection which was refractory to steroids and anti-lymphocyte antibody preparations. High levels of antibodies to AT_1R were identified using a bioassay. The patients were treated by AT_1R antibody removal by plasmapheresis and AT_1R pharmacologic blockade. Subsequently, a cell-based enzyme-linked immunosorbent assay (ELISA) was developed and validated [5]. This assay has now become commercially available, allowing for sera from large cohorts of patients from multiple transplant centers and several organ groups to be tested with reliability and reproducibility.

1. G protein coupled receptors, AT_1R and ET_AR: structure and function

Philogene et al. present in this issue an overview of AT_1R and ET_AR structure, function, and expression [31]. The crystal structure of human AT_1R has been elucidated [32,33] revealing the extracellular domains and potential molecular target for therapeutic intervention. Antibodies to AT_1R and ET_AR have been shown to activate their targets and affect signaling pathways. The authors provide a comprehensive outline of the AT_1R receptor function in homeostasis and dysfunction. With the wide distribution of these GPCRs throughout the body, including vascular endothelium smooth muscle cells, immune cells, kidney, lung, heart, and placental tissues [34], the wide spread effect of antibodies to these receptors becomes apparent. Pathogenic features including increased blood pressure, enhanced fibrosis, and immune cell recruitment contribute to poor transplant outcome. Also of importance is the issue of complement activation. The AT_1R antibodies have been characterized as IgG1 and IgG3, which typically bind complement with highest affinity. However, these antibodies are often associated with C4d-negative biopsies [35], implicating vascular remodeling as another mode of action. Consideration must also be given for the possibility that the GPCR antibodies may precede development of HLA specific antibodies.

2. Immunological risk stratification

The GPCR antibodies are often present in healthy subjects based on age and gender. The categories of binding reported in the ELISA test usually indicate negative binding at 10–12 U/ml and lower, intermediate binding at 12–17 U/ml, strong binding at > 17 U/ml, and saturation binding at > 40 U/ml. Philogene et al. present data relevant to strategizing pretransplant screening for non-HLA antibodies in patient groups including re-transplanted patients, males, Caucasians, patients with FSGS, and younger patients [36]. The incidence of GPCR antibodies in the pretransplant population varies depending on the binding designation used to define a positively binding antibody, organ type, and parameters listed above; however, ranges have been reported between 15 and 40% [37]. Mechanical circulatory support device implantation also significantly increases AT_1R antibody levels with saturated levels being associated with lower patient survival post-

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implantation [38].

In addition to the population listed above, pediatric patients appear to be at higher risk for immune complications in the presence of antibodies to AT_1R. In this issue, Pearl presents the current literature on the role of AT_1R antibodies in pediatric solid organ transplant outcome [39]. There are issues specific to the pediatric population including various immune complications and transplant outcomes due to differences in the immature versus mature immune system, complication of viral infections, and the implications of variations in immunosuppression drug metabolism [40]. The range of AT_1R antibody levels pretransplant appears to be higher in pediatric populations versus adult populations, and pediatric patients with levels of binding > 17 U/ml are more likely to develop increasing levels of AT_1R antibodies posttranplant [41]. Although no standardized treatment strategy is utilized in the pediatric transplant population, the authors raise many questions regarding mechanisms of injury, possible synergy with HLA and other non-HLA specific antibodies, and the need to identify which pediatric patients are at high risk for AT_1R antibody mediated injury. Taken together, an algorithm for pretransplant screening for GPCR antibodies should focus on those patients at high risk for immune complication and those more likely to have endothelial cell damage.

3. Preemptive treatment of angiotensin receptor antibodies for patients at high immunological risk for immune complications

Cases of severe AT_1R antibody mediated rejection have been described early posttransplant in kidney and lung transplantation [3,42,43]. Given the pretransplant prevalence of AT_1R antibodies along with the development of *de novo* DSA and acute rejection, prospective studies have been proposed to determine the appropriate immunosuppression protocol, including the use of plasmapheresis and AT_1R blockade, warranted to decrease the negative impact of these antibodies on graft outcome. In this issue, Carroll et al. describe patients at risk for acute cellular and humoral rejection with AT_1R antibodies levels > 17 U/ml and a low risk group with AT_1R antibody levels < 17 U/ml [44]. Since previous studies have shown that patients with > 17 or > 25 U/ml can experience high levels of microvascular injury [45], a proactive protocol utilizing candesartan (AT_1R blocker) and perioperative plasmapheresis was used to manage the at-risk patient group. The plasmapheresis also has the advantage of removing any HLA specific antibodies. These important studies show excellent long-term graft survival without significant complications. Further, 4-year survival was observed to be excellent at 94% and patients with saturated levels of AT_1R antibody binding > 40 U/ml did not experience any episodes of acute graft thrombosis. By identifying patients at risk for immune complications due to high levels of AT_1R antibodies, prospective clinical protocols can be implemented to improve graft outcome.

4. Negative impact of AT_1R antibodies on non-renal transplant outcome

The negative impact of GPCR antibodies in renal transplantation has also been observed in non-renal transplantation [11,46,6,47,13,48,5]. In this issue, Zhang and I review the impact of AT_1R antibodies in thoracic transplantation [49]. Sensitization to AT_1R presents a unique challenge in patients with advanced heart failure who are implanted with mechanical circulatory support (MCS) devices. Zhang et al. have shown that patients who receive MCS experience a significant increase in sensitization to AT_1R [38]. Also, these patients experience lower survival rates subsequent to the implantation of MCS. These studies provide insight into sensitization to AT_1R due to stress or shear rate caused by the MCS. Zhang et al. postulate that this increased stress can dislodge the von Willebrand factor from the cell surface, can clip off the second extracellular loop of the AT_1R protein thereby generating neoantigens, and can increase shedding of AT_1R proteins. MCS patients who eventually receive transplants showed lower survival rates, but this did not reach significance.

AT_1R antibodies in heart transplantation are associated with microvasculopathy [11]. In lung transplantation and various pulmonary diseases and pulmonary arterial hypertension, AT_1R signaling has been shown to be involved in the regulation of inflammation, proliferation and fibrosis. As in kidney transplant populations, studies of heart and lung transplant populations have shown recipients with antibodies to both AT_1R and donor HLA specific antibodies (DSA) have worse graft survival compared to either antibody alone [9,12,47,50,51]. Thus, antibodies to the GPCRs and to donor specific HLA appear to have impact both independently and in concert with each other. Concomitant studies have shown acute and chronic rejection can be associated with other non-HLA antibodies reviewed in the following manuscripts.

5. Impact of collagen V and K-α1 tubulin antibodies on lung transplant outcomes

Various non-HLA antigens such as Collagen-V and K-α1 tubulin represent sequestered self-antigens important in the autoimmune responses associated with loss of peripheral tolerance, thereby promoting allograft rejection. In lung transplantation the focus on these autoimmune responses has been on the development of bronchiolitis obliterans syndrome (BOS) but they may also be involved in the acute AMR process. In this issue, Hachem presents an overview of these autoimmune processes as well as the potential for therapeutic intervention [52].

Survival at 5-years after lung transplantation is significantly worse than after other solid organ transplants, with chronic lung allograft dysfunction (CLAD) being the leading cause of death after 1 year posttranplant. Besides studies of GPCR antibodies, the focus of investigation in lung transplantation has been on antibodies to collagen V and K-α1 tubulin. Both self-antigens are sequestered under normal conditions but are exposed with inflammation and tissue repair [53]. As in studies in kidney, heart and lung exploring the association between GPCR antibodies and DSA, a similar association between the development of DSA and antibodies to collagen-V and K-α1 tubulin has been recognized. The majority of patients who developed DSA to HLA also developed antibodies to collagen V and K-α1 tubulin, with some patients only developing DSA or antibodies to the non-HLA antigens [54]. The development of DSA preceded the development of antibodies to the non-HLA antigens which, in some cases, persisted longer than the DSAs.

The management of non-HLA antibodies in lung transplantation is hampered due to lack of randomized clinical trials and limited interest by the pharmaceutical industry in lung transplantation. Also, commercially available Luminex-based reagents have only recently become available. Nonetheless, a study was designed to test the effect of antibody depletion for both HLA DSA and non-HLA antibodies on clinical outcomes [25]. The study utilized rituximab and monthly doses of intravenous immune globulin (IVIG) or IVIG alone if the patients developed DSA. More patients developed antibodies to the non-HLA antigens than to donor HLA. The development of non-HLA antibodies was associated with a higher risk of BOS and death. The antibodies to non-HLA were less likely to be cleared from the system than antibodies to HLA with the methods used.

The role of non-HLA antibodies in AMR in lung transplantation has been more difficult to define. The definition of AMR in lung transplantation remains elusive; however, a definition based on allograft dysfunction, circulating DSA, abnormal lung pathology and C4d deposition has been recently proposed by the International Society for Heart & Lung Transplantation (ISHLT) [55]. As in kidney transplantation, many cases of AMR in lung are C4d negative [56,57], thus making cases of AMR due to non-HLA antibodies very difficult to detect. A recent report of AMR due to antibodies to collagen V, collagen I, and K-α1 tubulin has been described in recipients transplanted from the same donor [58]. Similar to reports in kidney transplantation of acute

rejection associated with GPCR antibodies, no DSA was detected. These studies find similar results to those of GPCR antibodies and highlight the need for commercially available reagents validated and used by multiple laboratories, a consistent and clear definition of the clinical pathology results, and studies with randomized controlled design, to identify effective therapies.

6. Role for exosomes in development of antibodies to self-antigens and allograft dysfunction

Exosomes, small membrane vesicles, are produced by endocytic pathway and secreted by fusion with the cell membrane [59,60]. Recently, collagen V and K-α1 tubulin along with donor HLA were identified on the surface of exosomes isolated from serum and bronchioalveolar lavage fluid from lung recipients with acute cellular rejection and BOS but not from stable recipients [61]. In this issue, Ravichandran and Akbarpour described the role of donor-derived exosomes and the clinical relevance of lung-restricted antibodies such as collagen-V and K-α1 tubulin in lung transplantation [62]. These exosomes are released due to stress and express allo-antigens and self-antigens, such as collagen V and K-α1 tubulin, and thereby may play an important role in lung rejection [61,63]. Further, the exosomes released subsequent to rejection or other stress such as ischemia reperfusion injury are different from those released during stable lung function. The circulating exosomes isolated from patients who have *de novo* DSA appear to express higher levels of lung self-antigens [60]. The authors postulate that the exosomes released following DSA development can enhance the immune responses and lead to chronic rejection. Recent studies have demonstrated the potential role of exosomes in the induction of tolerance in the transplant setting and may help define the role of circulating exosomes in induction and maintenance of tolerance posttransplant [64,65]. Important to the different approaches to immune intervention, the authors outline technologies such as *ex vivo* lung perfusion shown to reduce lung injury and to achieve successful transplantation [66]. Approaches such as blocking exosome formation and release of pharmacological agents during *ex vivo* lung perfusion are now potentially feasible interventions.

In this issue, Akbarpour et al. discuss investigations into the mechanisms of development of lung restricted antigens and the pathogenesis of the associated lung allograft injury [67]. Once again, the possible role of interplay between alloimmunity and autoimmunity is proposed, as alloimmunity has been shown to induce development of lung restricted autoimmunity [68]. Processes such as viral respiratory infections can lead to the expansion of lung restricted T cells and the development of cellular and humoral autoimmunity [69]. In lung transplantation, several disease processes have been attributed to lung restricted self-antibodies. The initial insult which affects over 50% of lung transplant recipients is primary graft dysfunction. Several aspects associated with this process are ischemia reperfusion injury, neutrophil infiltration, and alveolar edema, as are also observed in antibody mediated rejection. In some recipients, C4d deposition on the biopsies in the absence of any DSA has also been observed. Perhaps pre-existing non-HLA antibodies are involved in this process. Further, self-antigens can be released early in the posttransplant period, which may lead to development of antibodies to the newly exposed antigens. Thus, both preformed and *de novo* non-HLA antibodies may play an important role in hyperacute rejection and early acute antibody mediated rejection. The development of these auto antibodies is also associated with chronic rejection as has been previously addressed. The loss of T regulatory cells together with the lung injury may result in the exposure of these self-antigens. Studies have shown that recipients with gastroesophageal reflux and respiratory infections have diminished T regulatory cell levels and tend to develop *de novo* lung restricted autoimmunity [70,71]. The mechanisms of the resulting lung injury continue to be under investigation. The lung restricted antibodies are reported to be IgG antibodies capable of activating complement [70,58], which differs from that reported for several other non-HLA antibodies. Further investigation of complement pathways in the lung restricted auto antibody lung injury could lead to use of complement inhibitory drugs such as Eculizumab. These studies emphasize the need to investigate the mechanisms involved in autoantibody mediated immune injury and potentially the different clinical interventions needed depending on the specificity of these autoantibodies. Since the lung allograft is subject to stimuli from the environment, conventional immunosuppression therapies may not be optimal to address the immunity against these self-antigens.

7. Role of anti-vimentin antibodies

Vimentin is an intermediate filament protein which forms the basis of the cytoskeleton in fibroblasts, smooth muscle cells, and endothelial cells, and is the target of several kinases involved in signal transduction, cell motility, and differentiation. Antibodies to vimentin are associated with several autoimmune diseases such as rheumatoid arthritis as well as types of cancer. More recently, vimentin antibodies have been reported in the development of posttranplant renal and cardiac dysfunction [72,73,22]. In this issue, Divanyan et al. present studies on the generation and pathogenesis of anti-vimentin antibodies and their role in various immunological diseases [74]. In transplantation, the presence of anti-vimentin antibodies has been associated with cardiac vasculopathy and worse graft outcome [75]. The vimentin may be exposed during various stages of acute rejection, coronary artery vasculopathy (CAV), and with the presence of DSA. As seen in the immune response in lungs, there may be a breakdown of tolerance leading to inflammation [20]. However, unlike in lung transplantation, pretransplant levels of anti-vimentin antibodies do not seem to play a role in the development of antibody mediated rejection or CAV [24]. Therapeutic intervention has found mycophenolate to be more effective than azathioprine and tacrolimus is more effective then cyclosporine in decreasing anti-vimentin antibodies [76].

In renal transplantation, the presence of anti-vimentin antibodies has also been associated with chronic allograft dysfunction. Patients with chronic allograft dysfunction had higher levels of IgM anti-vimentin antibodies, which shows there is a role for anti-vimentin antibodies in the development of chronic allograft dysfunction with an additive effect in the presence of anti-HLA antibodies [77]. Although an increase in IgG anti-vimentin antibodies was observed with chronic allograft dysfunction, no difference in IgM concentration was noted [78]. In recent studies, higher concentration of anti-vimentin antibodies was observed in patients with graft dysfunction < 5 years posttransplant, and higher pretransplant concentrations conferred a 2-fold higher risk of early dysfunction [79]. Anti-vimentin antibodies have been shown to be present in dialysis patients prior to transplant, suggesting the concept of endothelial injury. Further, renal transplant recipients appear to develop anti-vimentin antibodies earlier than cardiac transplant recipients.

8. Role of anti-LG3 antibodies, an immunogenic fragment of Perlecan, in transplantation

In this issue, Dieude et al. present a potential role for apoptosis as a trigger for autoantibody production in the absence of membrane permeabilization, which may initiate various modes of intercellular communication important in local homeostasis and tissue remodeling leading to graft dysfunction [80]. Perlecan proteolysis and LG3 release can result from infiltrating leukocytes and aggregating platelets present at sites of vascular damage and/or inflammation. Serum cathepsin-L and LG3 have been found to be elevated in kidney transplant recipients with Banff grade 2 and 3 acute vascular rejection compared to controls with acute tubulointerstitial rejection or stable graft function [27]. In addition, elevated levels of urinary LG3 have also been observed in kidney transplant recipients with chronic rejection [81] and in patients

with severe IgA nephropathy [82] suggesting that LG3 is associated with vascular injury both in transplanted and native kidneys. The authors report a novel type of membrane vesicle they term “apoptotic exosome-like vesicle” (ApoExo) which, like classical apoptotic bodies, are released through a caspase-3-dependent pathway. The ApoExo ultrastructure, enzymatic activity and functions are strikingly different from those of apoptotic bodies [83]. It is of interest that both the studies in lung and these in kidney report the importance in the disease process of exosome-like vesicles which promote autoantibody production. In kidney transplantation, vascular injury can result from uremia, which is known to promote endothelial dysfunction, apoptosis, and the potential production of anti-LG3 antibodies. An association between increased pretransplant levels of anti-LG3 and the risk of subsequent vascular rejection (Banff grade ≥ 2) has been reported [28]. However, anti-LG3 antibodies have not been found associated with the presence of autoimmune disease prior to transplantation. In kidney transplantation, LG3 IgG autoantibodies appear almost exclusively to be of the complement fixing subclasses (IgG1 and IgG3). Elevated posttransplant anti-LG3 levels have been found in kidney transplant recipients with Banff grade ≥ 2 rejection compared to patients with normal allograft function and those with Banff grade I rejection. In addition, higher anti-LG3 IgG have been found in patients at the time of acute vascular rejection and also prior to transplantation. Pretransplant anti-LG3 antibodies titers have been associated with an increased risk of delayed graft function (DGF) [84]. Also, in patients with DGF but not in those with normal immediate function, anti-LG3 antibodies titers pretransplant predicted lower 1 year graft function. However, anti-AT_1R and anti-vimentin were not associated with DGF or graft function 1-year posttransplant in their studies [84]. Use of common immunosuppression to target T cell immunity appears to decrease humoral immunity specific to LG3. Use of bortezomib may inhibit ApoExo formation and also inhibit development of anti-LG3 antibodies. The benefit of plasmapheresis remains to be investigated.

9. Role of anti-endothelial cell antibodies

In addition to the non-HLA antibodies outlined above, other anti-endothelial cell antibodies (AECA) have been described as associated with increased acute and chronic rejection in multiple organ types [37]. Breimer et al. reported a prospective multicenter clinical trial utilizing flow cytometric crossmatches with endothelial precursor cells isolated from donor blood as target cells [85]. The results indicated a significantly higher rejection rate within the first 3 months posttransplant among patients with positive crossmatches compared to patients with negative crossmatches. This approach allowed the detection of donor specific proteins which may be polymorphic and differ among donors [86]. Heterogeneity of endothelial cell antigens has been reported using gene expression data from 53 different endothelial cells isolated from different tissues [87]. Further, specific AECA response to renal microvascular endothelial cells confined to the allograft highlight the need for tissue specific endothelial cell sources. Utilizing cell extracts to immunoprecipitate Ig-antigen complexes, followed by mass spectrometry, antigen targets have been identified [17]. These important studies revealed an array of both polymorphic and non-polymorphic non-HLA antigens and raise the question as to the optimal target and approach to use when investigating clinical impact. In this issue, Jackson et al. describe multiple studies that have demonstrated associations between pretransplant and posttransplant development of various AECAs at the time of acute and chronic rejection [88]. Further, the upregulated endothelium plays an active role in propagating immune responses [89]. Jackson et al. used endothelial cell absorption-elution approaches to isolate AECAs obtained at the time of rejection and showed that this fraction contained the capacity to upregulate adhesion molecules and inflammatory cytokine production of cultured endothelial cells [17]. Also observed was that AECA stimulation could enhance the expression of HLA, suggesting a role for synergy between AECAs and DSA, as seen with other non-HLA antibodies. With these various considerations, as well as the understanding of AECA characteristics and the detected targets, a global approach to investigation including transplantation proteomics, genomics and imaging will help enhance our understanding of the immune response to the allograft.

10. Future directions

A common theme becomes apparent throughout these studies of non-HLA specific antibodies. That is, there is a negative impact of these antibodies in all solid organ allograft outcomes which can work independently or in concert with the presence of DSA. The studies presented in this issue show potential points for intervention and provide future direction for other studies. In the past, studies of non-HLA antibodies have been limited due to lack of commercially available reagents. During the past ten years, commercial reagents have become available for the detection and binding strength of GPCR antibodies. These reagents have allowed for the validation of the assay and proficiency testing by multiple laboratories. As a result, large cohorts of patients from multiple transplant centers can be tested reliably and with reproducibility. These results lead to new insights into mechanisms of C4d positive and negative rejection. New approaches have been used to identify patients at risk for acute rejection and to identify new treatments involving AT_1R blockade and plasmapheresis to decrease the impact of these non-HLA antibodies. Recently, Luminex-based platforms have been developed to identify many more of the non-HLA antibodies, beyond the GPCR antibodies. These reagents provide the potential to validate testing and to expand studies of large patient cohorts at several transplant centers. Although management of non-HLA specific antibodies in organ transplantation has been limited due to lack of randomized clinical trials and limited interest by the pharmaceutical industry, perhaps with these newly validated assays important clinical trials can be developed. The overwhelming evidence to date indicates multiple mechanisms in the immune response to transplanted organs including both T cell and B cell responses along with antibodies to HLA and non-HLA targets. Immunological risk stratification encompassing both HLA and non-HLA specific antibodies provides the most comprehensive assessment of the patients’ immune responses.

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