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Moving Beyond HLA: A Review of nHLA Antibodies in Organ Transplantation

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Abstract

Given the finite graft life expectancy of HLA identical organ transplants and the recognition of humoral graft injury in the absence of donor directed anti-HLA antibodies, the clinical impact of antibodies against non-HLA (nHLA) antigens in transplant injury is being increasingly recognized. The recognition of the impact of nHLA antigen discrepancies between donor and recipient on transplant outcomes is timely given the advances in rapid and lower cost sequencing methods that can soon provide complete maps of all recipient and donor HLA and nHLA mismatch data. In this review, we present a summary of recent reports evaluating the role of nHLA antibodies and their relevance to the field of organ transplantation.

Keywords

organ transplantation; antibody; nHLA; biomarkers; transplant; rejection

Introduction

Early recognition and mitigation of transplant injuries due to immune and non-immune related injuries is a critical unmet need for improving the long-term management of transplant recipients [1]. The injurious role of circulating and graft deposited antibodies is well recognized in the context of donor- specific HLA antigens (DSA), acute humoral rejection and graft vasculopathy. Though most of this literature is in renal transplantation, a similar association has been observed in other solid organ transplants, such as heart, lung and intestine [2–6]. There has been an increased recognition of a causal and associative role of antibodies against non-HLA (nHLA) antigens such as MICA [7], anti-endothelial cell specific antibodies (AECAs), protein kinase zeta [8], with injury in both native organs and after organ transplantation [7, 9–16]. Given that organ transplant injury in the form of both acute and chronic rejection can occur in the absence of demonstrable donor specific HLA-antibodies, rejection can also occur in HLA identical transplants, and there is an unmet need to better define immunogenic epitopes other than HLA, that drive the evolution of chronic

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rejection after organ transplantation, this review discusses recent work in the field of nHLA antibody analysis in organ transplantation. To better understand the causal role of nHLA antibodies in human organ injury, the review also discusses relevant work on mapping of the nHLA antibody repertoire in failing organs, prior to transplantation, to evaluate if the existing repertoire of specific nHLA antibodies in the pre-transplant sera in the recipient could also contribute to post-transplant pathology.

1. nHLA Antibodies in Organ Transplantation

The finding of nHLA antibodies directed against donor antigens was reported as early as 1995 [17] and subsequent studies suggested that specific nHLA antibodies may bear relevance to transplant injury, irrespective of the impact of donor specific HLA antibodies. In a seminal study by Terasaki *et al* [18] UNOS Registry graft survival records were evaluated to assess the percentage of graft failures from immunological or non-immunological factors in HLA identical kidneys and HLA mismatched living donors and deceased donors. This study found that in recipients of cadaveric organs, the factors contributing to graft loss could be attributed in 38% of cases to nHLA factors, 18% to HLA factors, and in 43% to non-immunologic factors, thus clearly highlighting the importance of identifying and studying clinically relevant nHLA antibodies after organ transplantation. Nevertheless, prior to ascertaining clinical relevance to any detected post-transplant nHLA antibody, 2 important questions remained to be answered: the presence and clinical relevance of pre-formed nHLA antibodies and the impact of the transplant surgical process and the inherent ischemia-reperfusion injury process on the nHLA antibody repertoire, in the absence of detectable post-transplant organ injury. Addressing these two important variables is critical to fully understand if persistence or *de novo* generation of different nHLA antibodies after organ transplantation could subsequently be ascribed to be causally associated with specific clinical and pathology variables of graft injury. In the following paragraphs we have segregated published literature to address the evolution of the nHLA antibody repertoire in a patient, prior to transplantation, and after transplantation, in the absence of substantive injury and at the time of acute and chronic graft rejection. Rodent models better explain the pathogenic role of some of these nHLA antibodies and the support the importance of further validating the pathogenic relevance of selected nHLA antibiodies in additional associative human studies.

1.1 Can nHLA Antibodies be Detected at the Time of Organ Injury?—

Tissue and organ injury is associated with the detection of new nHLA antibodies, and though the direct pathogenic role of these antibodies has been difficult to define, a causal association is suggested with disease severity. A clear example of this is the detection of anti-GAD and anti- insulin antibodies in diabetic patients with pancreatic injury and insulin resistance. In patients with end stage renal disease, regardless of the etiology of renal failure, common panels of nHLA antibodies can be detected in patient sera to target antigens [9], though it is unclear if this is simply due to altered recognition of cognate self-antigen or if it relates to specific polymorphisms in these nHLA genes [19]. Similarly, in progressive IgA nephropathy, there is new detection of nHLA antibodies to specific panels of antigens (matriline 2, ubiquitin-conjugating enzyme E2W, DEAD box protein, and protein kinase D1) [20, 21], that correlate with functional decline and progressive histological injury.

Interestingly, nHLA antibodies may also be implicated in other diseases such as Moyamoya disease, as rare cerebrovascular occlusive disorder of uncertain etiology [22]. These data suggest that during organ injury, damage to tissue from stressors such as senescence, starvation, hypoxia or redox stress may alter the conformation of existing proteins or cause the “unmasking” of self-proteins making them immunogenic, with a resultant “new” or higher titer antibody response. One of the key issues in the detection of new nHLA antibodies rests with the inability to predict the target antigen that could drive a clinically relevant antibody response. The use of high density protein array platforms has allowed investigators to address this issue, as the nHLA antibody repertoire in patients' sera sample can now be rapidly screened by its ability to bind with any one of 9000 target proteins/antigens in a high-throughput multiplexed manner. This approach was initially utilized by Butte *et al* [9] to evaluate the panel of nHLA antibodies in the sera of patients with chronic renal failure. An increase of antibody levels against a total of 38 novel antigens was observed in these patients, inclusive of antibody to Angiotensinogen, which correlated with the incidence and severity of renal hypertension.

1.2. Do nHLA Antibodies occur with Organ Engraftment?—Mapping the nHLA antibody responses prior to transplantation is a helpful aspect for planning future experiments to interrogate nHLA antibodies after organ engraftment. To normalize for pre-existing nHLA antibodies in organ failure, the patients' day 0 (pre-transplant) sera sample can be used as a normalizing control. Recent studies have suggested that the process of organ transplantation, even with a well-functioning graft, results in the generation of new nHLA antibodies, the majority of which are likely to bear no physiological significance to the transplanted organ [10]. It can be postulated that the immune and non-immune related insults that the graft faces during surgical implantation and ischemia reperfusion, contribute to perturbing the nHLA antibody to a variety of tissue antigens repertoire in a transplant recipient, most of which are not likely to be functionally relevant. To determine the most common reactive nHLA antigens Bilalic *et al* assayed lymphocytic extracts from 20 healthy volunteers and tested with sera collected of 28 patients on the transplantation waiting list by Western blotting for selected nHLA antigens [23]. nHLA antibodies could be detected to a subset of nHLA targets studied (tubulin beta chain, vimentin, lamin-B1, and Rho GDP-dissociation inhibitor 2) but the clinical relevance of these antibodies remained to be better defined[23].

A study of stable post-transplant recipients, by Li *et al* [10, 14] conducted an analysis of pre and post-transplant sera samples from children transplanted with excellent post-transplant function over the first post-transplant year. The authors demonstrated a host of nHLA antibodies with the vast proportion being specific for proteins highly enriched in the kidney. The majority of which are not likely to be pathogenic, as all patients had normal histology and excellent graft function without proteinuria. Interestingly, in this analysis, the authors also conducted an analysis of nHLA antibodies detected *de novo* and specific to antigens that are specific to different compartments of the kidney (outer and inner renal cortex, outer and inner renal medulla, renal pelvis, renal papillae and glomerulus [10]) by doing an integrative analysis against kidney compartment specific gene expression data, mapped to the protein platform. Microarray gene expression profiles were mapped back to the

GenBank identifiers of the proteins on the protoarray, using a novel approach of *integrative antibiomics* (antibody combined with genomics; [10]. Interestingly, the most immunogenic region of the kidney, was identified to be the renal pelvis, the renal cortex and the glomerulus [14]. The finding of pelvis specific nHLA antibodies in the post-transplant setting was an unexpected finding; the authors hypothesized that the renal pelvis may sustain greater injury, possibly also because it is the reservoir of renal stem cells; the pelvis may be more susceptible to ischemia it has a separate blood supply from the rest of the kidney, and likely has greater exposure to urinary tract pathogens

Studies have also identified that *de novo* nHLA antibodies after transplantation vary with time post-transplantation, even in patients with stable graft function [10]. It is important to realize, that despite the stability of the serum creatinine, most patients who are currently identified in cohorts as “stable”, when analyzed by serial histology of the graft even the early post-transplant period (the first 2 years), show accrual of interstitial fibrosis and tubular atrophy [21, 24]. This injury has been previously hypothesized to be due to non-immune causes such as drug toxicity [25], but more recently Naesens *et al* [21, 24] have shown that the these recipients experience low grade, sub-clinical immune injury, at a lower intensity to the extent of immune injury observed for the same genes in acute rejection. It is thus not surprising to find that there is an increased response towards specific immune epitopes with increased post-transplantation time. When the approach of integrative antibiomics was utilized [10] to interrogate the tissue specificity of this response, the immunogenic proteins were found to be highly expressed in the outer renal cortex [14]. These data suggest that with increasing time post-transplantation, the renal cortex may be most susceptible to injury from sub-clinical immune and non-immune triggers of injury, and may explain the progressive cortical fibrosis and tubular atrophy observed in renal transplant recipients [21, 24, 26].

2. nHLA Antibodies and Acute Transplant Rejection

Antibodies against nHLA antigens are involved in hyperacute rejection, most of these inferred to be targeted against the vascular endothelium [27, 28] and called anti-endothelial cell antibodies (AECAs) Vascular endothelial cells are considered as primary targets for allograft injury for both both cellular and humoral AR [29]. A recently study by Jackson *et al* looked at 60 living-donor kidney transplant patients, by using flow cytometry and solid-phase bead immunoassays on donor-derived endothelial cell precursors (ECPs). They showed that 14 patients tested positive for donor-reactive IgG AECAs and this finding correlated with a higher incidence of cellular rejection during the early post-transplant period. [30]. Fredrich *et al* used cellular ELISA [31] to map AECAs and found a significant correlation between the AECA level and humoral AR [31]. This data was confirmed by Breimer *et al* who used flow cytometric crossmatch to demonstrate an association of both IgG and IgM AECAs with graft rejection during the early post-transplant period [32].

The angiotensin type 1 receptor (AT_1-receptor) is associated with acute renal allograft rejection, and it is proposed to be causative for acute rejection through complement-mediated and antibody dependent cell-mediated cytotoxicity. These AT_1-receptor antibody

are associated with refractory vascular rejection and involve phosphorylation of ERK ½, AP-1 activation and NF-κB activation in vascular cells [33].

The MHC class I-related chain A and B (MICA and MICB) have also been proposed as correlative nHLA antibody in acute transplant rejection [11, 34]. MICA and MICB genes share limited sequence homologies with HLA class I molecules and are expressed by a number of cells types including endothelial cells[35]. In a recent multicenter, randomized clinical trial, the evolution of humoral immunity was investigated in 130 low-risk pediatric patients over the first 2 years after renal transplantation by analyzing sera and protocol biopsies collected at 0, 6, 12 and 24 months post-transplantation. Among the study cohort 22% developed anti-HLA-DSA and 6% anti-MICA antibody, with increased overall risk of acute rejection ($p= 0.015$), chronic graft injury ($p=0.02$) and decline in graft function ($p=0.018$) [36]. Protein microarray technology was also applied to analyze the nHLA antibody repertoire in pediatric renal transplant recipients during allograft rejection and confirmed the association of MICA with humoral rejection with increased expression in the glomerular compartment of the rejecting kidney[7]. Using a hypothesis generating approach by protein array scanning of sera from patients with acute rejection, nHLA antibody against a novel antigen- protein kinase C zeta (PKCzeta) was identified as a significant correlate with specific types of acute rejection, with high expression of this protein in the mononuclear cell infiltrate of acute rejection. This antibody was found to causally associate with steroid-resistant acute rejection and an increased risk of graft loss [8].

The identification of a correlation of specific nHLA antibodies with organ rejection is extended to liver transplantation where antibody to liver sinusoidal endothelial cells correlated with acute rejection and proliferation of alloreactive T cells [37]. It is likely that many of these nHLA correlative responses in acute rejection in organ transplant recipients are patient specific; a review of published literature on this subject finds that apart from certain nHLA targets such as MICA and AECAs, it is likely that there are many patient specific nHLA antibody responses, which makes the discovery of a common list of pathogenic nHLA antibodies difficult to define, without the use of large patient cohorts.

3. nHLA Antibodies and Chronic Transplant Injury: Predicting the Event Before Organ Engraftment?

nHLA antibodies have also been implicated in chronic transplant injury in various organs. In heart transplant patients, there is a reported association of nHLA antibodies against myosin and vimentin with cardiac allograft vasculopathy (CAV) [38, 39]. In another study of heart transplant recipients, levels of anti-heterogeneous nuclear ribonucleoprotein K antibodies (anti-hnRNP-K antibodies) four years post-transplant were found to be statistically significantly associated with CAV disease [40]. Additionally, another study of 616 adult cardiac transplant recipients found that patients with pre-transplant nHLA IgM antibodies had significantly lower survival rates at 1, 2, 5, 10 year post-transplant and their presence in the pre-transplant sera could be a major risk factor for allograft failure [15].

Research into the role of nHLA antibody in lung allograft survival is scarce, but AECAs and anti-K-α1 tubulin antibody [41] have been found to play a role in allograft failure in lung transplant recipients by development of the lesion of chronic injury in bronchiolitis

obliterans syndrome (BOS). The binding of these nHLA antibodies to their antigens on airway epithelial cells is presumed to lead to increased expression of fibrogenic growth factors, activation of cell cycle signaling, and fibroproliferation, all of which are critical events leading to development of BOS post lung transplantation [42]. Though much of the data discussed so far relates to humans, nHLA antibodies have also been studied in vitro for their cellular pathogenicity and mechanisms. Tiriveedhi *et al* used in vitro analysis of normal human bronchial epithelial cell lines to demonstrate an increased level of hypoxia inducible factor (HIF-1α) protein and its antibody through c-JNK/MAP Kinase activation [43] and proposed that the HIF-1α signaling pathway contributes to upregulated expression of pro-fibrotic growth factors such as VEGF, HB-EGF, bFGF and TGF-β which drive the injury of chronic rejection and BOS. Collagen V antibodies have also been implicated in the development of BOS [44] [45].

nHLA antibodies to nucleolar and spindle-associated protein 1 (NuSAP1) and chromatin assembly factor 1, subunit B (CHAF1b), have been implicated in the development of chronic graft versus host disease (GVHD) [46]. In renal allograft recipients, specific antibody against peroxisomal-trans-2-enoyl-coA-reductase (PECR) were correlated with transplant glomerulopathy [47]. Human protoarrays were used to interrogate 172 longitudinally collected sera samples in an unbiased discovery approach from renal transplant patients without interval acute rejection, and the results of common panels of nHLA antibodies were correlated with histopathological scores for chronic allograft injury (CAI) [13]. A panel of 30 nHLA antibodies in the pre-transplant sera sample of these patients significantly correlated with the development of biopsy confirmed CAI ($P<0.01$), many of these recognizing immunogenic antigens such as MIG, ITAC, IFN-γ, and glial-derived neurotrophic factor (GDNF) positively correlated with chronic graft injury scores at 24 months post transplantation [13]. Similar to the adverse impact of high titer HLA-antibody in the pre-transplant sera in highly sensitized patients on acute rejection, specific nHLA antibodies in the pre-transplant sera in heart and kidney transplant recipients may be used to stratify patients for post-transplant risk, prior to organ transplantation.

Conclusions

With improvements in immunosuppression and a reduction in the incidence of early acute rejection, and limited improvements in graft life expectancy, attention has turned to nHLA antibodies as unrecognized triggers of acute and more importantly, chronic graft injury. These antibodies have been difficult to identify, given the inherent difficulty of identifying the immunogenic antigens *in vivo*. Though these have been traditionally identified by flow cytometry and ELISA against selected proteins, the advent of high-throughput screening methods such as protein arrays, allow for an unbiased selection of the most correlative proteins with clinical phenotypes of graft injury [48]. A review of the published literature reveals that there are significant advances as yet to be made in additional discovery of new nHLA antibody targets and their validation. It is likely that many associative nHLA antibodies in organ transplant recipients are bystander effects of previous injury, and may be highly specific in different patients/ patient groups. Given this observation, large scale validation studies will have to be planned to test the applicability of nHLA monitoring methods or patient selection will need to stringent. The identification of a predictive

potential for these antibodies in chronic rejection is an exciting application of nHLA antibodies as they may uncover novel drug targets and injury mechanisms and also provide a method to personalize immunosuppression to injury risk. Considerable validation work in required, in the form of prospective clinical trials, so that specific nHLA antibody measurements before and after transplantation that can help develop predictive risk profiles for different transplant injury phenotypes. By combining datasets generated through gene, transcripts, proteins, and antibodies, researchers can combine new insights from studies in the nHLA field and begin to uncover the relevant mechanisms driving the altered immunogenicity of the corresponding specific antigenic epitopes and be able to exploit their potential for rational drug design.

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Table 1

Organ	Author	Phenotype (Hyper Acute Rejection)/AR/Chronic Allograft Injury	Study Size	nHLA antibody (nHLA-Ab)	References
	Bialic *et al*	chronic hemodialysis (pre-txp)	48	tubulin beta chain, vimentin, lamin-B1, and Rho GDP-dissociation inhibitor 2	[23]
Kidney	Sutherland *et al*	Acute Rejection	15	Protein Kinase Cζ	[8]
	Li *et al*	Chronic Allograft Injury	33	Angiotensinogen (AGT) and Speedy homologue A (SPDYA)	[14]
	Li *et al*	Acute Rejection	15	MICA	[7]
	Li *et al*	Post-transplant Ab response	18	IgG's against 5,056 unique protein targets	[10]
	Butte et al	ESRD	95	angiotensinogen and PRKRIP1	
	Sigdel *et al*	Chronic Allograft Injury	98	MIG (CXCL9), ITAC (CXCL11), IFN-γ, glial-derived neurotrophic factor	[13]
	Chaudhuri *et al*	Acute Rejection	124	MICA	[36]
	Dragun *et al*	Refractory vascular rejection (AR)	33	Angiotensin II type 1 receptor antibody	[33]
	Dinavahi *et al*	Chronic Allograft Injury	39	peroxisomal-trans-2-enoyl-coA-reductase	[47]
	Zou *et al*	Acute Rejection	1910	MICA	[35]
	Narayan *et al*	Acute Rejection	1		[34]
	Jackson *et al*	Acute Rejection	60	anti-endothelial cell antibodies (AECAs)	[30]
	Breimer *et al*	Rejection	147		[32]
	Sun *et al*	Acute Rejection	226		[49]
Heart	Ismail *et al*	Acute Rejection	60		[50]
	Fredrich *et al*	cardiac allograft vasculopathy (CAV)	80		[31]
	Acevedo *et al*	Cardiac allograft vasculopathy (CAV)	48	anti-hnRNP-K antibodies	[40]
Lung	Smith *et al*	Hyperacute rejection, Acute rejection	616	IgM Abs to nHLAs	[15]
	Otten *et al*	Bronchiolitis obliterans syndrome (BOS)	11	PSMC4, F3, LOC284058, PLUNC, ZNF33A, XP_931864	[51]
	Jaramillo *et al*	Bronchiolitis obliterans syndrome (BOS)	27	anti-AEC Abs	[41]
	Goers *et al*	Bronchiolitis obliterans syndrome (BOS)	36	anti-K-alpha1 tubulin Abs	[42]
	Tiriveedhi *et al*	Bronchiolitis obliterans syndrome (BOS)	N/A	anti-K-alpha1 tubulin Abs	[43]
	Burlingham *et al*	Bronchiolitis obliterans syndrome (BOS)		collagen V	[44]
	Tirivedhi *et al*	Bronchiolitis obliterans syndrome (BOS)	12	collagen V epitopes	[45]
Liver	Sumitran-Holgersson *et al*	Rejection	95	Liver sinusoidal endothelial cells (LSECs)	[37]
BMT	Wadia *et al*	GVHD	190	NuSAP1, CHAF1b	[46]
	Porcheray *et al*	Rejection/tolerance	5	PSMA4	[52]

N/A* *in vitro* cell culture