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Anti-HLA DSA and Beyond: deciphering the immunological mechanisms driving chronic rejection

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Table of contents

Brief Research Report

Mini Review

Original Research

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SPECIAL ISSUE EDITORIAL
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Editorial: Anti-HLA DSA and Beyond: Deciphering the Immunological Mechanisms Driving Chronic Rejection

Federica Casiraghi[1]*, *Maarten Naesens*[2], *Aravind Cherukuri*[3], *Sandra Lindstedt*[4], *Candice Roufosse*[5] and *Olivier Thaunat*[6,7]*

[1]Department of Molecular Medicine, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Bergamo, Italy, [2]Department of Microbiology, Immunology and Transplantation, KU Leuven, Leuven, Belgium, [3]Thomas E. Starzl Transplantation Institute, University of Pittsburgh, Pittsburgh, PA, United States, [4]Department of Cardiothoracic Surgery and Transplantation, Lund University Hospital, Lund, Sweden, [5]Department Immunology and Inflammation, Imperial College London, London, United Kingdom, [6]Department of Nephrology, Transplantation and Clinical Immunology, Hospices Civils de Lyon, Groupement Hospitalier Centre, Lyon, France, [7]Lyon-Est Medical Faculty, Claude Bernard University (Lyon 1), Lyon, France

Keywords: donor-specific antibodies, innate immune cells, microvascular injury, natural killer cells, non-HLA antibodies

Editorial on the Special Issue

Anti-HLA DSA and Beyond: Deciphering the Immunological Mechanisms Driving Chronic Rejection

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***Correspondence**
Federica Casiraghi,
federica.casiraghi@marionegri.it
Olivier Thaunat,
olivier.thaunat@chu-lyon.fr

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FROM ANTI-HLA DSA TO INTEGRATED MODELS OF CHRONIC REJECTION

Solid organ transplantation remains the optimal therapy for patients with end-stage organ failure. Yet, despite major advances in therapeutic immunosuppression over the past two decades, chronic immune-mediated injury of allograft continues to be a leading cause of late graft failure.

Anti–donor-specific HLA antibodies (DSA) have long occupied a central place in this process, consistent with the strong association between the presence of circulating DSA, microvascular inflammation in graft biopsy and poor long-term outcomes. However, several observations challenge a strictly DSA-centric model: chronic microvascular rejection–like lesions may arise in the absence of detectable circulating DSA; pathological patterns vary widely across organs and patients; and graft injury may progress even when humoral activity appears controlled. Together, these findings support a more integrated framework in which multiple pathways of allorecognition and immune effector mechanisms—adaptive and innate—converge on the graft microvasculature, driving endothelial injury, remodeling, and fibrosis (**Figure 1**).

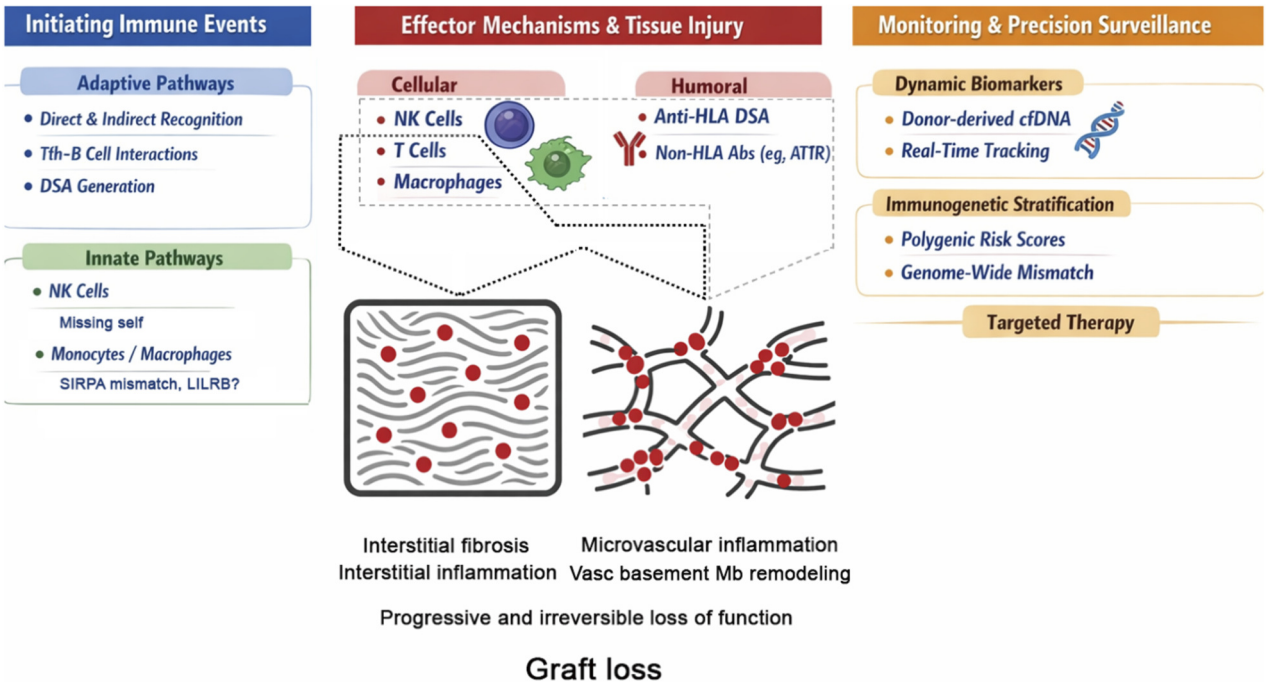

FIGURE 1 | Integrated Models of Chronic Rejection. Chronic rejection results from multiple innate and adaptive allorecognition pathways that activate effector mechanisms, causing interstitial and microvascular inflammation and progressive fibrosis. Dynamic biomarkers, including donor-specific HLA antibodies (DSA) and donor-derived cell-free DNA (dd-cfDNA), may early detect graft injury, guiding the biopsy. Integrating immunologic, histologic, and genetic data should enable mechanism-informed monitoring and targeted interventions before irreversible damage occurs.

This Special Issue follows this conceptual trajectory, from allorecognition to chronic tissue damage, highlighting how adaptive and innate immunity intersect to shape late transplant outcomes.

PROGRESS IN ELUCIDATING THE MECHANISMS UNDERLYING ALLORECOGNITION

Indirect Allorecognition, Tfh–B Cell Interactions, and the Maintenance of Pathogenic Antibody Responses

Basu et al. review the pivotal role of the indirect pathway of allorecognition—presentation of donor-derived peptides by recipient antigen-presenting cells in the context of self HLA—in sustaining helper T-cell responses, DSA generation, and chronic rejection. They detail how donor-specific B cells present processed alloantigen to $CD4^+$ T cells, frequently adopting a T follicular helper phenotype that maintains germinal center reactions, affinity maturation, and pathogenic antibody production. Importantly, the review also outlines endogenous regulatory mechanisms, describing how distinct regulatory T- and B-cell subsets can suppress cytokine release, plasmablast differentiation, antibody secretion, and germinal center maturation. These insights identify specific cellular and molecular targets for future immunoregulatory or cell-based therapeutic strategies.

Expanding Allorecognition Pathways and Innate Contributions

Charmetant et al. revisit the classical direct and indirect pathways of allorecognition by incorporating mechanisms that better explain persistent alloimmunity. They emphasize the semi-direct pathway—where recipient antigen-presenting cells acquire intact donor MHC molecules from graft cells—and introduce the concept of the "three-cell cluster" as a functional refinement sustaining long-term T-cell activation. The review also discusses the inverted direct pathway, whereby donor $CD4^+$ T cells provide help to recipient B cells, potentially supporting early DSA generation. Beyond adaptive immunity, the authors highlight antibody-independent roles of NK cells and describe how innate myeloid cells contribute to non-self recognition and initiation of adaptive responses, positioning innate immunity as a potential trigger and amplifier of late graft injury.

Innate Non-Self Recognition by Myeloid Cells

Focusing specifically on innate discrimination mechanisms, Palvair et al. examine non-self recognition by monocytes and macrophages, proposing leukocyte immunoglobulin-like

receptors (LILRs)—particularly LILRB3—as candidate mediators of allograft injury. Building on murine data on paired immunoglobulin receptor–dependent recognition, they describe LILRs as human orthologs capable of modulating myeloid activation in transplantation. The review summarizes evidence that LILRB3 may bind HLA class I molecules and interact with complement fragments and other ligands, and suggests that receptor polymorphism and the balance between inhibitory and activating counterparts (e.g., LILRA6) may polarize myeloid responses toward inflammatory or regulatory phenotypes. This work provides a conceptual bridge between innate recognition and chronic graft injury beyond a purely antibody-centered paradigm.

EFFECTOR MECHANISMS DRIVING ALLOGRAFT INJURY

Beyond HLA: Mapping the Non-HLA Antibody Landscape

Addressing antibody-mediated mechanisms beyond HLA, Schmidt et al. present an original study profiling 60 non-HLA antibodies in serial serum samples from 77 pediatric kidney transplant recipients. Substantial pre-transplant reactivity was observed, with more than half of patients displaying over 15 positive antibodies, and overall antibody burden remained relatively stable early after transplantation. While cumulative non-HLA antibody profiles did not clearly predict late antibody-mediated rejection (AMR), specific targets emerged as potential signals, including SNRPB2 pre-transplant and ACTIN/CGB5 at 1–2 years post-transplant. These findings suggest that selected non-HLA specificities—rather than overall antibody load—may hold clinical relevance, pending independent validation.

AT1R Antibodies and Endothelial Activation

Martin et al. focus on antibodies against the angiotensin II type 1 receptor (AT1R), among the most extensively studied non-HLA specificities associated with AMR-like phenotypes. They describe how AT1R antibodies can function as receptor agonists, sustaining endothelial signaling through pathways including β-arrestin and mTOR, thereby promoting vascular inflammation and impaired repair, often with limited complement deposition. Clinically, the review synthesizes heterogeneous observational evidence and underscores key challenges to implementation, including assay standardization, threshold definition, and prospective validation. The authors conclude with a pragmatic message: AT1R antibodies may be relevant in selected contexts, but routine testing requires stronger and more consistent evidence.

NK Cells as Central Effectors of Chronic Vascular Injury

Chambon et al. position NK cells as central drivers of chronic vascular rejection, acting both downstream of antibodies and through antibody-independent mechanisms. They organize NK activation into three major pathways: missing-self recognition due to reduced inhibitory HLA class I signaling, induced-self recognition of stress ligands, and antibody-dependent cellular cytotoxicity via FcγRIIIA engagement. Importantly, the review emphasizes that NK cells require priming to become fully pathogenic and can, in experimental models, recruit additional effectors such as T cells and macrophages through cytokines including IFN-γ, thereby sustaining inflammatory circuits that promote chronic vascular remodeling.

MECHANISTIC INSIGHTS FROM ALLOGRAFT BIOPSIES

Tissue-Level Evidence of NK Cell Involvement in ABMR

Providing biopsy-level support for NK cell involvement, Diebold et al. combine CD16 and T-bet immunostaining with histologic and molecular analyses. NK cell infiltration, particularly within peritubular capillaries, correlates strongly with microvascular inflammation (Banff glomerulitis plus peritubular capillaritis scores), while showing little association with tubulitis or chronic tubulointerstitial lesions. NK burden also aligns with Molecular Microscope Diagnostic System–derived AMR classifiers and NK transcript burden scores, but not with T cell–mediated rejection probability. The authors discuss timing-dependent mechanisms and propose that ADCC may be especially relevant in late AMR, linking tissue patterns to effector biology.

Rethinking Rejection Histology Through a Mechanistic Lens

Terinte-Balcan et al. reframe kidney transplant rejection histology by moving beyond a strict TCMR-versus-AMR dichotomy and examining which immune cells dominate specific lesion patterns. Using Banff lesions as a reference, they illustrate how multiplex immunofluorescence and bulk, single-cell, and spatial transcriptomics are redefining inflammatory landscapes across active AMR, chronic active AMR—including transplant glomerulopathy—and acute and chronic active TCMR. A key message is the marked heterogeneity within diagnostic categories and the increasing recognition of antibody-independent and non-HLA–related processes shaping histologic phenotypes and prognosis.

Microvascular Inflammation Beyond Antibody-Mediated Rejection

Varol et al. focus on microvascular inflammation—glomerulitis and peritubular capillaritis—traditionally considered a hallmark of AMR, and challenge its exclusivity. Through a systematic review and re-analysis of a local biopsy cohort, they demonstrate that microvascular inflammation can also occur in acute TCMR, albeit with lower frequency and severity. They discuss how these findings complicate Banff-based categorization and relate them to evolving Banff concepts,

including DSA- and C4d-negative microvascular inflammation phenotypes and potential contributions of non-HLA antibodies and innate activation.

Transplant Glomerulopathy as a Convergent Phenotype

Using transplant glomerulopathy as a paradigmatic lesion of chronic rejection, Chutani et al. explicitly question a one-to-one equivalence with DSA-mediated mechanisms. After reviewing defining morphologic features and prognostic implications, they broaden interpretation by discussing DSA-negative transplant glomerulopathy and multiple contributors converging on endothelial injury, including non-HLA antibodies, complement-related pathways, cell-mediated mechanisms, and podocyte stress. The review emphasizes transplant glomerulopathy as a histologic endpoint integrating diverse upstream immune processes.

FROM INJURY BIOMARKERS TO PRECISION SURVEILLANCE

Donor-Derived Cell-Free DNA for Dynamic Monitoring

Akifova et al. discuss donor-derived cell-free DNA (dd-cfDNA) as a practical tool to reduce diagnostic uncertainty in AMR, particularly in DSA-positive recipients. Given the limited sensitivity of creatinine and proteinuria for subclinical injury and the frequent absence of histologic AMR in for-cause biopsies, dd-cfDNA is presented as a dynamic injury-linked biomarker with a short half-life. The review summarizes technical considerations and associations with microvascular inflammation and molecular AMR signatures, while highlighting the need to prospectively define appropriate contexts of use.

In a brief research report, the same group Osmanodja et al. presents longitudinal dd-cfDNA monitoring in two sensitized kidney transplant recipients with biopsy-proven AMR treated with daratumumab. In both cases, dd-cfDNA trajectories closely mirrored histologic activity, decreasing with clinical improvement and aligning with post-treatment biopsy findings. Although limited by sample size, these cases provide proof-of-concept for integrating dd-cfDNA into treatment-response monitoring and follow-up strategies in difficult-to-treat AMR.

Toward Immunogenetically Informed Surveillance

Finally, Helanterä et al. extend monitoring strategies into a precision-medicine framework by examining how immunogenetics may stratify risk and guide post-transplant surveillance. They summarize emerging evidence linking polygenic risk scores and genome-wide mismatch concepts to post-transplant phenotypes, while acknowledging that current predictive gains remain modest. Importantly, the authors outline a translational perspective in which validated genetic signals could support individualized immunosuppression and targeted monitoring in high-risk subsets.

CONCLUSION

Collectively, these contributions portray chronic rejection as the result of layered and interacting immune pathways, in which humoral, cellular, and innate mechanisms converge on the graft microvasculature to drive progressive remodeling and fibrosis. Improving long-term graft survival will depend on mechanism-informed diagnostics and surveillance, enabling earlier intervention and paving the way for targeted therapies capable of interrupting chronic injury before it becomes irreversible.

AUTHOR CONTRIBUTIONS

FC drafted the manuscript. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

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ORIGINAL RESEARCH
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Development of Non-HLA Antibodies and Their Association With Antibody-Mediated Rejection in Pediatric Kidney Transplant Recipients

*Franziska Schmidt[1], Murielle Verboom[2], Michael Hallensleben[2], Alexander Braumann[3], Jens Drube[1], Lena Brunkhorst[1], Dieter Haffner[1], Anette Melk[1] and Nele Kanzelmeyer[1]**

[1]Department of Pediatric Kidney, Liver, Metabolic and Neurological Diseases, Hannover Medical School, Hannover, Germany, [2]Institute of Transfusion Medicine and Transplant Engineering, Hannover Medical School, Hannover, Germany, [3]Institute for Mathematical Stochastics, Technische Universität Braunschweig, Braunschweig, Germany

Antibody-mediated rejection (ABMR) is the leading cause of long-term graft loss in pediatric kidney transplantation (KTx). While donor-specific HLA antibodies are established contributors, emerging evidence suggests a role for non-HLA antibodies in ABMR pathogenesis. In this descriptive study, we analyzed 60 non-HLA antibodies in 77 pediatric KTx recipients using serum samples collected pre-transplant, post-transplant, and at ABMR diagnosis. During a median follow-up of 4.83 years, 29.8% developed ABMR, with a median onset of 3.67 years. Non-HLA antibody presence prior to KTx was not influenced by pre-transplant dialysis; over half of the patients already had >15 positive non-HLA antibodies. The cumulative antibody profile remained stable 1–2 years post-KTx, with no association between late ABMR and antibody strength or breadth. However, ACTIN (higher risk) and CGB5 (lower risk) at 1–2 years post-KTx, as well as SNRPB2 pre-transplant, were significantly associated with ABMR ($p < 0.05$). IL-21 levels increased in controls over time ($p < 0.05$), although driven by five patients with notably high levels. Our findings support a potential involvement of non-HLA antibodies in pediatric ABMR. Nevertheless, larger studies are needed to validate the predictive value of individual non-HLA antibodies for clinical application.

Keywords: non-HLA antibodies, antibody-mediated rejection, pediatric kidney transplantation, graft loss, pediatric kidney failure

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***Correspondence**
Nele Kanzelmeyer,
kanzelmeyer.nele@mh-hannover.de

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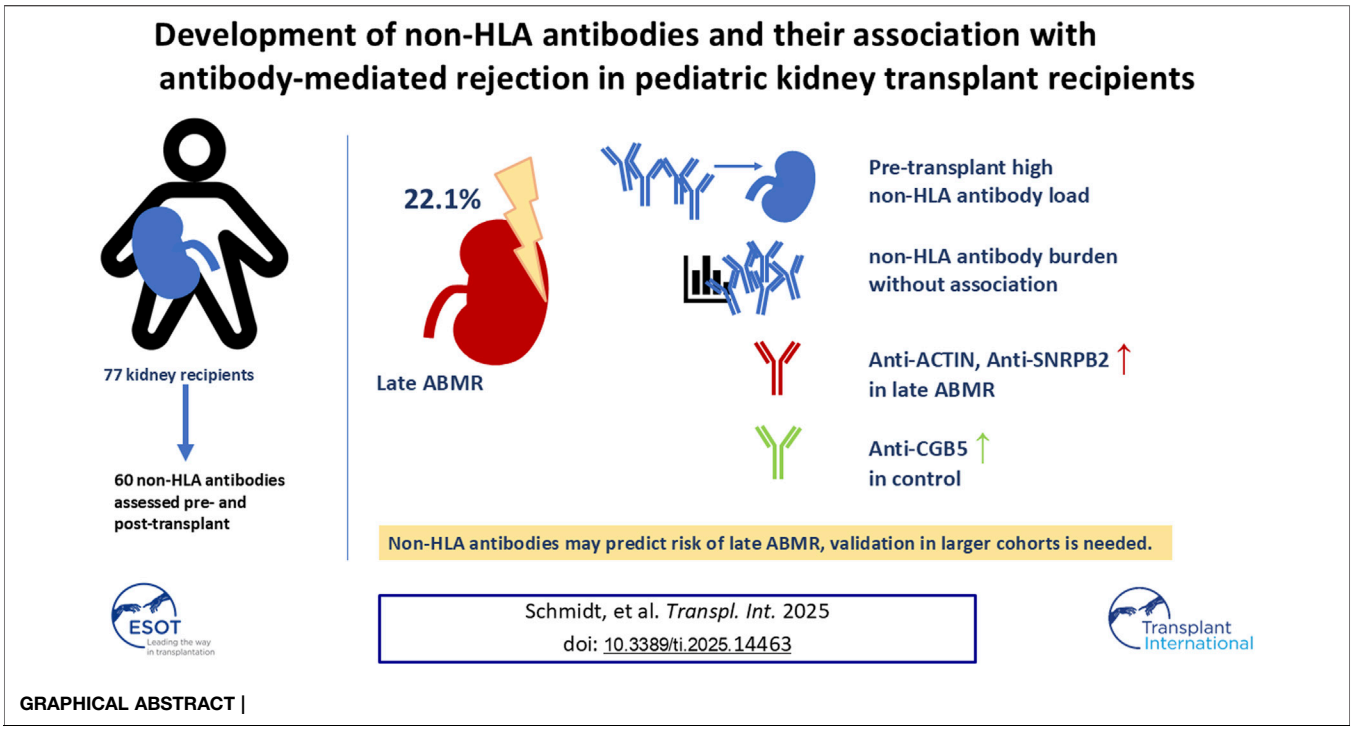
INTRODUCTION

Kidney transplantation (KTx) is regarded as the treatment of choice for children and adolescents with kidney failure [1–4]. Antibody-mediated rejection (ABMR) remains a leading cause of graft dysfunction and loss of allograft in both pediatric and adult kidney transplant recipients [5–8]. In a study of 337 pediatric KTx patients, 12.5% developed ABMR, of which 45% of the children experienced ABMR within 5 years after KTx. Twenty-five percent of the affected children experienced graft loss within 1 year following the diagnosis of acute



GRAPHICAL ABSTRACT |

ABMR, while approximately 50% of patients lost their graft within 1 year after chronic ABMR diagnosis [5].

While some risk factors for adults are also applicable to children, there are age-specific aspects, including immunological naivety in young children and difficulties with adherence to immunosuppressive therapy in adolescents [9–11]. Donor-specific antibodies (DSAs) directed against human leukocyte antigens (HLAs) have been identified as pivotal targets in the humoral immune response to renal allografts, contributing to antibody-mediated rejection [5, 12]. Nevertheless, the precise underlying causal mechanisms of these associations remain unclear [13–15]. Numerous studies have identified non-HLA antibodies as potential mediators of transplant rejection [16, 17].

Non-HLA antibodies play a complementary role in allo- and autoimmunity [18–20]. Their development is initiated by endothelial injury and subsequent exposure to neoantigens or polymorphic antigens differing between donor and recipient. Moreover, cells of the basal membrane and extracellular matrix exposed by vascular injuries can activate immunological processes. Consequently, a considerable number of non-HLA antibodies associated with transplant rejection are directed against antigens on endothelial cells, as well as transmembrane and extracellular proteins [18, 19]. In addition to vascular injuries, mismatches of non-HLA antigens between donor and recipient can increase the development of non-HLA alloantibodies. Among non-HLA antibodies, antibodies against MHC class I polypeptide-related sequence A (MICA) and autoantigens such as angiotensin II receptor type 1 (AT1R), endothelin-1 receptor type A (ETAR), and vimentin have recently been in research focus [21–28]. Non-HLA antibodies are implicated in autoimmune processes, with anti-AT1R and ETAR antibodies identified in several vasculopathic conditions, including peripheral arterial occlusive disease and essential hypertension [29–31]. In fact, anti-AT1R antibodies are detectable in 20%–40% of patients with kidney failure, yet only in 7%–15% of healthy individuals [23, 24].

It has been observed that 72% of pediatric patients with DSAs against the HLA surface antigens of the donor kidney additionally exhibited non-HLA antibodies [32]. Regardless of HLA-DSAs, the presence of AT1R antibody, ETAR antibody, and MICA antibody in pediatric kidney transplant recipients is associated with an elevated risk of acute ABMR and deterioration of graft function [32]. The incidence of acute ABMR was found to be approximately 3% higher in cases where non-HLA antibodies, particularly those directed against MICA, were identified prior to KTx [33]. Nevertheless, a considerably broader spectrum of non-HLA antibodies targeting endothelial and epithelial cells, in addition to various proteins, has been associated with unfavorable outcomes in kidney transplantation [34–36]. The role of non-HLA antibodies in the context of the developing immune system in pediatric patients remains poorly characterized [37, 38]. The existing literature indicates that non-HLA antibodies influence the trajectory of allograft function in pediatric KTx patients. Comprehensive studies, particularly those involving very young transplant recipients, are still required to elucidate the interplay between non-HLA antibodies and the onset of ABMR.

To fill these gaps, we investigated the development of non-HLA antibodies and their association with ABMR in a cohort of 77 pediatric KTx patients.

MATERIALS AND METHODS

Study Design and Study Population

We conducted a retrospective descriptive cohort study including pediatric patients with the following inclusion criteria: a) age <18 years at time of KTx, b) KTx performed at Hannover Medical School between May 2014 and June 2021, c) availability of pre- and post-transplant biobanked serum samples for HLA-DSA analysis. Exclusion criteria were defined as: a) combined organ transplantation, b) graft loss due to recurrence of the underlying disease, or c) loss to follow-up. Donor-recipient matching prior to KTx was based on blood group and HLA typing. The majority of patients received tacrolimus, mycophenolic acid or everolimus, and corticosteroids as baseline immunosuppression. Basiliximab induction was administered in patients treated with tacrolimus, prednisolone, and everolimus (initiated 4 weeks post-KTx). HLA antibodies were assessed annually and upon biopsy indication. ABMR was diagnosed via kidney allograft biopsy, which was performed when serum creatinine increased rapidly or progressively by >20% above baseline without an alternative explanation or as part of a surveillance protocol six or more months post-transplantation. Biopsies were screened ABMR according to the most recent available Banff consensus [39]. Rejection within the first-year post-transplant was defined as early ABMR; later on as late ABMR. Patients harboring histological abnormalities apart from ABMR, such as borderline, T-cell-mediated rejections, or inconclusive results, are included in the control group. Patients diagnosed with biopsy-proven ABMR received anti-humoral therapy, including plasmapheresis, intravenous immunoglobulin G, and rituximab. Non-HLA antibodies were analyzed retrospectively from biobanked serum samples obtained at several stages: pre-transplant (taken for the last evaluation of HLA alloantibodies before KTx), and post-transplant (twice within the first 2 years after KTx and whenever a kidney biopsy was performed).

Parents' and, if appropriate, patients' consent and the ethics committee approval of the Hannover Medical School were obtained and all investigations were performed in accordance with the Declaration of Helsinki and the Good Clinical Practice guidelines.

Detection of Non-HLA Antibodies

The pre- and post-transplant serum samples were retrospectively analyzed for 60 non-HLA antibodies using the LIFECODES non-HLA autoantibody assay (Werfen), performed according to manufacturer's instructions. Raw data were collected via Luminex 200 with hlaSYSTEM software (AVALAS). After background fluorescence subtraction, ratios of the measured Mean Fluorescence Intensity (MFI) to vendor-defined cut-offs were calculated for each non-HLA target; antibodies were defined as positive if ratios exceeded 1.0.

Statistical Analysis

Descriptive statistics for categorical variables were reported as frequencies and percentages; continuous variables were summarized using means with standard deviations or medians and interquartile ranges, as appropriate. Baseline characteristics included sex, age, donor type, pre-transplant dialysis or preemptive transplantation status, *de novo* versus repeat transplantation, and the presence of DSAs pre-transplant and at routine follow-up (1–2 years post-transplant). For each patient, the broadness, number of positive non-HLA antibody targets with ratio >1, and the strength of non-HLA sensitization, sum of positive antibody ratios with a value >1 per patient, were calculated. Group differences were analyzed using Student's t-test for normally distributed data and Wilcoxon signed-rank test otherwise. Differences between more than two groups were assessed by ANOVA. Fisher's exact test was used for associations between categorical variables. Pearson correlation analyses assessed the relationship between identical non-HLA antibodies pre-transplant and 1–2 years post-transplant, and between different non-HLA antibodies 1–2 years post-transplant (weak or no correlation: $r < 0.4$; moderate: $r = 0.4–0.8$; strong: $r > 0.8$). Temporal antibody dynamics were evaluated by mean MFI ratios per group at four time points: pre-transplant, up to 1 year post-transplant, 1–2 years post-transplant, and at rejection. Patients were classified as controls or ABMR cases, with or without HLA-DSA at rejection. For each group, the five antibodies with the greatest net increase were identified by averaging absolute changes in MFI ratios between pre-transplant and either 1–2 years post-transplant or time of rejection, irrespective of direction. Multiple logistic regression analyzed associations between late ABMR occurrence, baseline characteristics, and cumulative antibody broadness and strength. Individual non-HLA antibody associations were assessed via L1-penalized logistic regression [40]. The hyperparameter of the L1-penalization was determined by cross-validation and AIC was used for the stepwise selection algorithm. Statistical significance was defined as $p < 0.05$. Throughout all analyses, the control group was defined as patients who never fulfilled diagnostic criteria for ABMR during follow-up.

RESULTS

Demographic Characteristics of the Study Population

A total of 77 patients were eligible for further analysis in this study. The mean age at the time of transplantation was 10.06 years, with a male predominance (61.04%), **Table 1**. Most underlying conditions were renal, with congenital and genetic kidney diseases accounting for 75%, including CAKUT (n = 32) as the most frequent subtype, (**Table 2**). Degenerative nephritic or nephrotic disorders were observed in 13%. Living donor transplants were performed in 21%, and 19% were re-transplantations. Prior to KTx, 56% of patients underwent dialysis. Median follow-up was 4.83 years (IQR 3.08–6.96).

During the follow-up period, 23 patients (29.87%) were diagnosed with ABMR: five early (6.5%), 17 late (22.1%) one with early and late ABMR (1.3%). Late ABMR occurred after a median of 6.1 years, IQR (3.68–6.41). Within the late ABMR group, 52.9% of patients exhibited the presence of HLA-DSA

TABLE 1 | Baseline characteristics for the different patient groups (total, control and ABMR, further categorized as early onset, late onset an early and late onset.

Baseline characteristics								
Variables			**Total**	**Control**	**Early ABMR**	**Late ABMR**	**Early and late ABMR***	**p**
			n = 77	**n = 54**	**n = 5**	**n = 17**	**n = 1**	
Sex	f	n/total	30/77	20/54	1/5	8/17	1/1	0.53
		(%)	(38.98)	(37.04)	(20)	(47.06)	(100)	
	m	n/total	47/77	34/54	4/5	9/17	0/1	
		(%)	(61.04)	(62.96)	(80)	(52.94)	(0)	
Age at time of KTx		mean	10.06	9.5	15	10	6	0.06
		+/− SD	+/−5.37	+/−5.6	+/− 0.8	+/− 4.39	+/− 0	
Dialysis prior to KTx	yes	n/total	43/77	33/54	2/5	9/17	0/1	0.60
		(%)	(55.84)	(61.11)	(40)	(52.94)	(100)	
	no	n/total	34/77	21/54	3/5	8/17	1/1	
		(%)	(44.16)	(38.89)	(60)	(47.06)	(0)	
Donor type	LD	n/total	16/77	13/54	2/5	0/17	1/1	0.05
		(%)	(20.78)	(24.07)	(40)	(0)	(0)	
	DD	n/total	61/77	41/54	3/5	17/17	0/1	
		(%)	(79.22)	(75.93)	(60)	(100)	(100)	
Repeated KTx	yes	n/total	62/77	44/54	5/5	13/17	0/1	0.50
		(%)	(80.52)	(81.15)	(100)	(76.47)	(100)	
	no	n/total	15/77	10/54	0/5	4/17	1/1	
		(%)	(19.48)	(18.52)	(0)	(23.53)	(0)	
DSA pre KTx	yes	n/total		2/54	0/5	3/17	0/1	0.11
		(%)		(3.7)	(0)	(23.53)	(100)	
	no	n/total		52/54	4/5	14/17	1/1	
		(%)		(96.3)	(100)	(76.47)	(0)	
DSA after 1–2 years post KTx	yes	n/total		0/54	1/5	8/17	0/1	<0.001
		(%)		(0)	(20)	(47.06)	(100)	
	no	n/total		53/54	3/5	9/17	1/1	
		(%)		(98.15)	(60)	(52.94)	(0)	
	n/a[a]	n/total		1/54	1/5	0/17	0/17	
		(%)		(1.9)	(20)	(0)	(0)	

Abbreviations: p, p-value; f, female; m, male; KTx, kidney transplantation; LD, living donor; DD, deceased donor; n/a, not available.
[a]Excluded from ANOVA, analysis due to low number of cases.

TABLE 2 | Underlying causes of end-stage kidney disease in the study cohort, (N = 75).

Etiology	Diagnosis	Number of patients
Congenital and Genetic Kidney Diseases (75%)	CAKUT (Congenital anomalies of the kidney and urinary tract)	32
	Nephronophthisis	6
	Cystinosis	4
	Denys-Drash syndrome	4
	ARPKD (Autosomal recessive polycystic kidney disease)	3
	Joubert syndrome	3
	Primary hyperoxaluria type 1	3
	aHUS (Atypical hemolytic uremic syndrome)	3
	Congenital nephrotic syndrome	2
	Prune-Belly syndrome with bilateral renal dysplasia	1
	Mayer-Rokitansky-Küster-Hauser syndrome	1
	Renal coloboma syndrome	1
	Renal hypoplasia associated with branchio-oto-renal syndrome	1
	Spina bifida with neurogenic bladder	1
	Nephrocalcinosis	1
Degenerative Nephritic and Nephrotic Conditions (13%)	FSGS (Focal segmental glomerulosclerosis)	3
	Rapidly progressive glomerulonephritis	2
	Nephrotic syndrome (secondary ESRD)	2
	Tubulointerstitial nephritis	2
	Anti-GBM glomerulonephritis	1
Secondary Non-Renal Cause (1%)	Post cardiopulmonary bypass surgery (heart-lung machine operation)	1

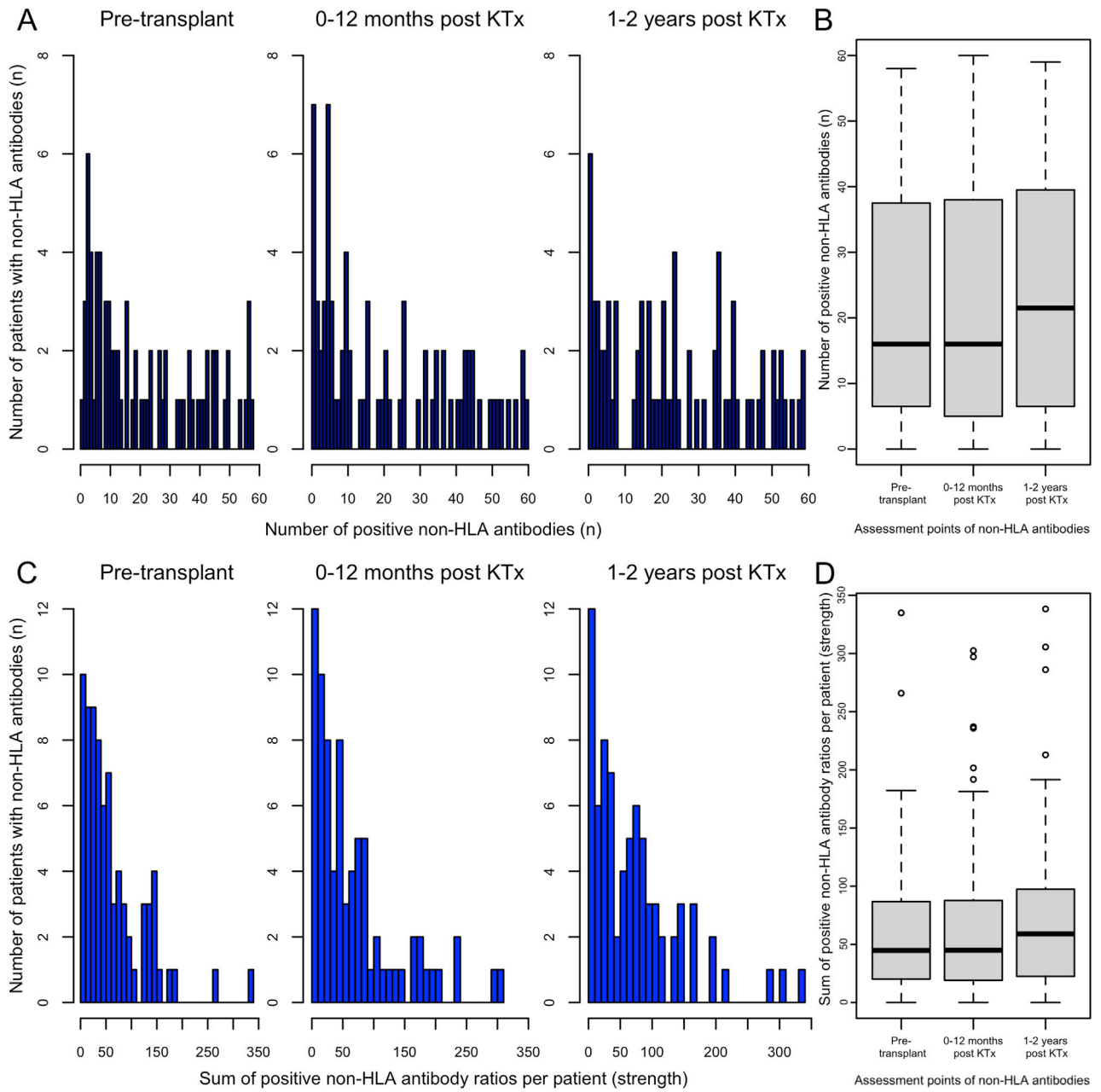


FIGURE 1 | Distribution of patients by the number of positive pre-transplant non-HLA antibodies. **(A)** The broadness of the non-HLA antibody profile, defined as the count of positive non-HLA antibodies per patient, is shown for the following periods: pre-transplant, 1–12 months, and 1–2 years post-transplant (from left to right). **(B)** ANOVA on differences between the broadness of the non-HLA antibodies pretransplant, 1–12 months and 1–2 years post KTx (from left to right). **(C)** The strength, defined as the sum of all non-HLA antibody ratios >1 per patient (total positive ratio), is shown for the following periods: pre-transplant, 1–12 months, and 1–2 years post-transplant (from left to right), bars represent categories constructed in 10-unit increments. **(D)** ANOVA on differences between the cumulative strength of the non-HLA antibodies pretransplant, 1–12 months and 1–2 years post KTx (from left to right).

antibodies at the onset of rejection. Pre-transplant DSA were more frequent in late ABMR (23.5%) than controls (3.7%), and 1–2 years post-transplant, DSA were detected in 47.1% of late ABMR patients versus none in controls ($p < 0.05$). Donor type also differed significantly between groups. No other significant differences were observed for these characteristics between those who developed early, late or both forms of ABMR and the control group, (**Table 1**).

In the control group, indication biopsies were performed in 41 of 54 patients (76%) within 2 years post-transplantation. In cases with multiple histopathological findings, the predominant lesion was used for classification, (**Supplementary Table S1**). In

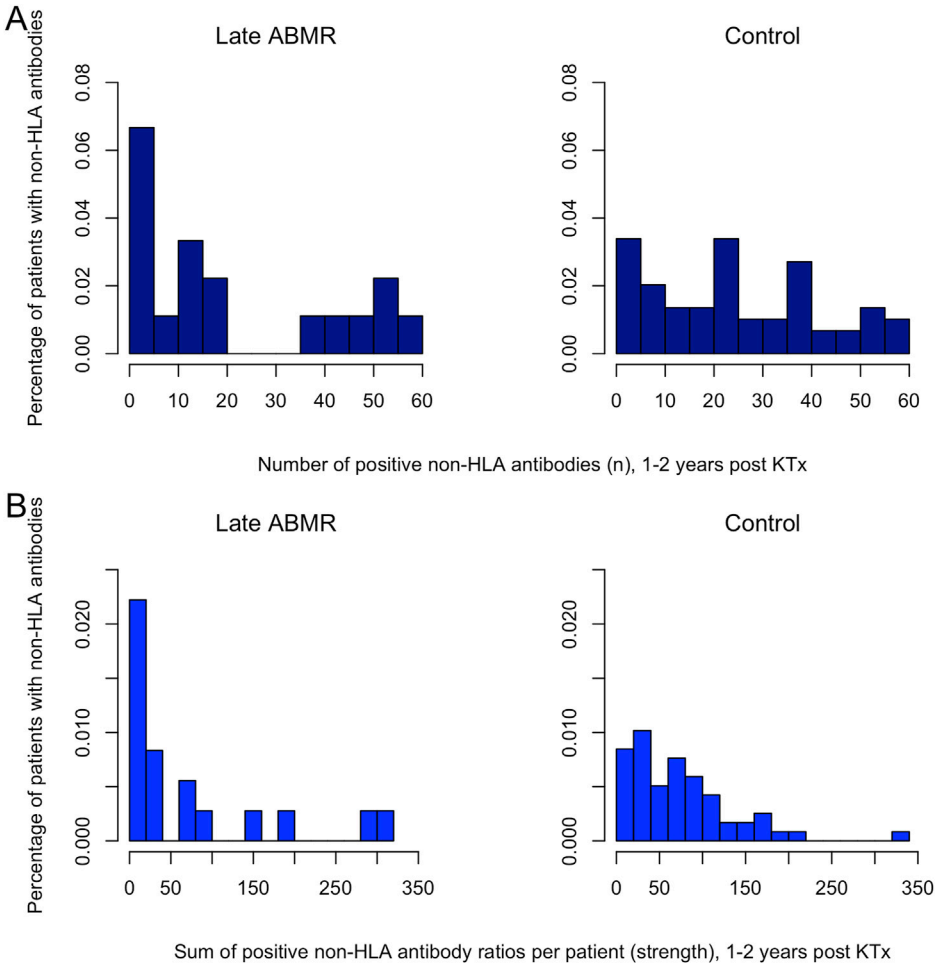


FIGURE 2 | Distribution of the relative frequencies of broadness and strength in the non-HLA antibody profile. **(A)** Distribution of the relative frequencies of broadness in the non-HLA antibody profile. The x-axis displays the number of antibodies for the period 1–2 years post-transplant. The y-axis shows the relative frequency, expressed as the percentage of patients exhibiting the respective number of positive antibodies, bars represent categories constructed in 5-unit increments. **(B)** Distribution of the relative frequencies of strength in the non-HLA antibody profile. The x-axis displays the strength, defined as the sum of all non-HLA antibody ratios >1 per patient for the period 1–2 years post-transplant. The y-axis shows the relative frequency, expressed as the percentage of patients exhibiting the respective strength value, bars represent categories constructed in 20-unit increments.

48.8% of the cases and thus most prominent were findings consistent with chronic allograft dysfunction, including tubular atrophy, interstitial fibrosis, and calcineurin inhibitor (CNI) toxicity. T cell–mediated rejection and borderline changes were observed in 31.7% of cases (n = 8 and n = 5, respectively).

Strength and Broadness of Non-HLA Antibodies

The distribution of positive non-HLA antibodies per patient, reflecting the broadness of non-HLA immunity, is shown in **Figure 1A**; cumulative proportions of all positive non-HLA antibodies per patient, indicating the total strength of the non-HLA antibody response, are shown in **Figure 1B**. Two patients were excluded from analysis due to missing values. Data are presented for three time periods: pre-transplant, up to 1 year post-transplant, and 1–2 years post-transplant. At pre-transplant stage, 53% of patients (40/75) demonstrated more than 15 positive non-HLA antibodies. Although a tendency toward an expanded spectrum of positive non-HLA antibodies was observed between 1 and 2 years post-transplant, this shift did not reach statistical significance. Furthermore, no significant changes were observed in the cumulative strength of positive non-HLA antibodies throughout the post-transplant period.

Within the late ABMR group, one subgroup showed broad and intense antibody responses, while another had only few positive antibodies. In contrast, antibody diversity in controls appeared more evenly distributed, though individual outliers with high antibody intensity were noted in both groups, (**Figure 2**). Pre-transplant levels of individual detectable non-HLA antibodies, stratified by those who underwent dialysis prior to KTx and those receiving a preemptive transplant

TABLE 3 | Tests of association between the event late ABMR and baseline characteristic variables as well as cumulative broadness and cumulative strength measured pre-transplant and 1–2 years post-transplant respectively.

Test of association	Variable	p-value
Chi-squared test of independence	Sex	0.559
Chi-squared test of independence	Donor type	0.067
Welch two sample t-test	Age at KTX	0.577
Chi-squared test of independence	Re-KTX	0.480
Chi-squared test of independence	Dialysis prior to KTX	1
Welch two sample t-test	Cum. broadness pre-transplant	0.225
Welch two sample t-test	Cum. strength pre-transplant	0.331
Welch two sample t-test	Cum. broadness 1–2 years post-transplant	0.427
Welch two sample t-test	Cum. strength 1–2 years post-transplant	0.932

(**Supplementary Figure S1**), revealed no significant impact of dialysis on average broadness of non-HLA antibodies ($p = 0.8$).

Association Between Non-HLA Antibodies and Late Antibody Mediated Rejection

To assess whether variables of non-HLA immunity may serve as potential predictors for the occurrence of late ABMR, we performed multiple logistic regression analyses. Univariable analyses revealed no statistically significant differences in baseline clinical characteristics between the late ABMR and control groups, including sex, age at transplantation, dialysis prior to transplantation, or retransplantation. Donor type (living vs deceased) approached significance ($p = 0.07$) but was excluded from multivariable models due to the absence of living donors in the late ABMR group. Cumulative non-HLA antibody measures (broadness and strength) showed no significant association with late ABMR either pre- or 1–2 years post-transplant, (**Table 3**). Multiple logistic regression based on the same variables confirmed these findings. Two models were conducted: one with pre-transplant cumulative variables and baseline characteristics, and another using the same variables 1–2 years post-transplant, (**Table 4**). After model reduction by retaining only variables from the initial models with p-values ≤0.17, only cumulative broadness at 1–2 years post-transplant approached significance ($p = 0.09$), showing a negative association, (**Supplementary Table S3**). Due to the absence of HLA-DSA positive individuals in the control group at the 1–2 years post-transplant interval, associations between DSA status and late ABMR could not be assessed using regression analysis.

Individual non-HLA variables were analyzed to evaluate their potential association with late ABMR. To address model overfitting and estimation instability due to the high number of predictors (60 non-HLA antibodies) and limited sample size ($n = 75$), a multiple logistic regression with Lasso regularization was applied. Cross-validation was used to determine the regularization parameter. This analysis was performed separately for non-HLA antibodies assessed at the pre-transplant and 1–2 years post-transplant timepoints. For pre-transplant non-HLA profiles, Lasso regression excluded 51 of 60 predictors. Backward selection using the Akaike Information Criterion (AIC) identified a reduced model including three non-HLA antibodies with potential relevance for late ABMR: SNRPB2 (positive coefficient, $p = 0.03$), ARGN (negative, $p = 0.08$), and ARHGDIB (negative, $p = 0.09$), the latter two showing significance only at the 10 percent level. For post-transplant profiles, 52 of 60 predictors were excluded by Lasso regression. AIC-based backward selection retained five non-HLA antibodies. Of those, CGB5 (negative, $p = 0.02$), ACTIN (positive, $p = 0.05$), and COLLAGEN V (negative, $p = 0.08$) showed potential relevance (**Table 5**). Complete Lasso results for both timepoints are provided in **Supplementary Table S4**.

Development of Non-HLA Antibodies Over Time

Pearson correlations of the same non-HLA antibodies pre- and 1–2-year post-transplant revealed strong correlations ($r > 0.8$, $p < 0.05$) for 3 antibodies in the ABMR DSA-positive group, 9 in the DSA-negative group, but none in controls. Moderate correlations ($r = 0.4–0.8$, $p < 0.05$) were found for 11 antibodies in the ABMR DSA-positive, 8 in the DSA-negative, and 26 in the control group (**Supplementary Table S5**). At 1–2 years post-transplant, correlations between pairs of different non-HLA antibodies revealed strong correlations ($r > 0.8$, $p < 0.05$) in 825 pairs (46.6%) in the late ABMR DSA-positive group, 720 pairs (40.7%) in the DSA-negative group, compared to 33 pairs (1.9%) in controls, **Supplementary Figures S2-S4**. Dataset analysis showed that none of the non-HLA antibodies included in the panel were completely absent in all pre-transplant samples. Analysis of the trajectory of the non-HLA antibody profile indicated distinct antibody dynamics between ABMR and controls. In the late ABMR cohort, particularly those with concomitant HLA-DSA, non-HLA antibody levels increased post-transplant and declined in at the onset of rejection. The control cohort showed a more stable profile, characterized by less fluctuation, (**Figure 3**), apart from Interleukin-21 (IL-21), increasing gradually and reaching the highest mean ratio in the control group (mean: 7.44), compared to lower levels in the ABMR DSA-positive (2.44) and DSA-negative (2.34) groups, ($p < 0.05$). This difference was primarily driven by five outliers in the control group, (**Supplementary Figures S5, S6**). IL-21 was not among the most prominent antibodies in late ABMR cohorts.

TABLE 4 | Multivariate logistic regression analyses for estimating the relationships between different patient factors and the event late ABMR, model a) uses pre-transplant, model b) 1–2-year post-transplant antibody broadness and strength.

Model a)	Reference	Estimate	SE	Statistic	p-value
Intercept		–1.125	0.86	–1.30	0.19
Sex (female)	(Male)	0.982	0.68	1.45	0.15
Age at KTX		0.001	0.06	0.02	0.98
Re-KTX (yes)	(No)	0.836	0.75	1.12	0.26
Dialysis prior to KTX (yes)	(No)	–0.192	0.61	–0.31	0.75
Cumulative broadness		–0.027	0.04	–0.74	0.46
Cumulative strength		–0.001	0.01	–0.06	0.95
Model b)	**Reference**	**Estimate**	**SE**	**Statistic**	**p-value**
Intercept		–0.748	0.93	–0.80	0.42
Sex (Female)	(Male)	0.684	0.66	1.04	0.30
Age at NTX		–0.003	0.06	–0.06	0.95
ReKTX (Yes)	(No)	0.595	0.69	0.86	0.39
Dialysis before NTX (Yes)	(No)	–0.417	0.60	–0.69	0.49
Broadness		–0.058	0.04	–1.65	0.10
Total strength		0.011	0.01	1.41	0.16

TABLE 5 | Multiple logistic regression model with Lasso regularization after backward model selection using the Akaike Information Criterion (AIC) was performed.a) uses pre-transplant, model b) 1–2-year post-transplant antibody broadness and strength.

Model a)	Estimate	SE	Statistik	p-value
Intercept	–1.001	0.432	–2.318	0.020
AGRN	–1.438	0.833	–1.726	0.084
ARHGDIB	–0.828	0.482	–1.717	0.086
CXCL9	0.121	0.088	1.373	0.170
SNRPB2	0.980	0.452	2.167	0.030
Model b)	**Estimate**	**SE**	**Statistik**	**p-value**
Intercept	–0.167	0.521	–0.320	0.749
ACTIN	0.979	0.501	1.955	0.051
ARHGDIB	–0.343	0.257	–1.332	0.183
CGB5	–2.589	1.117	–2.318	0.020
COLLAGEN V	–1.964	1.114	–1.762	0.078
IFNG	0.427	0.449	0.950	0.342

Given the low prevalence of DSA in the control group pretransplant (2/54) and post-transplant (0/54), further stratification by DSA status was not pursued in the control cohort.

The five non-HLA antibodies with the strongest increase over time were identified separately for each study group, (**Figure 4**). Between pre-transplant phase and 1–2 years post-transplant, IFNG and ROR1 showed the highest mean increase between in the ABMR DSA-negative group. CXCL9 showed a similar temporal pattern in the ABMR DSA-positive group with significant differences to the other cohorts. However, after exclusion of one outlier with markedly elevated CXCL9 levels, the difference between groups was no longer statistically significant. From the pre-transplant phase to the time of rejection CXCL11 showed the most distinct upward trend in the DSA-positive ABMR group, without showing significance.

DISCUSSION

To our knowledge, this is the first explorative study assessing a comprehensive range of non-HLA antibodies in pediatric KTx recipients encompassing both pre- and post-transplantation periods. We demonstrated that 53% of pediatric patients with CKD stages 5–5D, listed for KTx, showed non-HLA autoantibodies prior to transplantation. This indicates a substantial degree of non-HLA autoantibody immunity even prior to KTx, which did not significantly change over time. Despite this, no statistically significant association between the presence of non-HLA antibodies and the development of ABMR was observed during a median follow-up of 4.83 years (IQR = 3.08–6.96).

Vascular damage, particularly in the kidneys themselves, caused by chronic kidney injury and its systemic consequences, appears to be a major factor in the pre-transplant development of non-HLA antibodies, functioning as alloantibodies. Notably, dialysis prior to transplantation did not exert a significant impact on the burden of non-HLA antibodies. This may be at least partly explained by the finding that advanced cardiovascular damage, such as vascular calcifications, is predominantly observed in adolescents and young adults within the pediatric population undergoing dialysis [41]. The observation that none of the tested non-HLA antibodies were consistently absent in all pre-transplant samples suggests that they do not represent purely alloimmune responses. Contrary to studies in adult KTx patients [42], our pediatric cohort did not show a decrease in non-HLA antibody levels post-transplant despite immunosuppressive therapy. Moreover, the highest proportion of at least moderate correlations between pre- and post-transplant non-HLA antibody levels was observed in controls (43%), suggesting a sustained antibody profile independent of ABMR development.

Several studies have indicated the potential involvement of non-HLA antibodies in the pathogenesis of ABMR in adult KTx

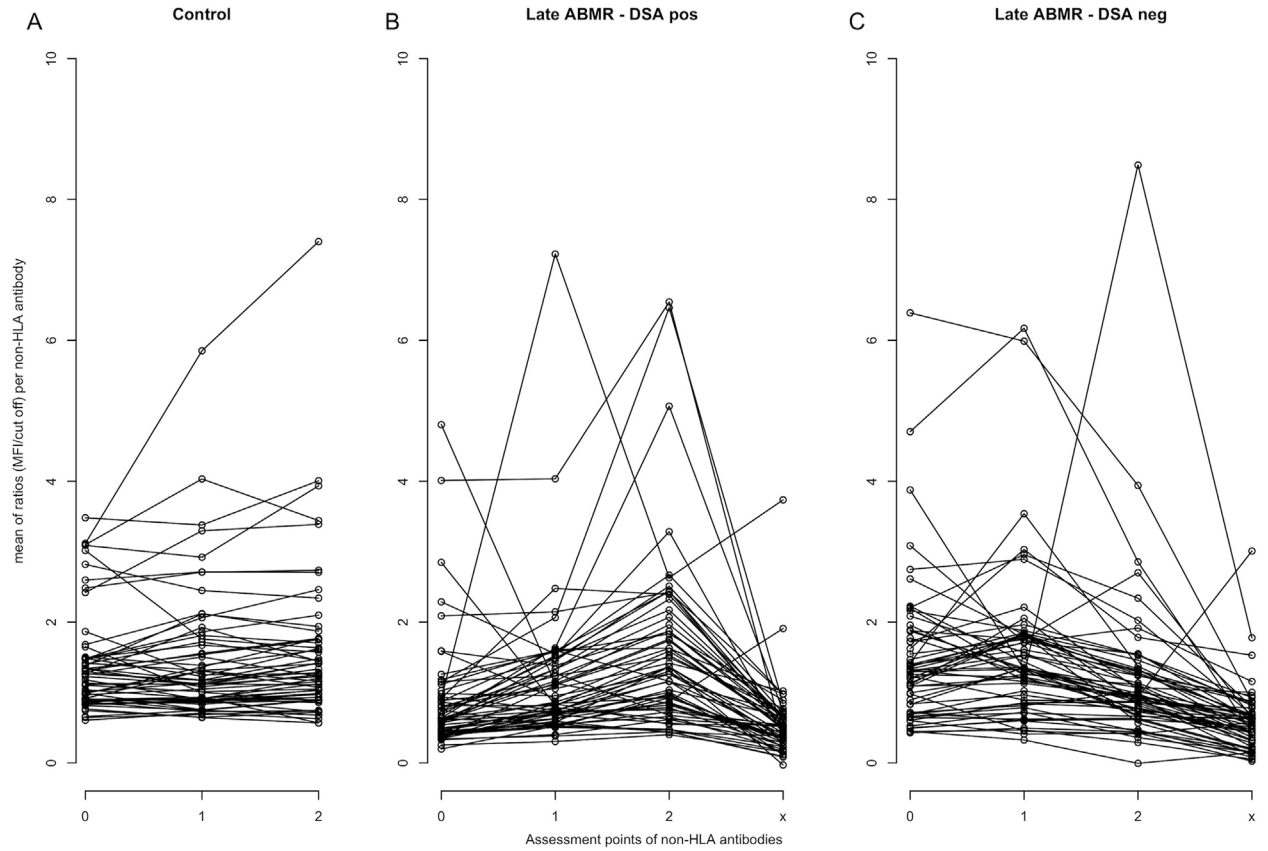


FIGURE 3 | Trajectory of individual non-HLA antibody profiles over time. Each line represents the mean MFI-to-cut-off ratio over all patients for a specific non-HLA antibody. Antibody trajectories are shown separately for the control cohort **(A)**, late ABMR DSA-positive **(B)**, and late ABMR DSA-negative **(C)**. Assessment points on the x-axis represent: 0 = pre-transplantation, 1 = 1–12 months post-transplantation, 2 = 1–2 years post-transplantation, and x = time of rejection diagnosis. The y-axis indicates the mean MFI-to-cut-off ratio.

patients [16, 17, 43]. However, there are currently no established diagnostic protocols for routine testing of non-HLA antibodies in KTx patients, thus precluding a conclusive assessment of their individual clinical relevance and their overall burden when screening for ABMR.

In this present study, the aggregated non-HLA antibody burden, defined by broadness and strength, showed no predictive value for the development of late ABMR. A negative association between post-transplant antibody broadness and ABMR risk was observed, yet the result narrowly failed to reach statistical significance ($p = 0.08$), highlighting limitations of relying on cumulative measures in capturing immune dynamics associated with ABMR. This prompted a focused analysis of individual non-HLA antibodies, assessed both pre- and post-transplant, considering the already elevated pre-transplant antibody burden.

Pre-transplant antibodies against SNRPB2 were significantly positively associated with late ABMR, prior only associated with the recurrence of focal segmental glomerulosclerosis in kidney transplants [44]. Conversely, antibodies against ARGN and ARHGDIB showed negative marginal associations with ABMR occurence, ($p < 0.1$), although prior studies linked these antibodies to graft dysfunction and transplant glomerulopathy, warranting cautious interpretation [45, 46]. Post-transplant, antibodies against ACTIN were significantly positively associated with late ABMR. While a direct link between anti-ACTIN antibodies and graft loss has not yet been established, related cytoskeletal mechanisms have been implicated in chronic ABMR pathogenesis. Notably, increased phosphorylation of actin-associated proteins such as ARPC2 has been observed in peripheral blood mononuclear cells from patients with chronic ABMR, suggesting possible cytoskeletal dysregulation in immune cells [47, 48]. Although anti-CGB5 antibodies have previously been associated with post-transplant recurrence of FSGS(44), our findings indicate a negative association with late ABMR, leaving their broader role in transplant immunology to be further clarified.

Due to the high pre-transplant non-HLA antibody burden and to overcome the limitations of analyzing single time-points, a trajectory-based analysis was applied to capture non-HLA antibody dynamics. They revealed distinct temporal patterns between patients with and without late ABMR. In

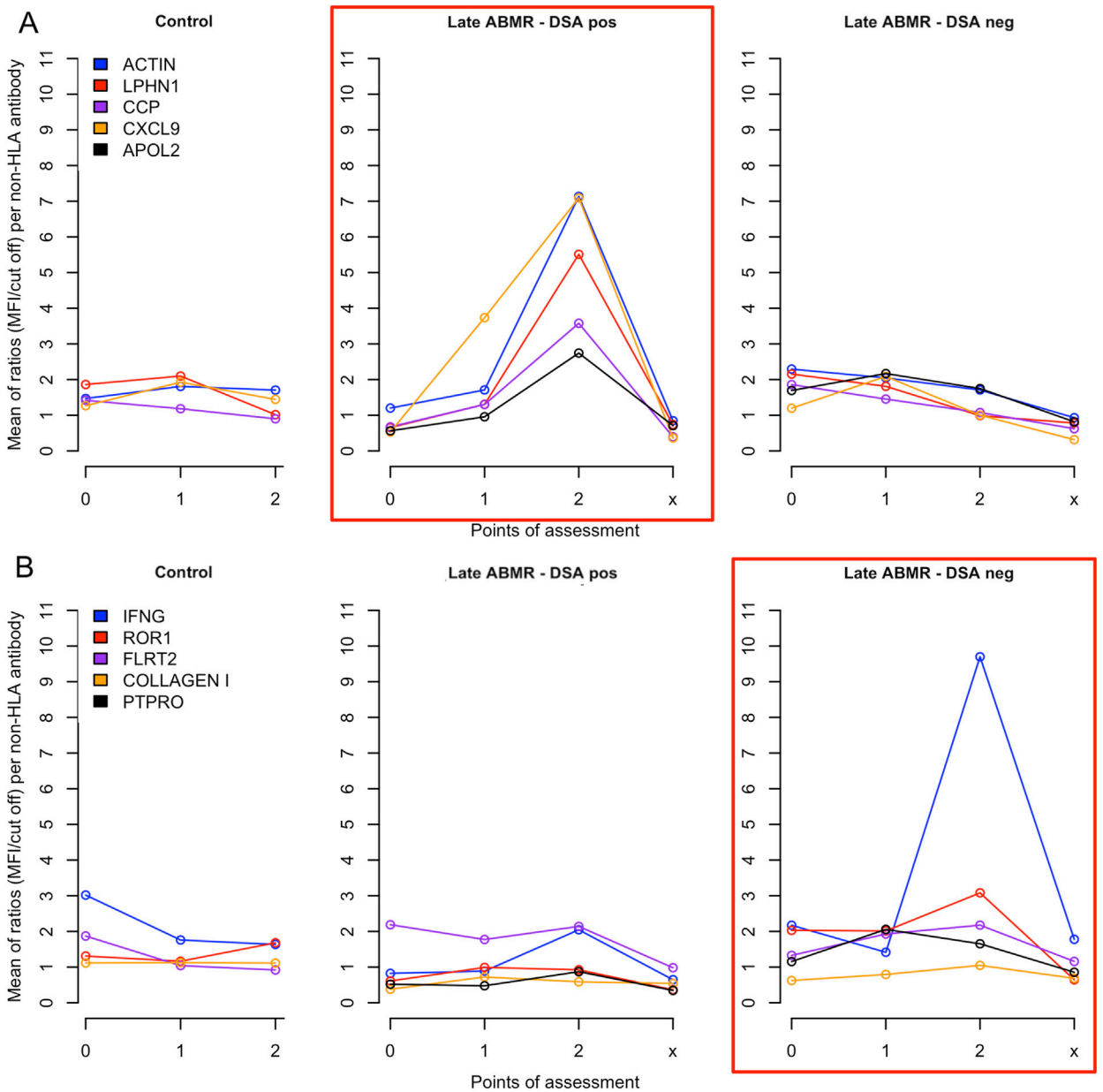


FIGURE 4 | Temporal evolution of the five non-HLA antibodies showing the strongest relative increase in patients with late ABMR. **(A)** Five non-HLA antibodies showing the strongest relative increase in patients with late ABMR -DSA-positive at the time of rejection. The central panel (highlighted in red) shows the course of the levels of antibodies against ACTIN, LPHN1, CCP, CXCL9, and APOL2 in the group late ABMR DSA-positive (Late ABMR–DSA pos). For comparison, the same antibodies are displayed in the control (left panel) and DSA-negative ABMR group (right panel, Late ABMR-DSA neg). **(B)** Five non-HLA antibodies showing the strongest relative increase in patients with late ABMR–DSA-negative at the time of rejection. The right panel (highlighted in red) shows the course of the levels of antibodies against IFNG, ROR1, FLTR2, COLLAGEN I, PTPRO in the group late ABMR DSA-negative (Late ABMR–DSA neg). For comparison, the same antibodies are displayed in the control (left panel) and DSA-positive ABMR group (central panel, Late ABMR–DSA pos). For both assessments the x-axis represents antibody measurement time points: 0 = pre-transplantation, 1 = 1–12 months post-transplantation, 2 = 1–2 years post-transplantation, and x = time of rejection diagnosis (not for the control cohort). The y-axis indicates the mean MFI-to-cut-off ratio.

ABMR cohorts, particularly those with HLA-DSA, an initial post-transplant increase in parts of the non-HLA repertoire suggested subclinical alloimmune priming. This was followed by a marked decline at the time of rejection, coinciding with manifest histopathological injury. The observed drop in circulating non-HLA antibodies may reflect compartmentalization of immune mediators into the graft, potentially accumulating at the site of rejection. Concurrent assessment of these mediators and their antibodies in graft tissue or urine could offer further insight into their spatial

dynamics and potential pathogenic roles. Conversely, controls exhibited more stable antibody levels, except for a few distinct outliers, seen in a marked post-transplant increase in anti-IL-21 antibodies, predominantly driven by five strong outliers. IL-21 has been implicated in pro-inflammatory pathways in transplantation [49–51]. The observed anti–IL-21 pattern with extreme levels in single patients could reflect an adaptive response to elevated IL-21 levels—potentially indicating subclinical alloimmune activity—or a modulatory mechanism interfering with IL-21–mediated signaling.

The five most prominently increasing non-HLA antibodies varied between ABMR subgroups, suggesting that HLA-DSA status may influence distinct trajectories of antibody development and reflect divergent underlying immune activation pathways. In DSA-negative ABMR, anti-IFNG and anti-ROR1 antibodies exhibited the strongest increases, whereas anti-ACTIN and anti-CXCL9 antibodies rose most prominently in the DSA-positive group. Despite the within-group increases, intergroup differences were not statistically significant, partly due to individual outliers. Anti-CXCL9 showed a transiently significant association, primarily driven by a single DSA-positive case with markedly elevated levels, thus precluding definitive conclusions regarding its predictive relevance, underscoring the need for validation in larger, balanced cohorts.

An objective of this study was to determine whether broad-spectrum screening could identify clinically relevant non-HLA antibodies in pediatric kidney transplant recipients, independent of confounding factors. The predominance of CAKUT limited the detection of disease-specific antibody patterns. Moreover, post-transplant biopsies in controls revealed diverse histopathologies. Modifications in immunosuppression due to CNI toxicity or viral infections may have further contributed to interindividual variability. While our data suggest that HLA-DSA status may influence the evolution of non-HLA antibody responses as reflected in varying correlation patterns, no consistent differences in the most increasing non-HLA antibodies between DSA-positive and DSA-negative patients were observed.

A strength of our study is the analysis of both pre- and post-transplant non-HLA antibody immunity. This provided new insights into the impact of alloimmunity and autoimmunity on the development of the tested non-HLA antibody profile in pediatric patients. The high pre-transplant antibody burden observed underscores the relevance of autoimmune mechanisms in the development of non-HLA antibodies.

Our study is limited by several factors, most notably the retrospective study design, the relatively small number of patients exhibiting ABMR and the sensitivity of the analysis to individual outliers. The latter was partially addressed through targeted outlier analyses and potential impacts on results were reported. The etiology of ABMR is multifactorial, which presents a challenge in identifying individual risk parameters for developing an ABMR. Although patient age was not identified as an independent risk factor in the present group, future studies should also account for factors such as adherence to immunosuppressive therapy, particularly in adolescents.

Large-scale studies are necessary to determine the prognostic relevance of specific non-HLA antibodies in pediatric renal transplant patients. Given the high prevalence of non-HLA antibodies already prior to transplantation, more studies on non-HLA alloimmunity in renal-healthy children and those with renal insufficiency should be conducted. This would forward more detailed information regarding specific alterations in the antibody profile under investigation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving humans were approved by MHH Ethikkommite, Hannover Medical School. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

AUTHOR CONTRIBUTIONS

Conception of the study was done by NK and FS. FS, NK, DH, JD, LB, and AM oversaw the clinical aspects of the study or contributed clinical information. MV and MH conducted the detection of non-HLA Antibodies, and were responsible for the acquisition and processing of the biochemical data. Data analysis and interpretation were done by FS and AB. FS, NK, and DH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2025.14463/full#supplementary-material

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ORIGINAL RESEARCH
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Expanding the Scope of Microvascular Inflammation: Unveiling Its Presence Beyond Antibody-Mediated Rejection Into T-Cell Mediated Contexts

Hilal Varol[1,2†], Anne Wagenmakers[2†], Konrad Hoeft[3,4], Jasper Callemeyn[5,6], Roos Bodewes[2], Wichor Bramer[7], Andrew Stubbs[2,8], Rafael Kramann[3,4], Maarten Naesens[5,6] and Marian C. Clahsen-Van Groningen[1,2,3*]*

[1]Department of Pathology, Erasmus Medical Center Rotterdam, Rotterdam, Netherlands, [2]Erasmus MC Transplant Institute, University Medical Center Rotterdam, Rotterdam, Netherlands, [3]Division of Nephrology and Clinical Immunology, RWTH Aachen University Hospital, Aachen, Germany, [4]Department of Internal Medicine, Division of Nephrology and Transplantation, Erasmus University Medical Center Rotterdam, Rotterdam, Netherlands, [5]Division of Nephrology, University Hospitals Leuven, Leuven, Belgium, [6]Department of Microbiology, Immunology and Transplantation, Nephrology and Renal Transplantation Research Group, KU Leuven, Leuven, Belgium, [7]Medical Library, Erasmus MC, Erasmus University Medical Centre Rotterdam, Rotterdam, Netherlands, [8]Department of Pathology, Clinical Bioinformatics Unit, Erasmus MC, University Medical Center Rotterdam, Rotterdam, Netherlands

Microvascular inflammation (MVI) in kidney transplant biopsies is mainly associated with antibody-mediated rejection (AMR), sparking debate within the Banff Classification of Renal Allograft Pathology regarding its exclusivity. This study reviewed the literature on MVI in T cell-mediated rejection (TCMR) and analyzed MVI in our transplant population. We searched English publications in MEDLINE, Embase, Web of Science, Cochrane, and Google Scholar until June 2024, focusing on glomerulitis (g), peritubular capillaritis (ptc), or MVI in kidney transplant biopsies classified as TCMR. Additionally, we examined g, ptc, and MVI in 69 patients with AMR, TCMR, and no rejection. Our search yielded 541 citations, with 10 studies included, covering 810 TCMR and 156 AMR biopsies. The studies showed g, ptc, and MVI were present in TCMR but were less prevalent and severe than in AMR. In our cohort, AMR had significantly higher g, ptc, and MVI scores compared to aTCMR and ATN, however, aTCMR also displayed MVI. These findings confirm that MVI occurs in aTCMR and should not be exclusively linked to AMR. These findings highlight the need to further explore MVI's significance in TCMR and investigate the inflammatory composition. This could refine the Banff Classification, improving Classification accuracy of kidney transplant pathology assessments.

Keywords: kideny transplantation, banff classification, MVI, histology, TCMR

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***Correspondence**
Hilal Varol,
h.varol@erasmusmc.nl
Marian C. Clahsen-Van Groningen,
m.clahsen-vangroningen@erasmusmc.nl

†These authors have contributed equally to this work and share first authorship

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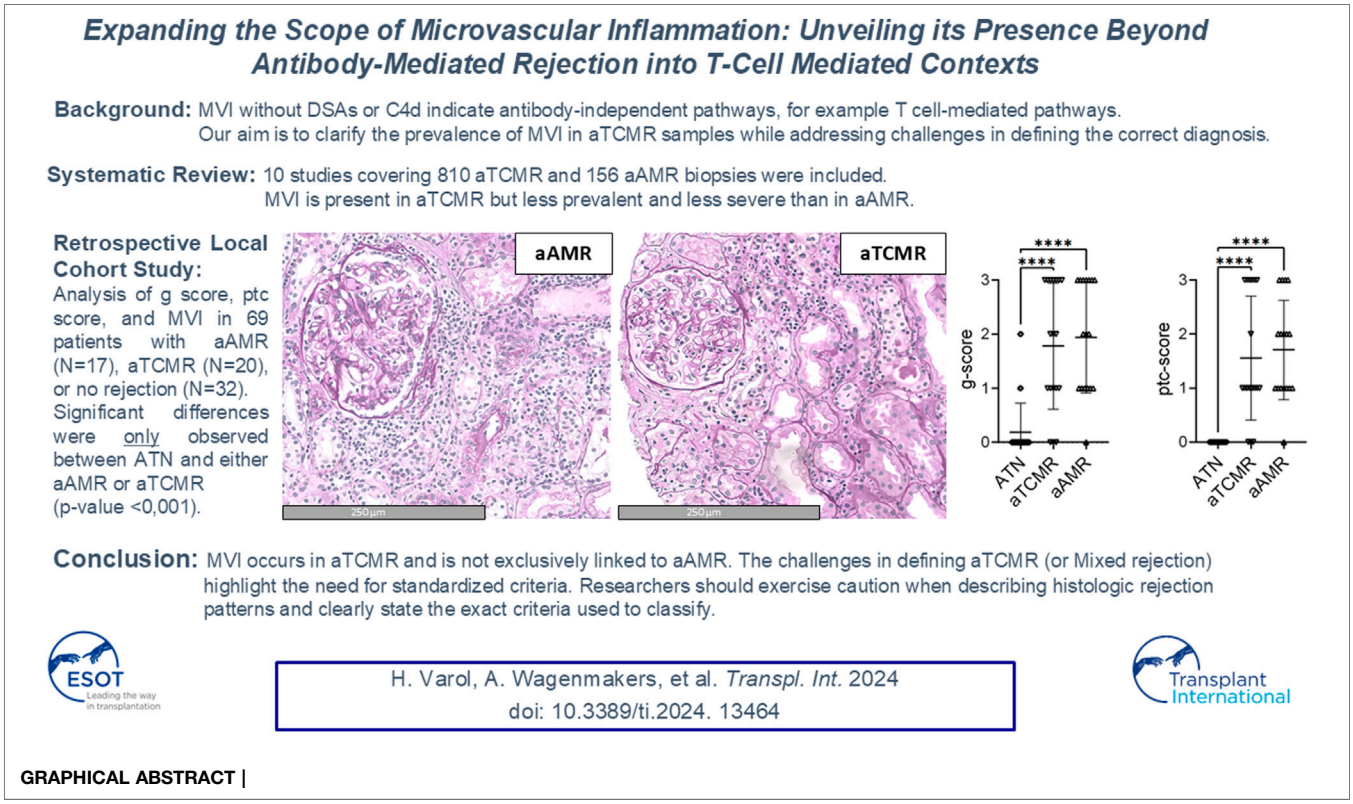
Abbreviations: AMR, antibody-mediated rejection; aAMR, active AMR; DSA, donor-specific antibodies; DSAST, DSA selective transcripts; ENDAT, endothelial-associated transcripts; g, glomerulitis; IFNγ, interferon-γ; MHC, major histocompatibility complex; MVI, microvascular inflammation; ptc, peritubular capillaritis; TCMR, T cell-mediated rejection; aTCMR, acute TCMR.



GRAPHICAL ABSTRACT |

INTRODUCTION

Since its inception in 1991, "the Banff Classification of Renal Allograft Pathology" provides diagnostic criteria for interpreting renal allograft pathology, evolving through biannual updates from experts [1]. It categorizes acute inflammatory lesions into specific clusters such as tubulitis (t), interstitial inflammation (i), glomerulitis (g) and peritubular capillaritis (ptc). The 2019 Banff update focused on histological evidence, primarily microvascular inflammation (MVI), and includes the presence of donor-specific antibodies (DSAs) and C4d deposition in peritubular capillaries as diagnostic criteria for antibody-mediated rejection (AMR). However, despite the comprehensiveness of the Banff Classification, it presents a challenge, as a considerable number of biopsies exhibit a high MVI-score ([g + ptc] ≥ 2) without detectable DSAs or C4d. The clinical implications of such findings are unclear [2]. The Banff Classification solely includes MVI in the category of AMR and does not provide a specific evaluation framework for MVI in other categories. Recently, there have been discussions about whether dichotomization of a complex histological image and thereby assigning MVI to AMR is valid. The 2022 Banff update addresses these complexities by identifying two new phenotypes of MVI and providing criteria for DSA- and C4d-negative cases, potentially involving various factors such as alloreactive T cell-mediated responses, non-HLA antibodies, primary NK cell activation through missing self, viral infection, other mechanisms of innate immune activation, and ischemia-reperfusion injury [3, 4]. In this report, we will conduct a systematic review investigating the literature's perspective on MVI within the context of TCMR. Additionally, we will investigate the relationship between MVI and aTCMR and active AMR (aAMR) in our transplant population. We investigate the prevalence of MVI in cases classified as acute rejection as defined by the Banff Classification and to address the challenges in defining the correct diagnostic category.

MATERIALS AND METHODS

Systematic Review

A systematic review of the literature was performed in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [5].

Study Eligibility

Patients and Biopsies

With a focus on MVI within TCMR cases lacking an antibody-mediated component, our inclusion criteria comprised studies that examined kidney transplant biopsies with TCMR. Citations exclusively investigating AMR or Mixed Rejection without a clear description of the diagnostic process were excluded from consideration. Also, articles solely focusing on cases classified as suspicious (borderline) for acute TCMR were excluded due to the heterogeneous diagnostic grouping and the difference in Banff scoring of this category over time.

Index Test

Studies reporting the MVI score in TCMR transplant biopsies were eligible for inclusion. Studies only reporting g and/or ptc in TCMR allograft samples were also eligible for inclusion. Citations that excluded TCMR samples with g and/or ptc were excluded for this systematic review, as that the Banff Classification never explicitly mentions excluding the diagnosis of TCMR if MVI is present. We did not impose a minimum sample size (TCMR) in our selection as our hypothesis is that little is published on MVI in TCMR.

Comparators

Studies with lesion scores (specifically g, ptc or MVI) in TCMR samples compared to samples with other rejection patterns were considered for inclusion. Studies that mentioned lesion scores in different TCMR samples were also considered for inclusion. Studies that focused on chronic damage and not specifically on inflammation were excluded from further analysis.

Outcomes

Studies reporting individual lesion scores and/or MVI in the results section or **Supplementary Material** were included.

Study Design

English case-series, cross-sectional studies, cohort studies and controlled trials focusing on g, ptc or MVI in TCMR allograft biopsies in KTx were eligible for inclusion in the systematic review. We excluded manuscripts reporting non-original data.

Information Sources and Search Strategy

We conducted a comprehensive literature search (**Supplementary Material**), regardless of language or publication status. An experienced information specialist (WB) developed database-specific search strategies for each of the following electronic databases (up to June 24, 2024): MEDLINE, EMBASE, Web of Science Core Collection, the Cochrane Central Register of Controlled Trials, and Google Scholar. The electronic search focused on MVI, allograft rejection, or failure. The electronic database searches were supplemented by manual scanning of the reference lists of relevant articles and reviews.

Study Selection and Data Collection

The electronic database search yielded citations that we downloaded into Endnotes reference manager for screening. The selection process for eligible studies involved two stages: firstly, screening of titles and/or abstracts based on pre-established eligibility criteria, and secondly, conducting full-text evaluations of citations that were not excluded in the initial step by applying the same criteria. Relevant information from electronic database searches and potentially relevant full-text articles were screened independently by two investigators (AW, RB). Any disagreements were resolved through consensus and, if required, a third reviewer (HV) was involved.

Data Items

After selecting relevant papers, two investigators (AW, HV) extracted the following data by reading the articles thoroughly: study design, sample size per rejection type (TCMR or AMR), sample size per biopsy type (indication or protocol) and Banff Classification used for diagnosis. Data on g, ptc and/or MVI were collected, as well as C4d and anti HLA-DSA status. Each article was evaluated on the method and details pertaining to the calculation of MVI. The quantitative representation of the g, ptc, MVI, C4d and non HLA-DSA status were assessed and evaluated whether they are comparable between the articles. Cases with BK nephropathy or (recurrence of) primary glomerulopathy were excluded from further analysis.

Retrospective Cohort Study

Sample Collection

A total of 69 for-cause kidney transplant biopsies diagnosed as aAMR (n = 32), aTCMR (n = 20), and acute tubular necrosis (ATN) (n = 17), obtained between 2009 and 2019 previously included in a study investigating transcriptomics were retrieved from the archives of the ErasmusMC, Rotterdam, the Netherlands (MEC-2019-0307 [6]). All biopsies were re-evaluated according to the Banff 22 Update [2], focusing on Banff lesion scores g and ptc. Cases with aTCMR were excluded if they showed concurrent DSAs and/or C4d positivity. To support the robustness of our findings, we refer to previous transcriptional analysis of the samples as previously described [6]. These analyses found no distinct subgroups within the aTCMR group denoting an AMR transcription al profile, highlighting the uniformity of sample characteristics and reinforcing the integrity of our comparative assessments.

Statistical Analysis

Statistical analysis was performed using SPSS software version 25 (IBM Corp., 2017). To assess whether there are significant differences in the Banff lesion scores for g, ptc and MVI between aAMR, aTCMR, and ATN, the Kruskal-Wallis test will be used. Post-hoc pairwise comparisons will be conducted using Dunn's test with Bonferroni correction to identify specific group differences if the Kruskal-Wallis test yields a significant result. Statistical significance will be set at $p < 0.05$.

RESULTS

The origin and timeline of MVI in renal allograft pathology according to the Banff Classification is depicted in **Figure 1**, with the most recent edition from 2022. **Supplementary Table S1** provides a more detailed description of the key updates in the Banff Classification over the years, focusing on g-lesion score, ptc-lesion score, and MVI-score.

Systematic Review

Our search retrieved 616 citations after removing duplicates (**Table 1**). Of the 96 potentially relevant papers, eight studies met our criteria (**Figure 2**). After reviewing these eight articles, we found two additional studies meeting our criteria, which were not

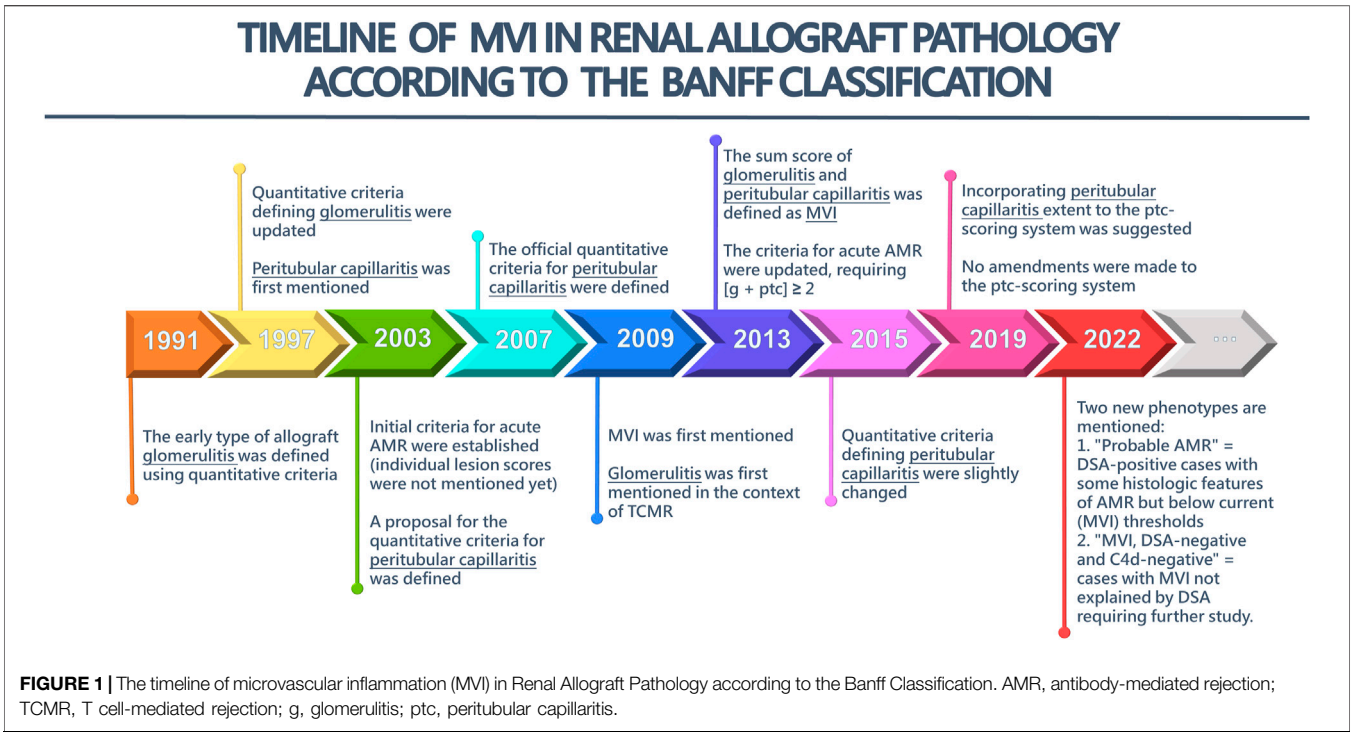


FIGURE 1 | The timeline of microvascular inflammation (MVI) in Renal Allograft Pathology according to the Banff Classification. AMR, antibody-mediated rejection; TCMR, T cell-mediated rejection; g, glomerulitis; ptc, peritubular capillaritis.

TABLE 1 | Systematic review search strategy.

Database searched	Platform	Years of coverage	Records	Records after duplicates removed
Medline ALL	Ovid	1946 - June 2024	242	241
Embase	Embase.com	1971 - June 2024	405	188
Web of Science Core Collection[a]	Web of Knowledge	1975 - June 2024	345	113
Cochrane Central Register of Controlled Trials[b]	Wiley	1992 - June 2024	18	11
Additional Search Engines: Google Scholar[c]			100	63
Total			1,110	616

[a]*Science Citation Index Expanded (1975-present); Social Sciences Citation Index (1975-present); Arts and Humanities Citation Index (1975-present); Conference Proceedings Citation Index- Science (1990-present); Conference Proceedings Citation Index- Social Science & Humanities (1990-present); Emerging Sources Citation Index (2005-present).*
[b]*Manually deleted abstracts from trial registries.*
[c]*Google Scholar was searched via "Publish or Perish" to download the results in EndNote.*
No other database limits were used than those specified in the search strategies.

part of the initial 541 citations. Eight studies reported individual lesion scores (g and/or ptc) in renal transplant biopsies, and two studies reported MVI scores. Four studies reported C4d and anti-HLA DSA status. The excluded articles solely focused on mixed rejection or suspicious (borderline) for acute TCMR. Mixed rejection articles were omitted due to the presence of an AMR component, while articles on suspicious TCMR were excluded due to inconsistent definitions within this diagnostic group. A total of 2119 biopsies were included, of which 810 were renal allograft biopsies with TCMR. Among them, 1785 were indication biopsies, 577 were surveillance biopsies, and 46 were preimplantation biopsies. Additionally, the included articles covered a variety of rejection patterns besides TCMR. Furthermore, the included articles included samples with a broad range of different rejection patterns besides TCMR.

Clinical and Methodological Heterogeneity Among studies

Table 2 outlines the study characteristics. Methodological variations were noted across the included articles, particularly in their use of different editions of the Banff Classification. Despite this, there was consistency in the criteria for g and ptc lesion scores and MVI scores. However, the quantitative representation of the lesion scores varied. Comparing MVI scores between articles and different rejection patterns posed challenges due to the absence of a defined framework beyond AMR diagnostic criteria. According to the 2019 Banff Classification, AMR requires at least (g + ptc) ≥ 2. However, in the presence of acute TCMR, borderline infiltrate or infection, ptc ≥2 alone is insufficient, and g must be ≥1. Additionally, it was unclear whether AMR cases also included a TCMR component.

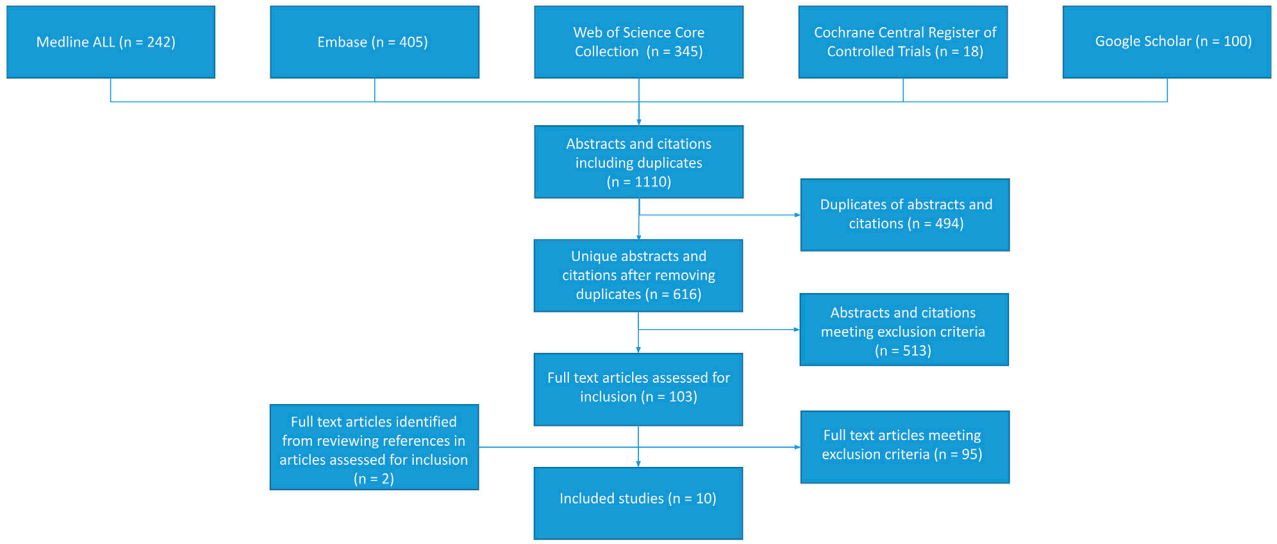


FIGURE 2 | Summary of study inclusion and exclusion process.

TABLE 2 | Heterogeneity among studies in sample size and used Banff Classification.

Author, year	Setting	Design	Total biopsies – TCMR biopsies	Indication biopsies – Protocol biopsies	Banff classification used for diagnosis
Bouatou et al. [7]	Assistance Publique - Hôpitaux de Paris, Paris, France	Cohort study	256–256	256–0	2017
Jung et al. [8]	Kyungpook National University School of Medicine, Daegu, Korea	Cohort study	106–6	24–82	2013
Zhao et al. [9]	Beijing Friendship Hospital, Capital Medical University, Beijing, China	Cohort study	42–18	42–0	2013
Sis et al. [10]	University of Alberta, Edmonton, Alberta, Canada	Cohort study	329–44	329–0	2009
Lee et al. [11]	Kyung Hee University, Seoul, South Korea	Cohort study	203–46	169–34	2007
Park et al. [12]	Keimyung University Kidney Institute, Daegu, Korea	Cohort study	139–48	139–0	Unknown (biopsies from 2006 till 2018)
Gibson et al. [13].[a]	MS4 Health Sciences Centre, Winnipeg, Manitoba, Canada	Cohort study	688–76	181–461	2003
Gupta et al. [14]	Albert Einstein College of Medicine, Bronx, New York, USA	Cohort study	356–27	356–0	2013
Kozakowski et al. [15]	Medical University of Vienna, Vienna, Austria	Cohort study	1,322–224	1,322–0	2005, the composition of ptc was assessed in a different quantitative manner.[b]
Batal et al. [16]	University of Pittsburgh Medical Center, Pittsburgh, PA	Cohort study	111–65	111–0	2007

Abbreviations: (g): glomerulitis, (ptc): peritubular capillaritis, MVI: microvascular inflammation, TCMR: T cell-mediated rejection, AMR: antibody-mediated rejection.

[a]*An additional 46 preimplantation biopsies were included in this study.*

[b]*The quantification of leukocytic composition of ptc was as follows: (i) predominantly mononuclear (>75% mononuclear cells), when mononuclear cells were at least three times as many as granulocytes; (ii) granulocytic dominated (>75% granulocytes), when granulocytes were at least three times as many as mononuclear cells; or (iii) mixed, if no dominant population was identified.*

There is notable heterogeneity among the included articles regarding the definition and interpretation of different rejection patterns in relation to C4d and/or anti-HLA-DSA. Bouatou et al. excluded mixed rejection cases, yet 28 TCMR samples were DSA-positive [7]. In Park et al. 's article two TCMR cases were DSA-positive [12]. It's unclear why they were not classified as mixed rejection. Sis et al. defined mixed rejection as TCMR plus AMR (C4d positive or negative), but did not provide the C4d and anti-HLA-DSA status for mixed rejection and TCMR cases [10]. Lastly, Zhao et al. selected 356 TCMR cases of which

TABLE 3 | Heterogeneity among studies in outcome variables in g, ptc and MVI in TCMR and AMR biopsies.

Author, year	Measurement	TCMR (n = 810)					AMR (n = 156)				
		g score	ptc score	MVI score (g + ptc)	C4d	Anti-HLA DSA	g score	ptc score	MVI score (g + ptc)	C4d	Anti-HLA DSA
Bouatou et al. [7] [a,b]	N (%)	0: 243 (94.9) 1: 13 (5.1) 2: 0 (0) 3: 0 (0)	0: 149 (58.2) 1: 42 (16.4) 2: 44 (17.2) 3: 21 (8.2)	-	0: 256 (100) >0: 0 (0)	28 (11)	-	-	-	-	-
Jung et al. [8] [c,d]	N (%)	-	-	0: 1 (9.1) ≥1: 5 (38.5)	-	-	-	-	0: 0 (0) ≥1: 5 (38.5)	2 (15.4)	-
Zhao et al. [9] [a, e]	Mean (SD)	0.25 (0.39)	0.72 (0.89)	-	0 (0)	0 (0)	-	-	-	-	-
Sis et al. [10] [f]	N (%)	0: 35 (79) >0: 9 (21)	0: 37 (84) >0: 7 (16)	0: 31 (71) >0: 13 (29)	-	In MVI>0: 0 (0)	0: 12 (40) >0: 18 (60)	0: 4 (13) >0: 26 (87)	0: 2 (7) >0: 28 (93)	30 (100)	In MVI>0: 28 (100)
							0: 20 (50) >0: 20 (50)	0: 14 (35) >0: 26 (65)	0: 11 (27) >0: 29 (73)	0 (0)	In MVI>0: 29 (100)
Lee et al. [11] [b,c]	Median (IQR)	0 (0.1)	0 (0.0)	0 (0.1)	-	-	2 (2.3)	2 (2.3)	4 (3.5)	-	-
Park et al. [12]	N (%)	0-1: 44 (91.7) 2-3: 4 (8.3)	0-1: 42 (87.5) 2-3: 6 (12.5)	>1: 18 (37.5)	>0: 13 (27)	2 (11)	0-1: 32 (55.2) 2-3: 26 (44.8)	0-1: 23 (39.7) 2-3: 35 (60.3)	>1: 54 (93.1)	>0: 40 (69)	41 (68)
Gibson et al. [13]	N (%)	-	>0: 76 (68.4)	-	-	-	-	-	-	-	-
Gupta et al. [14] [b,c,d]	N (%)	-	-	0: 8 (4) 1: 7 (10) ≥2: 12 (15)	-	-	-	-	0: 0 (0) 1: 2 (3) ≥2: 12 (15)	-	-
Kozakowski et al. [15]	N (%)	-	0: 137 (24.1) >0: 87 (48.1)	-	-	-	-	-	-	-	-
Batal et al. [16]	N (%)	0: 24 (37) 1: 16 (25) 2: 14 (21) 3: 11 (17)	-	-	-	-	0: 3 (30) 1: 3 (30) 2: 3 (30) 3: 1 (10)	-	-	-	-

Abbreviations: (g), glomerulitis; (ptc), peritubular capillaritis; MVI, microvascular inflammation; TCMR, T cell-mediated rejection; AMR, antibody-mediated rejection; SD, standard deviation; IQR, interquartile range.

[a]*This study focused on TCMR. Data on AMR was not included.*

[b]*This study specifically included acute TCMR cases.*

[c]*This study specifically included acute AMR cases.*

[d]*This study focused on MVI in different diagnostic groups. The outcome (N) represents the number of samples of acute TCMR or acute AMR within each MVI group. The percentages are specific to MVI groups, not diagnosis categories. Diagnoses other than acute TCMR and acute AMR are not covered in this systematic review therefore the percentages do not sum up to 100%.*

[e]*This study excluded all C4d and DSA positive TCMR cases, therefore the exact percentage of C4d and DSA positive TCMR cases is not clear.*

[f]*Glomerulitis, peritubular capillaritis and MVI scores of TCMR and AMR samples are presented in this table. Data on C4d-positive and C4d-negative AMR cases are given separately.*

197 were DSA-positive. Of the 159 DSA-negative TCMR cases a total of 131 cases were C4d positive [9].

Glomerulitis in TCMR

Glomerulitis scoring ranges from 0 to 3, reflecting the percentage of glomeruli affected [17]. Among the included studies, six discussed g lesion scores (**Table 3**). Bouatou et al. found that 94.9% of acute TCMR cases had g0, and 5.1% had g1, with no cases with g2 or g3 [7]. In Zhao et al.'s study, borderline TCMR had a g lesion score of 0.10 ± 0.31 (mean ± SD), while acute TCMR had 0.25 ± 0.39 [9]. Sis et al. showed that 79% of TCMR samples displayed g0, while the remaining 21% exhibited a g > 0. In contrast, among the C4d-positive AMR samples, 40% had g0, while 60% showed g > 0 [10]. Lee et al. reported a median of 0 (0.1) for g in TCMR, while acute AMR had a median of g2 [2, 3]. [11] Park et al. found 91.7% of TCMR cases with g between 0% and 1% and 8.3% between g2–g3, while for AMR, 55.2% had g0–g1, and 44.8% had g2–g3 [12]. Lastly, Batal et al. reported that 37% of the TCMR samples had g0, 25% g1 score, 21% g2 score and 17% g3 [16]. Additionally, in **Supplementary Table S3**, the i score, t score, and v score are provided per article, where available, alongside the g score.

Peritubular Capillaritis in TCMR

Peritubular capillaritis can be scored 0 through 3, according to the presence and severity of ptc seen [17]. Of the studies that were included for this systematic review, seven studies investigated the ptc individual lesion score (**Table 3**). Bouatou et al. showed that 58.2% of the samples with acute TCMR had ptc0, whereas 16.4% had ptc1, 17.2% had ptc2 and 8.2% had ptc3 [7]. Zhao et al., showed that the borderline TCMR group had a ptc of 0.10 ± 0.31 (mean ± SD), whereas for acute TCMR group, it was 0.72 ± 0.89 (mean ± SD). These values were also compared to the control group as mentioned in the previous paragraph [9]. Sis et al. found that a majority of TCMR samples (84%) had a ptc0, while the remaining 16% had ptc>0. In contrast, 13% of the C4d-positive AMR samples had ptc0 while the majority (87%) had ptc>0 with a median (interquartile range) of 2 [2, 3]. [11] Park et al. showed that 87.5% of TCMR cases had a lesion score of ptc0–ptc1 and 12.5% had a lesion score for ptc2–ptc3. Whilst, 39.7% of the AMR samples had ptc0–ptc1 and a majority (60.3%) had ptc2–ptc3 [12]. Gibson et al. found that 68.4% of the TCMR samples had ptc, with a preponderance of ptc2. However, 68.6% of focal C4d-positive and 88.2% of diffuse C4d-positive samples had ptc. Diffuse C4d-positive samples comprised the majority of ptc3 in this cohort [13]. Lastly, Kozakowski et al. reported that 24.1% of samples classified as TCMR had ptc0 and 48.1% of samples had ptc>0 [15]. The other included studies did not report the distribution of the cases across ptc0, ptc1, ptc2, and ptc3 in the results section or in **Supplementary Material**. Additionally, in **Supplementary Table S3**, the i score, t score, and v score are provided per article, where available, alongside the ptc score.

MVI in TCMR

Among the studies that were included for this systematic review, five studies investigated MVI (**Table 3**). In these studies, the MVI score was assessed by combining the Banff lesion scores g and ptc. Jung et al. reported that in cases with no MVI, there were no cases of acute AMR and only 9.1% had TCMR, while in cases with MVI≥1, the prevalence of both acute AMR and TCMR was 38.5%. It should be noted, however, that only six TCMR cases were analyzed in this study [8]. According to Sis et al., 71% of the TCMR cases did not show MVI, while the remaining 29% showed MVI>0. In contrast, among the C4d-positive AMR cases, 7% showed no MVI (MVI = 0) while the majority (93%) showed MVI>0 [10]. Lee et al. found a median (interquartile range) of 0 (0.1) for MVI in TCMR cases, while acute AMR cases had a median (interquartile range) of 4 [3, 5]. [11] Park et al. showed that 37.5% of the TCMR samples showed MVI≥1, compared to 93.1% of the AMR samples [12]. Lastly, Gupta et al. showed that in samples with no MVI, there were no cases of acute AMR and only 4% had acute TCMR, while in samples with MVI≥2, the prevalence of both acute AMR and acute TCMR was 15%. In cases with MVI = 1, 3% were acute AMR and 10% acute TCMR [14].

Retrospective Local Cohort Study

In our local study of 69 patients, cases with ATN had a g lesion score of 0.19 ± 0.54 (mean ± SD), a ptc lesion score of 0.00 ± 0.00 (mean ± SD), and an MVI score of 0.19 ± 0.54 (mean ± SD). Notably, two ATN cases demonstrated a g lesion score of g1 and two cases exhibited a g lesion score of g2. Cases classified as aTCMR, had a g lesion score of 1.60 ± 1.23 (mean ± SD), a ptc lesion score of 1.40 ± 1.19 (mean ± SD) and a MVI score of 3.00 ± 2.38 (mean ± SD). Interestingly, six cases of aTCMR had both a g lesion and ptc lesion score of 3 resulting in an MVI of 6. aAMR cases had a g lesion score of 2.00 ± 0.94 (mean ± SD), a ptc lesion score of 1.76 ± 0.83 (mean ± SD), and a MVI score of 3.76 ± 1.60 (mean ± SD). The g, ptc lesion and MVI scores per sample are depicted in **Supplementary Table S2**. Comparing the three groups, a significant difference was found between the g lesion score, the ptc lesion score and the MVI score (p-value < 0.001). However, upon conducting post-hoc pairwise comparisons, no significant difference was found between the aTCMR and aAMR groups in the g lesion score (p-value = 0.224), the ptc lesion score (p-value = 0.180) and MVI score (p-value = 0.224). Instead, significant differences were only observed between ATN and aTCMR or aAMR (p-value < 0.001), see **Figure 3**.

DISCUSSION

Our review reveals that the g and ptc score, along with the MVI score, are found in biopsies classified as aTCMR, albeit usually with lower frequency than in aAMR biopsies. Interestingly, our retrospective local cohort study also shows that MVI is present in aTCMR, additionally, we have found no differences between the degree of g, ptc and MVI between AMR and aTCMR.

Despite the elaboration in numerous studies, the correlation between scores for g, ptc, and MVI in TCMR or other renal transplant pathology remains unaddressed in the Banff Classification. Batal et al. showed that glomerulitis, commonly associated with AMR, was also detected in DSA-/C4d- TCMR (47%) and DSA/C4d-borderline samples (62%) [16]. Sis et al. showed that while glomerulitis occurs more frequently in AMR biopsies compared to TCMR or glomerulonephritis biopsies, its severity remains consistent across different types of renal allograft pathology, suggesting that glomerulitis is an ambiguous lesion [10]. Furthermore, Gupta et al. found that MVI is not specific for AMR and can also be seen in ATN, glomerulonephritis, and TCMR. In 19% of the for-cause transplant biopsies with MVI ≥2, there were no other histologic signs of rejection [14].

Interestingly, in our retrospective analysis, none of the cases with ATN had a ptc score greater than 0 (at least one leukocyte in <10% of cortical PTCs and/or maximum number of leukocytes <3), indicating that these cases either displayed no peritubular capillary inflammation or that any observed inflammation fell below the diagnostic threshold. This could be due to the highly stringent selection of the cases included. In addition to AMR and TCMR, it is important to recognize that some cases of ATN may also exhibit (ptc) or a lower score of MVI. In instances of ATN associated with MVI, it is crucial to carefully consider the implications and potential consequences of MVI. By closely monitoring these patients, we can gain valuable insights into their prognosis, as they might be at increased risk for developing rejection in some cases.

Recent research has expanded our understanding of MVI in organ transplantation, revealing multiple underlying causes beyond antibody-mediated mechanisms. Studies have shown

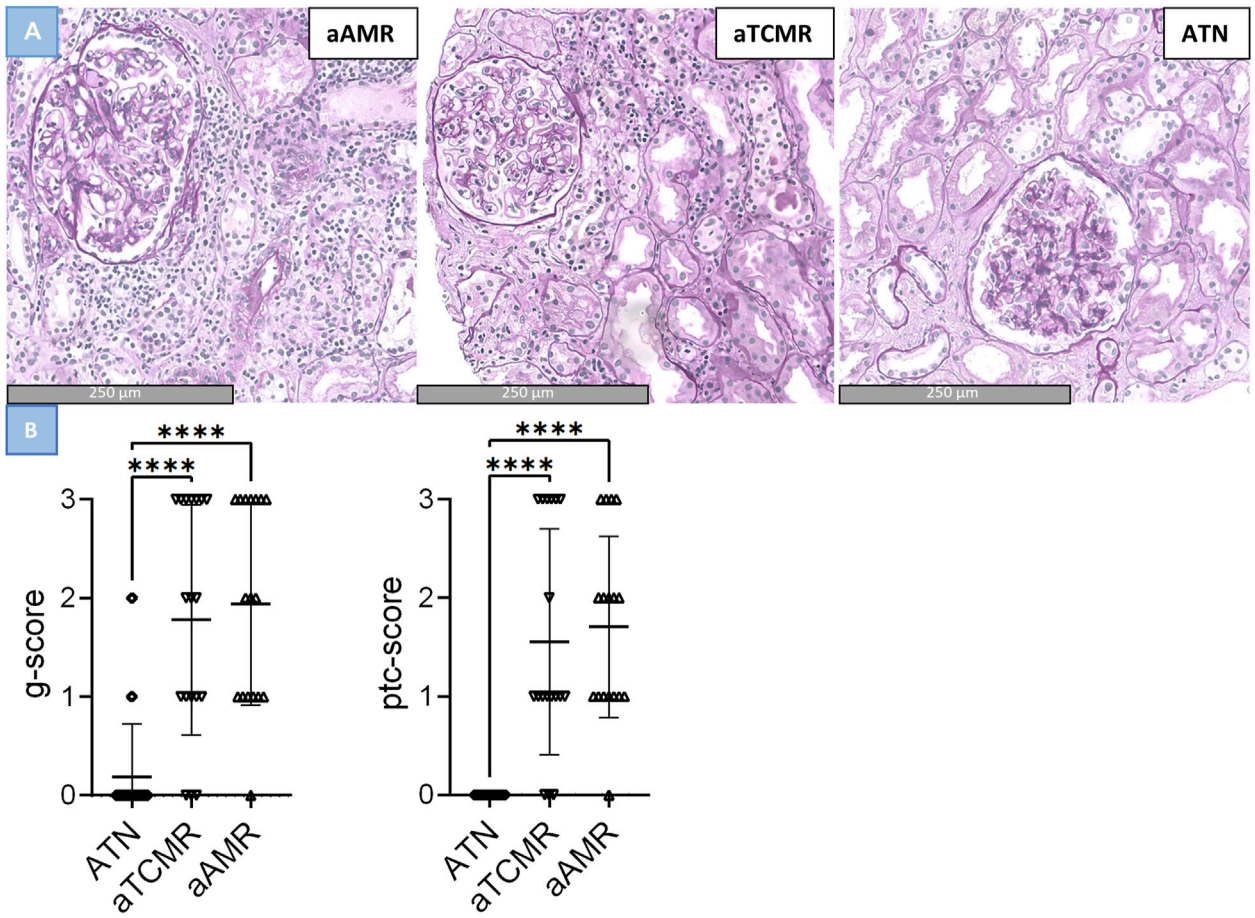


FIGURE 3 | Differences in glomerulitis, peritubular capillaritis and MVI in active antibody–mediated rejection (aAMR), acute T cell-mediated rejection (aTCMR) and acute tubular necrosis (ATN). **(A)** Histomorphologic images of aAMR, aTCMR and ATN, respectively, showing microvascular inflammation (PAS+, magnification 40x). (B) Lession scores in ATN, aTCMR and aAMR. Kruskal-Wallis. ***P < 0.0001.

significant NK cell presence in AMR cases with MVI, alongside specific AMR-related NK cell transcripts, including *SH2D1B*, *GNLY*, *FGFBP2*, and *CD160* [18–20]. Lamarthee et al. suggest that NK cells primarily interact with transplants through FCRL3 induction, triggering antibody-dependent cellular cytotoxicity [21]. Additionally, NK cells can recognize missing self by sensing the absence of HLA-I molecules through Killer Ig-Like Receptors, contributing to MVI [22–25]. Understanding these pathways holds therapeutic potential. Koening et al. showed that chronic vascular rejection depends on the mTORC1 pathway, with mTOR inhibitors showing promise in preventing development of histological lesions [25, 26]. Senev et al. suggested that antibody-independent NK cell activation mediated by the missing self is a mechanism through which HLA mismatch in the allorecognition pathway can lead to MVI. Interestingly, one-third of cases with this phenotype also showed concurrent TCMR, indicating possible primary T cell involvement in antibody- and complement-independent processes [27]. This relation between T cell activation and MVI has been demonstrated in preclinical research. T cells possess the ability to recognize all antigens, including HLA (class 2) antigens on endothelial cells, potentially leading to direct targeting of endothelial cells in transplanted tissue, causing microvascular injury and inflammation [28–30].

Understanding the diverse causes of MVI and their complex pathways is crucial for grasping their clinical effects on graft outcomes. In DSA+ AMR cases, MVI correlates with poor graft survival rates [31], especially when it progresses to chronic graft injury (cg), observed in both DSA-positive and DSA-negative cases [32]. Parajuli et al. discovered that DSA- AMR patients with MVI had similar poor graft survival rates as DSA+ AMR patients with MVI, but did not mention patients who had MVI alongside TCMR [24]. Our group has recently shown that cases showing transplant glomerulopathy lacking DSA and no C4d have superior transplant function to those cases classified as caAMR (ref Varol et al KI). Bouatou et al. identified factors independently associated with transplant loss in TCMR patients during the 3-month post-treatment assessment, which included GFR, proteinuria, i-IF/TA, anti-HLA DSA, and ptc score (HR, 2.27; 95% CI, 1.13-4.55; P = .022). However, the study did not

differentiate between cases with positive and negative DSA status [7]. So, while both antibody-independent and antibody-dependent mechanisms can contribute to MVI, their relative impact remains uncertain. To address this gap, it is important to understand distinct infiltrating cell types and underlying pathophysiological mechanisms. Immunophenotyping and transcriptomics could aid in elucidating the differences in MVI between TCMR and AMR. Such insights hold considerable potential for diagnostic classification and enhancing the precision of treatment decisions.

The findings of our systematic review come with limitations. Initially, we included eight articles and found two more by reviewing their references. However, these additional articles were not part of our initial search, revealing a limitation in our search strategy. Despite our specific and thorough search, we acknowledge that we may have missed some studies addressing MVI in TCMR. In particular, studies published before MVI and peritubular capillaritis became an area of interest and discussion within the context of TCMR, their MeSH terms might not have been used widely. Limitation arises from the relatively small number of available articles eligible for reliable data extraction and subsequent analysis. Including articles that only mention the "g" or "ptc" score without providing the complete MVI score presents a limitation. Without this comprehensive MVI score, it becomes difficult to thoroughly evaluate the influence of these variables. Nonetheless, these articles still provide valuable insights into aspects related to MVI. If these papers were excluded, it would reduce the number of eligible articles, potentially affecting the overall comprehensiveness of the study. Furthermore, most of the included manuscripts are observational and retrospective, inherently introducing a layer of selection bias. In order to maintain the integrity of this study and prevent another layer of selection bias, we excluded articles that excluded TCMR biopsies featuring g, ptc, or MVI, as the Banff Classification previously did not explicitly mention including the diagnosis of TCMR if MVI is present. Consequently, our pool of eligible articles became even more limited, with insufficient sample sizes for statistical pooling methods.

Another important limitation arises from the ambiguity in defining and interpreting various rejection patterns. In all studies included, in addition to the MVI score typically being represented as mean, median, or percentage values and without access to individual sample data, there is no information on individual DSA status or C4d staining. We therefore cannot be 100% sure that those cases that the authors diagnosed as "pure" TCMR are indeed so. However, as they did follow the Banff classification, we do assume that they did take the factors of DSA and C4d into account when diagnosing the cases of TCMR. Additionally, there was heterogeneity among the included articles in lesion interpretation methods and Banff Classification versions used. While modifications and updates to the Banff classification are necessary, they make comparing studies over time challenging. Therefore, inconsistencies in TCMR and mixed rejection interpretations, together with varying diagnostic criteria descriptions, make it unclear whether differences in TCMR cases with g, ptc, or MVI among studies stem from some studies categorizing TCMR with MVI as mixed rejection, while others classify this as TCMR. This diversity in terminology and methodologies posed challenges in establishing direct comparisons between the results. Additionally, some of the included articles mentioned samples classified as borderline TCMR or mixed rejection alongside TCMR cases. We chose not to incorporate data of these samples in our analysis due to insufficient descriptions regarding the precise definitions of these diagnoses. It is for example unclear how pathologists would diagnose TCMR with MVI; some might classify it as mixed rejection whilst others might consider the MVI as part of TCMR and label it as "pure" TCMR. In mixed rejection, it is not possible to attribute the presence of MVI to either AMR or TCMR or maybe even both, as little is known about the inflammatory cells present in both Banff categories. Finally, it is known that TCMR is a risk factor for AMR [33], which could therefore possibly explain the presence of MVI in TCMR. This decision was made to mitigate another potential factor for ambiguity in interpreting the results. Nevertheless, it is worth noting that excluding this information might have limited the findings of our study.

Future research efforts should focus on elucidating the role of MVI in aTCMR, identifying the main contributing inflammatory cells, and exploring the molecular profile of MVI within this context. Incorporating advanced immunohistochemistry and immunofluorescent staining techniques may provide deeper insights into the specific immune cell populations involved. By addressing these challenges and uncertainties, we can enhance our understanding of transplant pathology and ultimately improve patient outcomes. This comprehensive approach will help clarify the inflammatory composition and facilitate the identification of potential therapeutic targets.

To summarize, our systematic review and retrospective cohort study shows that g, ptc, and MVI can be present in for-cause kidney transplant biopsies diagnosed with aTCMR. It is crucial to underscore that in a clinical setting a more detailed clinicopathological correlation is necessary for an accurate diagnosis. Our analysis also revealed challenges related to the definition and classification of aTCMR and mixed rejection across various articles. These challenges underscore the importance of standardized and clear criteria in defining rejection patterns, especially given the biannual updates to the Banff Classification. Moving forward, it is imperative for researchers to exercise caution when including and describing different rejection patterns, ensuring a thorough description of the exact criteria used for their included samples.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving humans were approved by the METC, ErasmusMC Rotterdam Netherlands. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin because this is a non-WMO retrospective study for which METC approval is obtained.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13464/full#supplementary-material

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REVIEW
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Deciphering the Complexity of the Immune Cell Landscape in Kidney Allograft Rejection

George Terinte-Balcan[1,2,3†], *Emilie Lebraud*[3], *Julien Zuber*[4], *Dany Anglicheau*[3,4†], *Gener Ismail*[1,5†] *and Marion Rabant*[2,3*]

[1]Nephrology department, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania, [2]Department of Pathology, Necker-Enfants Malades Hospital, Assistance Publique—Hopitaux de Paris, Paris, France, [3]Centre National de la Recherche Scientifique (CNRS), Inserm U1151, Institut Necker-Enfants Malades, Université Paris Cité, Paris, France, [4]Department of Kidney and Metabolic Diseases, Transplantation and Clinical Immunology, Necker Hospital, Assistance Publique Hôpitaux de Paris (AP-HP), Paris, France, [5]Department of Nephrology, Fundeni Clinical Institute, Bucharest, Romania

While the Banff classification dichotomizes kidney allograft rejection based on the localization of the cells in the different compartments of the cortical kidney tissue [schematically interstitium for T cell mediated rejection (TCMR) and glomerular and peritubular capillaries for antibody-mediated rejection (AMR)], there is a growing evidences that subtyping the immune cells can help refine prognosis prediction and treatment tailoring, based on a better understanding of the pathophysiology of kidney allograft rejection. In the last few years, multiplex IF techniques and automatic counting systems as well as transcriptomics studies (bulk, single-cell and spatial techniques) have provided invaluable clues to further decipher the complex puzzle of rejection. In this review, we aim to better describe the inflammatory infiltrates that occur during the course of kidney transplant rejection (active AMR, chronic active AMR and acute and chronic active TCMR). We also discuss minor components of the inflammatory response (mastocytes, eosinophils, neutrophils, follicular dendritic cells). We conclude by discussing whether the over simplistic dichotomy between AMR and TCMR, currently used in clinical routine, remains relevant given the great diversity of immune actors involved in rejections.

Keywords: rejection, immune cells, infiltrates, heterogeneity, complexity

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***Correspondence**
Marion Rabant,
marion.rabant@aphp.fr

†ORCID:
George Terinte-Balcan
orcid.org/0000-0003-4063-8431
Dany Anglicheau
orcid.org/0000-0001-57936174
Gener Ismail
orcid.org/0000-0003-3306-189X

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INTRODUCTION

Transplantation remains the most efficient method of treating chronic kidney disease despite the high risk of adverse events such as rejection [1], infection or recurrence of disease. Regarding rejection, there is a growing consensus among the transplant community that the phenotypes are increasingly subtler and more complex [2]. The diagnosis of rejection is based on the Banff classification which takes into account (among other criteria) the lesions encountered in the graft such as glomerulitis, peritubular capillaritis, interstitial inflammation, tubulitis or arteritis [3, 4]. While the probability of being diagnosed with an episode of rejection remains relatively high at around 10%, considerable progress has been made in the last few years in reducing episodes of acute T-cell mediated rejections (TCMR) due to efficient therapy [5]. On the other hand, antibody-mediated rejection (AMR) still remains an unsolved problem with studies showing that chronic lesions (transplant glomerulopathy) are a major cause of late graft loss [6, 7]. One possible explanation for this mechanism could be that current therapies are predominantly focusing on

T lymphocytes, while other cell types (for example, NK cells and macrophages) and soluble factors (complement) that participate to the immune response are currently being insufficiently targeted [8, 9].

During the course of rejection, there is a very complex interaction between different immunological mechanisms and immune cell types [10]. Interestingly, both innate and adaptative immune cells participate to the cascade of events leading to rejection [11]. In the first report addressing the heterogeneity of cell populations involved in mixed rejection (i.e., both TCMR and AMR), using single cell RNA-sequencing, Wu et al. found up to 16 different immune and renal stromal cell types [10]. Among the immune cell category, the group described 2 types of monocytes, T cells, B cells, plasma cells and mast cells [10]. More recently, Lamarthée et al. studied 16 biopsies using the same technique and also identified 10 different immune cell clusters including various subtypes of $CD4^+$ T cells, $CD8^+$ T cells, NK cells, dendritic cells, $CD19^+$ B cells and monocytes/macrophages [12].

Although the Banff classification does not take into account the cellular composition of the inflammatory infiltrate, but only its intensity and localization (schematically within the interstitium for TCMR and within the capillaries for AMR), there is a growing belief that subtyping the leukocytes can help refine prognosis prediction and treatment tailoring, based on a better understanding of the pathophysiology of kidney allograft rejection [3, 13–17]. However, many hurdles have to be overcome in order to accurately identify and count cells in the setting of rejection. As a matter of fact, manually counting cells stained either by immunohistochemistry (IHC) or by immunofluorescence (IF) is very time consuming and raises the challenge of reproducibility [18, 19]. In the last few years, multiplex IF techniques and automatic counting systems have led to a large number of papers that focused on better describing the nature of the inflammatory infiltrates during rejection [20, 21]. Moreover, transcriptomics studies (bulk, single-cell and spatial techniques) have provided invaluable clues to further decipher the complex puzzle of rejection [10, 22, 23]. This is of particular importance in the setting of AMR where the pathophysiology becomes more complex with the description of antibody-independent and non-HLA donor specific antibody (DSA) mechanisms [9, 24].

In this review we aim to better describe the inflammatory infiltrate that occurs during the course of kidney transplantation, highlighting the different immune cell types involved and also their repartition. Therefore, we structured this review into active AMR, chronic active AMR and acute and chronic active TCMR. We also included a category of minor components of the inflammatory response that do not perfectly fit in the categories described. We conclude by discussing whether the over simplistic dichotomy between AMR and TCMR, currently used in clinical routine, remains relevant given the great diversity of immune actors involved in rejections.

ACTIVE AMR

Regarding the diagnosis of AMR, glomerulitis represents an important component of microvascular inflammation (MVI) [3]. Glomerulitis is associated with the infiltration of different cell types, with the most common being macrophages and T lymphocytes [19, 25]. Interestingly, it has been shown using IHC that the mean number of monocytes per glomeruli is higher in C4d positive-AMR compared to C4d negative-AMR, whereas T cells are predominant in the glomeruli, in C4d negative AMR [15]. This finding was confirmed by another center that used electron microscopy [26]. Moreover, using IHC for CD68, Tinckam et al. demonstrated that a mean glomerular monocyte infiltration ≥1 was associated with a worse graft survival and independently predicted graft function at 2 and 4 years independent of C4d status [27]. More recently, Mölne et al. developed a Glomerular Macrophage Index (GMI) using IHC as the mean number of macrophages in 10 glomeruli and demonstrated in a cohort of 1,440 biopsies that GMI was predictive of graft loss, independently of histological diagnoses [28].

The presence of inflammatory cells in the peritubular capillaries (PTC) represents the second lesion in the category of MVI [3]. Hidalgo et al. performed IHC for CD3, CD68 and CD56 on 18 biopsies that were diagnosed as C4d positive-AMR, C4d negative-AMR and TCMR and found an increased number of $CD68^+$ macrophages ($p = 0.03$) and $CD56^+$ NK cells ($p = 0.006$) in the PTC in cases of AMR, independently of C4d staining, as opposed to TCMR [17]. In a study conducted by our group using multiplex IF, we found in the PTC a higher proportion of T lymphocytes during AMR and TCMR (81.1% and 87.6% respectively), than macrophages (14% and 10.5%, respectively) and NK cells (4.8% and 2.0%, respectively). However, the density of NK cells and macrophages were significantly higher in AMR compared to TCMR (4.7 ± 1.2 vs. $1.5 \pm 0.5/mm^2$, $p = 0.01$ for NK cells and 11.6 ± 2.5 vs. $5.0 \pm 1.5/mm^2$, $p = 0.02$ for macrophages) [19]. These results were not aligned with those from an older study by Liptak et al. that used electron microscopy and showed that monocytes represented 59% of cells in the PTC, while granulocytes and lymphocytes represented 14% and 12% respectively in a series of 12 AMR biopsies [26].

Computer-assisted counting of immune cells (CD20 for B lymphocytes, CD138 for plasma cells, CD4 or CD8 for T lymphocytes, CD56 for NK cells, FoxP3 for T regulatory cells, CD68 for macrophages with pSTAT1 or cMAF, to distinguish M1 and M2 macrophages respectively) using IHC on serial sections was used to characterize inflammatory infiltrates in different types of rejection [20]. Aguado-Dominguez et al. showed that T cells and non-polarized $CD68^+$ macrophages represented 40% and 36%, respectively, of the total inflammatory cells found in the interstitium during AMR [20]. When further investigating T-cell subtypes, 21% were $CD4^+$, 15% $CD8^+$ and 4% FOXP3+. Even though this study analyzed only the interstitial compartment, clustering analysis revealed a correlation between NK cells and active AMR. Interestingly, they found that the cellular composition greatly varied across patients within the same diagnosis category, and failed to identify a unique profile associated with a given type of rejection [20].

Sicard et al. automatically quantified $CD20^+$ cells, CD3 + cells, $CD68^+$ cells and granulocytes using conventional IHC on serial slides in 52 AMR biopsies and showed that the extent of $CD68^+$

macrophage infiltration was the sole predictive factor associated with subsequent graft function. The more intense the macrophage infiltrate in the interstitium and in the PTC the greater the rate of graft loss [21]. Furthermore, patients with a high macrophage density also had higher expression of C4d and a higher score of interstitial inflammation and tubulitis according to the Banff classification [21].

The prognosis value of macrophages probably results from their instrumental role in the priming and polarization of the adaptive immune response [29–31]. From a functional point of view, macrophages have been classically divided into M1 with a pro-inflammatory phenotype, while M2 macrophages are rather considered as anti-inflammatory and pro-fibrotic [32]. Using IHC in a cohort of 55 AMR samples, Kim et al. stained M1 and M2 macrophages using MRP8/14 and CD163 markers respectively [33]. They found that glomerular M2 macrophages were associated with chronic transplant glomerulopathy and poorer graft function, whereas tubulointerstitial M2 macrophages were associated with lower MVI and lower arteritis than the M1 polarization group [33]. The group also found a trend toward longer graft survival in patients that had higher numbers of glomerular M1 ($p = 0.175$) [33].

NK cell contribution to rejection has long been overlooked given the scarcity of lineage-specific markers to accurately differentiate NK cells from activated T cells [34]. Hidalgo et al. were the first to highlight the importance of NK cells during AMR by using data obtained by transcriptomics and CD56 IHC staining [17, 35]. However, it is worth to note that CD56 may be expressed by some T cell subsets as well, and the lack of CD3 expression by CD56-expressing cells should be requested to assign the label of NK cells with certainty. Although IHC was performed on a small number of patients with AMR (C4d positive and negative) and TCMR, the group highlighted a large increase of $CD56^+$ cells in the peritubular capillaries of AMR patients when compared to TCMR ($p = 0.03$) [17]. Furthermore, they found a large number of NK-associated transcripts in biopsies that were done 1 year after transplantation with a diagnosis of either AMR of mixed rejection [35]. Moreover, in these biopsies, they found an important correlation between the presence of MVI, DSA positive status and NK specific transcripts [35]. In another study that used transcriptomic data and deconvolution analysis, obtained from 95 cases, 15 of whom had a diagnosis of AMR and 63 did not have rejection, Yazdani et al. found an increased number of NK cells in AMR cases compared to those without rejection [36]. Moreover, the presence of NK cells was correlated with MVI, DSA and C4d positivity. Out of all the cells types, NK cells were the best predictors of graft failure at 1 and 2 years, outperforming even the prognosis value of Banff classification ($p < 0.001$ vs. $p = 0.039$) [36]. Jung et al. used multiplex IF on a cohort of 39 for-cause biopsies (8 with no rejection, 11 TCMR and 20 AMR) and noticed that the highest density of NK cells was found in cases diagnosed with AMR (2.57 ± 2.58 cells/mm^2) compared to 0.12 ± 0.28 cells/mm^2 for non-rejection biopsies and 0.25 ± 0.34 cells/mm^2 for TCMR ($p = 0.002$) [37]. Interestingly, the density of NK cell infiltrate was correlated with the "i" and "ti" scores as well as with the "ptc" ($r = 0.489$, $p = 0.002$), yet not with glomerulitis scores. In the study from Aguado-Dominguez et al., NK cells were mainly found in the cases of active AMR, whereas they were only a minor component in other types of rejection [20]. In a multiplex IF study conducted by our group on a cohort of 20 TCMR, 20 AMR and 5 non-rejection biopsies, we used the NK lineage-specific marker NKp46 to emphasize that NK cells represented only $2.7\% \pm 0.7\%$ of the total inflammatory burden during AMR, as opposed to $0.6\% \pm 0.4\%$ in normal biopsies and $2.9\% \pm 0.6\%$ in TCMR [19]. More recently, Lamarthée et al. used single cell RNA-sequencing to show an increased density of FcγRIII+ NK cells in AMR and mixed rejection biopsies when compared to TCMR [12]. The same team also used deconvolution analysis of bulk transcriptomics data to demonstrate that NK cells and $CD14^+$ monocytes/macrophages are more common in DSA+ AMR cases, whereas $CD4^+$ memory T cells are more represented in DSA- AMR cases [11].

Graft-infiltrating B cells seem to play a minor role in the setting of active AMR, although a few studies have suggested an accumulation of B cells in the tertiary lymphoid structures that can develop in chronically rejected allografts [38]. This finding will be addressed later on. Aguado-Dominguez et al. showed that B cells represented 10% of the total interstitial inflammatory infiltrate [20]. In a multiplex IF study performed by our group from 125 rejection kidney biopsies, including 69 AMR, B cells accounted only for 3.4% of the infiltrating inflammatory cells (M1 and M2 macrophages, NK cells, T and B lymphocytes) during AMR (*unpublished data*). Importantly, the presence of $CD20^+$ B cells did not correlate with positive C4d staining, suggesting that the presence of $CD20^+$ cells in the allograft was independent of the presence of circulating DSA, produced by bone marrow or spleen-resident plasma cells [39, 40]. In another study, based on mRNA gene expression profiles in 21 cases with early AMR (diagnosed on average on the 9th day post-surgery), Viklicky et al. found that biopsies with a low expression of CD20, FoxP3, and TGF-β1 had an increased risk of graft failure in the next year [41].

CHRONIC ACTIVE AMR

Chronic active AMR (CA AMR) is suspected when there is persistent, ongoing MVI with added features of transplant glomerulopathy (TG) and lamellation of the lamina densa of PTC as demonstrated by electron microscopy [3]. The exact mechanisms that lead to this pattern of injury are not yet fully understood [42], although recent studies that looked at gene expression profiles are starting to decipher the involved molecular pathways [43]. Adam et al. studied a panel of 34 genes in 197 non-human primates renal transplant biopsies and found 3 endothelial genes (VWF, DARC, CAV1) that correlated with the development of chronic glomerulopathy [44]. Interestingly, expression of these 3 genes was associated with C4d positivity ($p < 0.001$) and DSA positivity ($p < 0.001$) when compared to C4d negative and DSA negative cases [44]. Another study, based on gene expression profiling of chronic AMR, identified genes suggestive of NK cells, cytotoxic lymphocytes and activation of macrophages [45]. Interestingly, in this study, C4d-negative

DSA-negative TG biopsies exhibited higher expression of cytotoxic T cell-associated transcripts, in keeping with enhanced T cell activation. A very recent study, based on the use of bulk RNA-sequencing, reported a significant increase in NK cell cytotoxic and T cells transcripts in biopsies with chronic AMR when compared to active AMR. Moreover, this study showed that CA AMR shared molecular features with TCMR, whereas neutrophils and monocytes-related pathways were predominantly involved in active AMR [46]. Deconvoluted RNA-sequencing data analysis also unveiled that the proportion of NK cells *in situ* was higher in CA AMR than in active AMR ($p = 0.0038$).

Recently, Cristoferi et al. investigated the differences between graft biopsies with either the diagnosis of TG C4d-/DSA- or TG C4d+/DSA+, through multiplex IF and bulk transcriptomics [47]. In line with the conclusion drawn by an above-cited study [46], C4d-/DSA-cases had higher numbers of $CD3^+$ T cells and a higher expression of cytotoxic T-cell-associated mRNA than their C4d+/DSA+ counterparts. In contrast, the C4d+/DSA+ group had a predominance of infiltrating macrophages, NK cells and neutrophils [46, 48]. In the above-cited study from Aguado-Dominguez et al., 18 biopsies were diagnosed with CA AMR and disclosed an increased number of T cells and macrophages in the interstitial and glomerular compartments, with 39% of $CD4^+$ T lymphocytes, 18% of $CD8^+$ 18%, 6% of M2 macrophages, 4% of M1 macrophages and 2% of FOXP3+ cells [20]. CD138+ plasmocytes were also readily detected in CA AMR, unlike in active AMR ($p < 0.05$).

Papadimitrou et al. studied the cellular composition of glomerulitis in 240 transplant biopsies performed after 1 year post transplantation using IHC for CD3, CD20 and CD68 and its impact on TG's outcome. They found a predominance of $CD68^+$ macrophages, followed by $CD3^+$ T lymphocytes. $CD20^+$ B lymphocytes were barely identified. A high number of $CD68^+$ macrophages (more than 12 in the most inflamed glomerulus) was strongly associated with TG, DSA and C4d [49]. Furthermore, the degree of macrophage infiltration in the glomeruli was also a strong predictor of subsequent graft dysfunction prompting the authors to hypothesize that the development of transplant glomerulopathy is preceded by the accumulation of macrophages. However, other studies have shown that T cells can also lead to transplant glomerulopathy in the absence of circulating DSA [47, 50].

Sablik et al. studied 20 biopsies with CA AMR using multiplex IF and evaluated T-cell subsets (CD3, CD8, FoxP3, Granzyme B), macrophages (CD68 and CD163), B cells (CD20) and NK cells (CD57) in the glomeruli (cells/glomeruli) and the tubulointerstitial compartment [cells/high-power field (HPF)] [51]. In the glomeruli, the main cell types were $CD3^+$ T cells and macrophages, with an average of 5.5 cells and 4 cells per glomerulus, respectively. $CD8^+$ T cells represented 61.7% of the total T cell population. Approximately 46% of $CD8^+$ T cells and 23% of $CD4^+$ T cells also expressed granzyme B, showing cytotoxic potential of these cell populations. NK cells, Treg and B lymphocytes were rarely found in the glomeruli. In the tubulo-interstitial compartment, the majority of cells were $CD3^+$ cells with a mean number of 116.3 cells/HPF, followed by macrophages (21.5 cells/HPF). Interestingly, B cells aggregates were frequent in the tubulo-interstitial compartment. Unexpectedly, patients with a lower density of Treg in this compartment had a longer graft survival than patients with a high density (5.3 years vs. 2.1 years, $p = 0.004$).

ACUTE AND CHRONIC ACTIVE TCMR

In cases of acute TCMR, T cells are known to predominate and to drive the inflammatory response [13]. However, as for AMR, there are a number of other different immune cell types (macrophages, B cells, plasmacytes, NK cells, dendritic cells) that can be found, with different effects on the severity and outcome [19]. Moreover, Girlanda et al. demonstrated that T cell accumulation did not correlate with the extent of graft dysfunction, whereas monocytes did, suggesting that other immune effectors could be involved than cytotoxic T cells [13]. Hancock et al. reported a large number of macrophages in the tubulo-interstitial compartment, accounting for 52, 38, and 60% of the total infiltrating cells in mild, moderate and severe episodes of TCMR, respectively [52]. Similarly, Bergler et al. showed that $CD68^+$ macrophages-rich infiltrates were found in severe cases of TCMR, associated with arteritis [14]. Furthermore, in this latter study, increased densities of macrophages correlated with reduced graft function at 3-year post transplantation [14]. Multiple studies have also shown that $CD68^+$ macrophages infiltration positively correlates with the extent of interstitial fibrosis/tubular atrophy and with subsequent graft function [16, 53, 54].

Using multiplex IF, our group has also shown that CD163+ macrophages are the second most common cell type (45.3% ± 5.8%) in TCMR after $CD3^+$ lymphocytes (51.8% ± 6.0%) [19]. A representative image from a case from this study is depicted in **Figure 1**. Furthermore, we showed a great heterogeneity in the composition of the cellular infiltrate across the 20 individual patients with TCMR. As a matter of fact, the frequency of macrophages ranged from 7.0% to 89.0% while the frequency of $CD3^+$ T lymphocytes varied from 10.0% to 92.7% of the total leukocytes infiltrating the graft [19]. On the other hand, we identified remarkable similarities, regarding the composition of the infiltrates, between patients with different pathological diagnoses (TCMR and AMR), as highlighted in **Figure 2** [19]. We failed to identify any clinical or pathological factors that could predict the proportions of $CD3^+$ T lymphocytes and macrophages in this series [19].

Macrophage accumulation in areas of interstitial fibrosis and tubular atrophy indicates a tissue repair-related universal phenomenon, independent of the pathogenesis process, with M2 outnumbering M1 [16, 32]. Notably, macrophages gain the ability to produce profibrotic mediators through M1 to M2 phenotype shift [30]. An IHC study by Ikezumi et al. showed that the number of infiltrating M2 $CD68^+$ CD163+ macrophages increased over time after transplantation and correlated with the loss of glomerular filtration rate ($p < 0.0001$) as well as with the extent of interstitial fibrosis and tubular atrophy ($p < 0.0001$), whereas T cell accumulation did not [32].

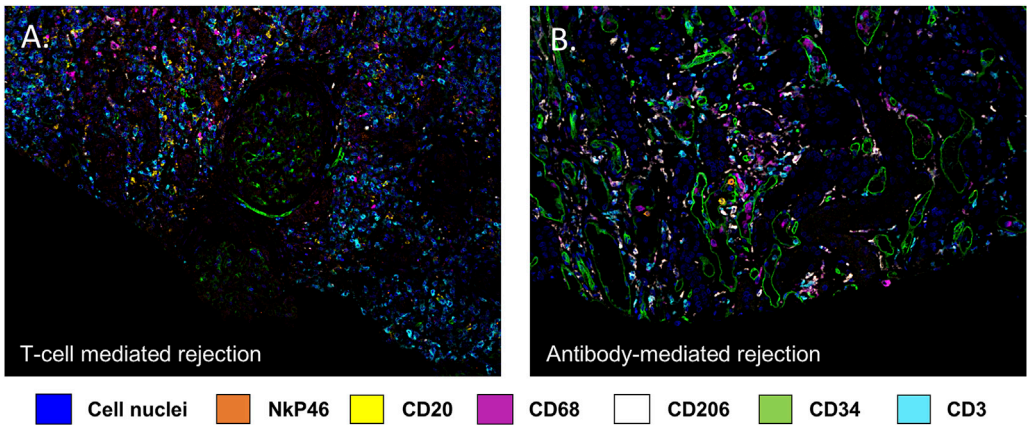

FIGURE 1 | Multiplex immunofluorescence image highlighting the diversity of immune cells involved in kidney allograft rejection. Endothelial cells are stained with an anti CD34 antibody (green), B lymphocytes by an anti CD20 antibody (yellow), T lymphocytes using an anti CD3 antibody (turquoise), macrophages using an anti CD68 antibody (purple) and NK cells using an anti NkP46 antibody (orange). DAPI (blue) stains for cell nuclei. **(A)** illustrates a T cell mediated rejection, **(B)** illustrates an antibody mediated rejection.

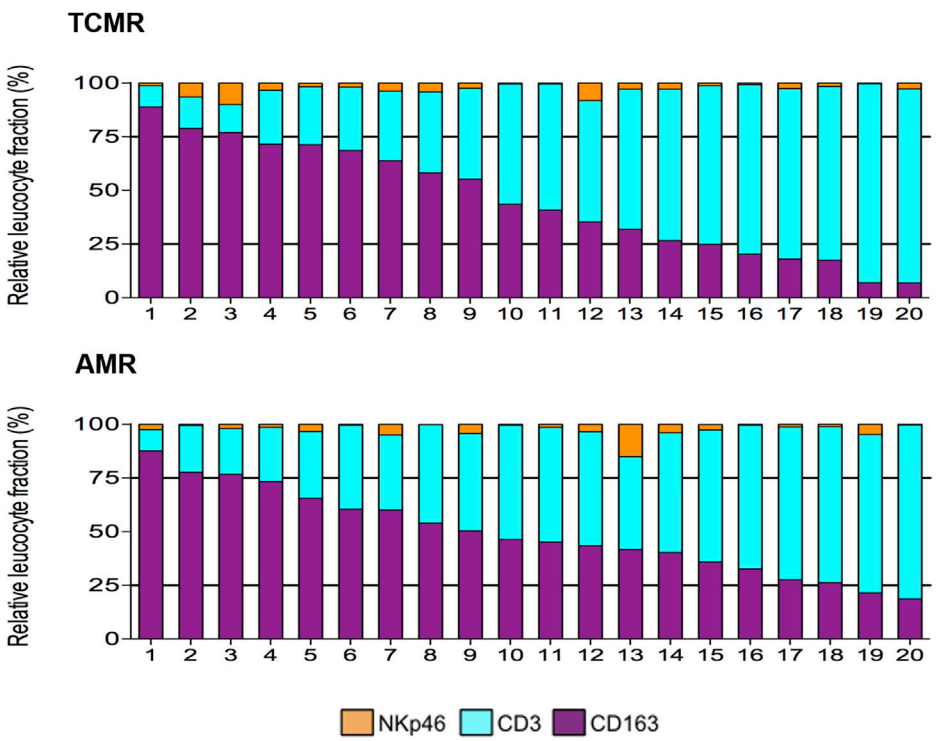

FIGURE 2 | Heterogeneity of the composition of the inflammatory infiltrate during TCMR and AMR (from [19]). During both AMR and TCMR, biopsies displayed a wide range of proportions of the main inflammatory cells ($CD3^+$ T lymphocytes, and CD163+ macrophages), and a small proportion of NkP46+ NK cells.

Regarding B lymphocytes, using IHC Hwang et al. revealed $CD20^+$ clusters (defined by more than 275 cells/HPF) in 37.3% of patients diagnosed with TCMR [55]. The presence of $CD20^+$ clusters seemed to be associated with a poor graft survival and with steroid resistance [40, 56, 57]. On the other hand, other studies have yielded conflicting results with $CD20^+$ infiltrates having no effect or even being associated with a better allograft survival [58, 59]. The implications of B-lineage cells in kidney allograft have been superbly reviewed by Filippone EJ and Farber JL [39].

Plasmocytes have also sparkled interest primarily in the context of plasma cell-rich TCMR, but also in some cases

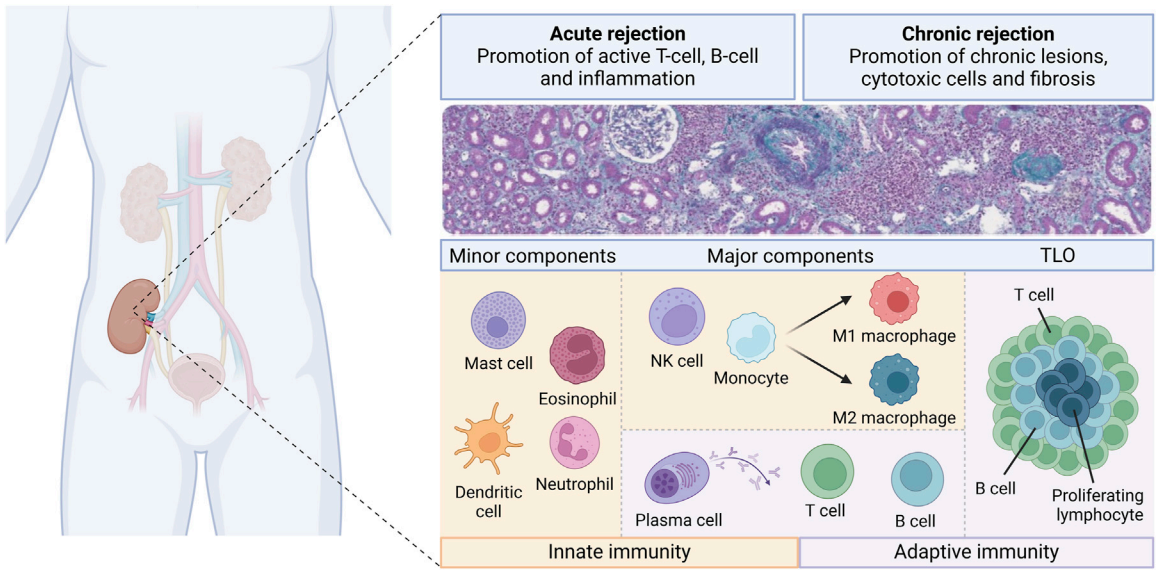

FIGURE 3 | Overview of the diversity of the immune cell landscape during kidney allograft rejection. Different immune cells from innate (orange boxes) and adaptive (purple boxes) immunity are involved during the course of kidney transplant rejection. There are classified as minor components, major components and tertiary lymphoid organs (TLO). These components may play a role into acute and chronic rejection, whether in T-cell mediated rejection or antibody-mediated rejection.

featuring AMR or mixed rejection lesions [60, 61]. Currently, this type of rejection is not individualized *per se* in the Banff classification, although an infiltrate with more than 5%–10% plasmocytes should be acknowledged by an asterisk after the inflammation score "i" [3]. Plasma cell-rich rejection is usually defined by the presence of plasmocytes in more than 10% of the cortical surface [60]. Using IHC, Mubarak et al. found plasma cells accumulating in the periglomerular area, in the perivascular space as well as at the cortico-medullary junction [62]. Interestingly, the number of plasma cells inversely correlated with the number of B cells [62]. The frequency of this rejection pattern varies between 2% and 14% [63]. Some studies have suggested that infections and poor adherence to the treatment can be risk factors for developing a plasma cell-rich infiltrate [64, 65]. Unfortunately, there is no standard treatment for this type of rejection and therefore these cases are usually refractory to treatment and have a very poor prognosis [60, 63]. These patients usually have low graft survival, with 40%–60% of cases promptly losing their graft following the diagnosis of plasma cell-rich episode of rejection [60, 64]. When considering the diagnosis of plasma cell-rich TCMR, the BK virus nephropathy is an important alternative diagnosis to rule out, since half of BKV-related renal inflammation exhibits plasma cell-rich infiltrates [66].

Over the past years, innovative tools have allowed a deeper understanding of the cellular composition during TCMR. Salem et al. were the first team using spatial transcriptomics in a single case of chronic active TCMR compared to a control case with no rejection [23]. The analysis focused on 5 regions of interest within the tubulo-interstitial space and emphasized an increase in genes related to T lymphocyte proliferation and activation. Interestingly, these findings were not correlated with the lesions scored according to the Banff classification. The authors also studied 3 glomerular areas and found no differences between controls and chronic active TCMR. In mice, single cell RNA-sequencing data obtained by Shen et al. showed that the inflammatory infiltrate evolves along with the progression of the rejection from the acute to the chronic phase. More specifically, the proportion of B cells, neutrophils and $CD8^+$ T cells decrease over time, while macrophages become more prevalent [67]. Another single cell RNA-sequencing study performed by Liu et al. performed on 2 biopsies with chronic lesions showed an increase in memory B cells, myofibroblasts and activated monocytes [68].

Recently, Vaulet et al. studied the infiltration of 9 different immune cells by investigating 3 different data sets of 909 biopsies obtained by bulk transcriptomics. This study highlighted that the greatest amount of infiltrating inflammatory cells was observed in TCMR, whereas DSA+ AMR, in contrast, had the lowest number of infiltrating cells, close to that of non-rejection biopsies [11]. When compared to the non-rejection group, TCMR cases had an increase of 16.5% ($p < 0.001$) in the number of inflammatory cells. Furthermore, there was an increase in CD4 naive cells (+1.8%, $p < 0.001$) and $CD14^+$ monocytes/macrophages (+5.9%, $p < 0.001$) when compared to non-rejection cases. NK cells had a lower contribution to the infiltrates when compared to AMR cases, while CD8 effector cells demonstrated the greatest frequency in TCMR and DSA-positive mixed rejection. Interestingly, the main Banff rejection diagnosis categories could not be individualized based on the estimation of immune cell composition alone.

Zhou et al. also showed interesting differences between cases diagnosed with TCMR and stable graft biopsies by analyzing transcriptomic data available from the public domain, obtained from 224 TCMR and 1,561 stable samples [69]. Similarly, as in the

study from Vaulet et al. [11], they estimated the relative proportion of 22 immune cell types by deconvolution analysis. The investigators found that biopsies with TCMR had a reduced infiltration by naïve B cells, M0 macrophages, neutrophils and resting dendritic cells, yet an increased proportion of memory B cells, $CD8^+$ T cells, $CD4^+$ T cells, follicular helper T cells, gamma delta T cells, monocytes, M1 macrophages, activated dendritic cells and eosinophils when compared with biopsies free of rejection [69].

OTHER MINOR COMPONENTS OF THE INFLAMMATORY INFILTRATE

Tertiary Lymphoid Organs

B cells may aggregate and form tertiary lymphoid nodules in the allograft which are composed of B lymphocytes, follicular dendritic cells (FDC), T follicular helper (TFH) cells and a rim of T lymphocytes, plasmocytes and plasmablasts [39]. These structures are supported by lymphoid vessels and high endothelial venules [39]. The main goal of these structures is to form antibodies after an interaction between TFH cells and B cells [70]. They have been proven to be very important in different scenarios such as autoimmunity, cancer and infection [71]. However, the study of lymphoid nodules is complicated by a lack of standardization and by the fact that the classification of these structures according to the Banff criteria is difficult [3]. Therefore, to date, it is not clear whether accurate diagnosis or treatment is needed in order to disrupt the formation of tertiary lymphoid organs, as they seem to have some potential to induce tolerance of the graft [39].

In the setting of kidney transplantation, tertiary lymph node formation has been demonstrated in acute and chronic rejection scenarios [38]. Using IHC, De Leur et al. showed that ectopic lymphoid structures were predominantly found in acute TCMR, unlike in AMR [70]. In a series of 26 cases of explants, 20 of which were diagnosed with chronic rejection, Thaunat et al. found tertiary lymphoid structures in almost all cases of chronic rejection [72].

Lee et al. showed in a large series of 214 patients with protocol biopsies without evidence of rejection that almost half of the biopsies (46.9%) had aggregates of lymphoid cells classified as tertiary lymphoid structures that formed as early as within the first month after transplantation [73]. Interestingly, only 3.8% of implantation biopsies demonstrated such structures. The further development of stage II tertiary lymphoid tissues, defined by the presence of FDC in these structures was gradual, from 1.4% in 0-h biopsies, to 3.6% at 1 month and 18.9% at 1 year [73]. In this cohort, the finding of FDC correlated with a subsequent decay of the graft function as well as with the presence of DSA, even though no patient developed a subsequent episode of AMR. However, other studies did not confirm the association between FDC and rejection, but in the contrary, suggested that tertiary lymphoid nodules could play a role in graft tolerance [74]. Using a mouse model of kidney allograft tolerance, Brown et al. demonstrated using IHC the presence of tertiary lymphoid structures in these kidneys [75]. They further showed that there was a mild correlation between the size of the lymphoid structures and graft function, with larger sized nodules being seen in better functioning grafts [75].

Mastocytes

Mastocytes represent a very versatile cell type, with the capacity to both increase or decrease the inflammatory processes that takes place during rejection, depending on whether they secrete anti-inflammatory factors or degranulate pro-inflammatory mediators. Their significant impact on the course of the inflammatory response contrasts with their minor contribution to the infiltrate [76]. Outside the field of transplantation, mastocytes have mostly been involved and described in the setting of allergy [77]. Regarding transplantation, they have been observed as a component of acute rejection, although not in all studies [76–78]. It is also hypothesized that mastocytes, recruited by Treg-produced IL-9 [79], could be involved in the maintenance of allograft tolerance, although the exact mechanisms remain ill-defined [76].

In a study performed by Varol et al. using IHC for tryptase in 53 biopsies diagnosed with borderline TCMR, mastocyte accumulation was correlated with delayed graft function ($p = 0.020$) and deceased donor status ($p = 0.035$) [77]. The authors found an average of 10.79 mast cells/mm^2 in the interstitial space of the cortex, with almost no mastocytes being found in the glomerular or vascular compartments [77].

While there are not many studies, mastocytes have been proven in some series to be important in the setting of chronic rejection, with the levels of mastocytes correlating with the extent of interstitial fibrosis and tubular atrophy, with the decline in graft function and also with the time after transplantation [80–82]. In a transcriptomic study from the Edmonton group that analyzed 129 for-cause biopsies from 104 patients, it was shown that there is a correlation between the levels of mastocyte-associated transcripts and the extent of chronicity Banff scores as well as a worse graft prognosis [83]. Moreover, biopsies that had a low level of mast cell transcripts had a better graft survival.

Eosinophils

Eosinophils are usually considered as aggressive cytotoxic leukocytes involved in the innate defense system, seen in diverse conditions such as allergic diseases, autoimmune diseases and parasite infection [84]. Their scarcity in kidney biopsies makes them difficult to study and therefore, their exact role in alloimmunity has remained controversial and poorly understood [85].

In the context of acute rejection, their role remains uncertain although some reports find a connection between blood eosinophilia and the diagnosis of rejection [84, 86]. Moreover, old studies have shown accumulation of eosinophils in the graft in a context of vascular rejection, although conflicting results have been published since then [87–89]. In an older study reported by Hongwei et al. using manual counting and a carbol chromotrope staining protocol, the density of eosinophils was much higher in cases with acute rejection (0.4–1.1 cells/μm^2) when compared to cases with no rejection (less than 1 cell/μm^2). Interestingly,

biopsies diagnosed with rejection that progressed to graft loss had a higher density when compared to those who did not (1.9 vs. 0.2 cells μm^2, p = 0.014) [88]. Vanikar et al. showed in a more recent study performed on 1,217 kidney transplant biopsies by using hematoxylin and eosin-stained slides that the presence of tissue eosinophilia (defined as ≥4% eosinophils in the interstitium) was associated with poor graft outcomes [90].

In a study done by Nolan et al. on allograft nephrectomy specimens using epifluorescence, the authors highlighted the presence of eosinophils in the intima, in the adventitia of vascular walls and in the interstitium in 73%, 80%, and 87% of chronic rejection cases respectively [91]. Furthermore, they showed that the medium from cultured eosinophils stimulates DNA synthesis of vascular smooth muscle cells, therefore indicating a potential role of eosinophils in the development of chronic vascular lesions in the allograft [91].

Neutrophils

Neutrophils can be seen as a link between the innate and the adaptative immunity [92]. Furthermore, they can elicit opposite functions in the immune response, from one extreme (anti-inflammatory and regulatory) to the other (pro-inflammatory) [92]. In acute rejection, neutrophils are activated by endothelial cells and then, after crossing the vessel walls, are involved in the destructive release of reactive oxygen species or in programmed cell death [93–95]. Neutrophil depletion experiments have indeed revealed the importance of neutrophils in promoting alloimmune responses. For example, in a mouse skin transplant model neutrophil depletion mitigated the acute rejection by attenuating the recruitment of alloreactive memory $CD8^+$ T cells [96]. Neutrophils may stimulate the recruitment of activated $CD8^+$ T cells through their expression Fas ligand, which can induce expression of the T cell chemoattractant CCL1, CCL2 and CCL5 [97]. In AMR, little is known about the mechanisms of neutrophil activation, even though neutrophilia has been observed as a sign of ongoing rejection [92]. The Banff classification specifies that an asterisk shall be added to Banff Lesion Score "i" (e.g., "i1*"), if there are more than 5%–10% of eosinophils, neutrophils or plasma cells [4].

In the setting of chronic rejection, neutrophils can sometimes be observed, but unfortunately data regarding their proportion and the exact mechanisms by which they promote inflammation are poorly described [92]. The current view is that neutrophils accumulation is driven by IL-8 and IL-17-dependent chemotactic pathways, and get activated by the exposure to damage associated molecular patterns [98, 99].

Follicular Dendritic Cells

FDCs are the most effective antigen presenting cells in mice and humans and can be found in both lymphoid and non-lymphoid tissues [100]. In the kidney, as for other tissues, FDCs are derived from bone marrow-derived hematopoietic stem cells [101]. Until now, only a few studies investigated the link between the presence of FDCs in transplanted kidneys and the allograft survival. Using IHC, Batal et al. stained CD209+ DCs in 105 allograft biopsies from kidney transplant recipients with various diagnosis (TCMR, AMR, mixed rejection and others) [102]. They found an association between a high dendritic cell density and a poor graft survival and localized these cells mainly in the interstitium, occasionally in the peritubular capillaries and rarely in the tubules, glomeruli or arteries [102]. Yazdani et al. found an increase in FDCs-associated genes in AMR and TCMR compared to patients without rejection, but no differences between AMR and TCMR patients [36]. In a murine model of kidney transplantation, Zhuang et al. confirmed that donor DCs were mainly replaced by recipient FDCs originating from non-classical monocytes 7 days after transplantation [103]. Depletion of these recipient FDCs by diphtheria toxin significantly prolonged graft survival compared to controls injected with PBS [103]. Although studies on animal models shed some light on the function of FDCs, studies on human kidney allografts are still lacking and will be necessary to truly understand their role in kidney allograft rejection.

Another aspect of FDCs is their capacity to induce rejection but also tolerance. Tolerogenic FDCs, also called FDCregs, can suppress the function of T cells or provide a weak stimulation. They are also involved in the generation of induced Tregs [104]. Therefore, cell-based immunotherapy with tolerogenic FDCs is now recognized as a promising approach to increase the survival time of grafts and to reduce the use of immunosuppressor treatments. Moreau et al. published in 2023 the results of the first phase I/II clinical trial using autologous tolerogenic FDCs (ATDC) immunotherapy in kidney transplant recipients [105]. Eight patients received ATDC the day before transplantation in conjunction with standard steroids, mycophenolate mofetil and tacrolimus immunosuppression. The control group composed of 9 patients received the same standard immunosuppression, with ATDC replaced by basiliximab induction. In both groups of patients no death occurred, the graft survival was 100% at 3 years and there were no adverse events related to ATDC infusion. Furthermore, monitoring of circulating immune cells in patients reported no increase of activated CD8 T cells in the ATDC group when compared to the reference group [105].

Involvement of FDCs in ischemia-reperfusion, rejection and tolerance makes them difficult to characterize and their role in each process still needs to be clarified. Upcoming studies will have to carefully choose the markers used to discriminate all the subpopulations of FDCs to clearly identify their specific role in each process. In rejection, a better characterization of their localization inside the nephron will also help to disclose their contribution to the alloimmune response.

IMPACT ON TREATMENT

The incidence of diagnosed TCMR episodes has significantly decreased due to advances in effective therapeutic options. Consequently, research efforts have shifted toward addressing AMR [5]. Despite ongoing advancements in our understanding of AMR, the development of novel therapies targeting acute episodes and preventing chronic lesion formation remains limited [106]. As highlighted earlier in this review, AMR involves a variety of cellular types, contributing to its complexity. This cellular heterogeneity presents both

opportunities and challenges for the development of effective therapies.

Notable progress has been made in targeting AMR, particularly with felzartamab, an anti-CD38 monoclonal antibody that acts on plasma cell and NK cells [107]. In a recent trial, 22 patients diagnosed with AMR were randomized to receive either felzartamab or a placebo. After 24 weeks, patients in the felzartamab group demonstrated significantly lower MVI scores (0 vs. 2.5) and reduced levels of donor-derived cell-free DNA (0.31% vs. 0.82%), compared to the placebo group [107]. Although these promising results have sparkled significant interest in CD38 targeting strategies, they have also raised critical questions regarding the underlying mechanisms of action. Notably, the marked improvement in intra-graft inflammation scores observed with treatment contrasts sharply with the minimal, if any, effect on DSA levels. This discrepancy suggests that the therapeutic efficacy may not primarily rely on modulating antibody responses, but rather on targeting other CD38-expressing effector cells, with NK cells emerging as main culprits. Further research is needed to elucidate the composition of AMR-associated inflammatory infiltrates before and after anti-CD38 therapies.

The interleukin-6 (IL-6) signaling pathway, which is critical in the maturation of B cells into plasma cells, has also been explored as a therapeutic target in AMR [108]. For instance, clazakimumab, an anti-IL6 antibody, was tested in patients with CA AMR. However, the trial was discontinued due to lack of efficacy [108, 109]. Additionally, a humanized anti-IL-6 receptor antibody, tocilizumab, is currently being evaluated in the INTERCEPT trial, which involves 50 patients with an established diagnosis of CA AMR [110].

SHOULD WE STILL CLASSIFY REJECTION INTO CELLULAR OR HUMORAL?

The current literature demonstrates that many immune cell types with various densities and proportions are involved in kidney allograft rejection (**Figure 3**). Different patterns of injury can be histologically distinguished, according to the localization of the cells (i.e., within the capillaries or mainly within the interstitium). However, several studies have demonstrated that the cell type composition and the molecular pattern may be similar across different histological types of rejection. Furthermore, for the same type of histological rejection, the nature and the proportion of the cells may be highly variable from an individual to another [19]. Given the fact that the presence or absence of different cell types may carry significative prognostic impact, this raises the question whether we should classify rejection only based on the localization of the cells (i.e., in the interstitial compartment or in the vascular compartment) rather than on the type and quantity of cells involved. For example, Azad et al. described a panel consisting of 3 genes that were common for both AMR and TCMR and that correlated with the degree of injury [111]. Using transcriptomic data obtained from 1,571 renal biopsies, the group found that pro-inflammatory macrophages correlated with the presence of a common rejection module [111]. This suggests that there are common pathways in both TCMR and AMR, further questioning the rationale and somehow arbitrary separation between these 2 types of rejection. In the transcriptomic study from Shah et al., the authors showed overlap and differences in the genes expressed in different rejection phenotypes [46]. Active AMR, chronic AMR and TCMR shared 117 genes while expressing also 231, 60, and 114 different genes respectively when compared to normal biopsies [46]. Interestingly, this study has shown that there are more similarities between chronic AMR and TCMR than between chronic and active AMR [46]. Furthermore, as already mentioned, our previous study using multiplex IF has shown similar composition of $CD3^+$ T cells, NK cells and CD163+ macrophages in biopsies with different types of rejection [19].

In conclusion, as different types of transcriptomic data and cell counting techniques emerge and become more readily available, further studies will probably further elucidate the common and different pathways encountered in different rejection settings, and help to better understand the pathophysiology of rejection. Moreover, the precise description of molecular pathways and cells involved in rejection episodes may help assess the prognosis more accurately after rejection and guide treatment, by developing cell-specific therapies rather than global immunosuppressive therapies. Whether cellular composition will be implemented in the Banff classification to refine the categorization of different rejection types has yet to be clarified.

AUTHOR CONTRIBUTIONS

GT-B and MR participed in the design of the manuscript. GT-B and EL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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REVIEW
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Novel Aspects of Immunogenetics and Post-Transplant Events in Kidney Transplantation

Ilkka Helanterä[1]*, *Salla Markkinen*[2], *Jukka Partanen*[2] and *Kati Hyvärinen*[2]

[1]Transplantation and Liver Surgery, Helsinki University Hospital, University of Helsinki, Helsinki, Finland, [2]Finnish Red Cross Blood Service, Helsinki, Finland

HLA typing and matching have been crucial in kidney transplantation, but methods for assessing tissue histocompatibility have advanced significantly. While serological-level HLA typing remains common, it captures only a small fraction of true HLA variation, and molecular matching is already replacing traditional HLA matching. Recent studies have expanded our understanding of genetic tissue compatibility beyond HLA loci. Candidate gene analyses and genome-wide association studies (GWAS) have identified genetic factors linked to post-transplant complications, though replication of these findings is challenging. An alternative approach involves genome-wide matching of genes or genetic variations. This method has shown promise in hematopoietic stem cell and kidney transplantation. For instance, homozygous gene deletions in LIMS1 or complement factor H (CFH) genes have been associated with acute rejection risk. This may be due to alloimmune responses against proteins absent in the patient but present in the graft, or due to the missing protein's function. Genetic studies in clinical medicine face challenges due to the interplay of genetic and environmental factors, necessitating large datasets for meaningful associations. International collaboration and large consortia, like iGeneTRAin, are essential for validating findings and advancing the field. This review highlights recent advancements in immunogenetics and tissue histocompatibility, emphasizing future research directions.

Keywords: kidney transplantation, HLA matching, genome-wide association study, polygenic risk score, candidate gene analyses

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***Correspondence**
Ilkka Helanterä,
ilkka.helantera@helsinki.fi

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INTRODUCTION

Although human leukocyte antigen (HLA) typing and matching have been key elements of kidney transplantation since the early days, the knowledge on HLA molecules and HLA matching, and methods used for assessing tissue histocompatibility have evolved enormously during the past decades. Serological-level HLA typing is still used for organ allocation in many programs, and although it is a highly simplified view of tissue histocompatibility, robust evidence still supports the association of serological HLA mismatch with kidney graft outcomes. For example, large registry reports from the Collaborative Transplant Study (CTS), including mainly European transplant centers, or from United Network for Organ Sharing (UNOS) from the US demonstrate a strong independent association between HLA-matching at the *HLA-A, -B* and *-DBR1* loci and graft and patient outcomes, independent of donor type, initial immunosuppression, or transplant era [1, 2].

With increasing frequency of high-resolution HLA typing, it is well known that traditional HLA matching captures only a small fraction of actual HLA variation. Immunogenicity of HLA molecules is mediated through epitopes, groups of amino acid residues recognized by HLA antibodies. Perhaps a biologically more plausible and relevant method to quantify differences between recipient and donor tissue types is to look at molecular mismatch of the smallest functional units of epitopes, the so called eplets [3]. Eplets are small clusters of amino acids identified on the surface across HLA molecules. Instead of directly comparing amino acid sequence differences between the whole HLA molecules, eplet or molecular mismatch can be quantified using a computer algorithm [4]. Evidence suggests that molecular mismatches, especially in HLA-DR and -DQ, are associated with many immunological outcomes after kidney transplantation [5, 6].

In addition to the traditional HLA typing or molecular matching, recent studies have shed light on more comprehensive aspects of genome level tissue histocompatibility, outside HLA genes and reaching far beyond. The aim of this review is to provide an overview of the novel aspects non-HLA-related alloimmunity, immunogenetics, and tissue histocompatibility in light of recent research findings, and discuss some future considerations that would help the field proceed forward with the ever-increasing amount of genetic information related to kidney transplant outcomes.

NON-HLA ANTIBODIES AND ALLOIMMUNE RESPONSES

Non-HLA-related molecules have significance in alloimmunity, as acute antibody-mediated rejection (AMR) has been described in HLA identical siblings [7], and as AMR-related changes have also been detected in patients without HLA antibodies [8]. Indeed, one way to analyze the significance of histocompatibility antigens in kidney transplantation is the ability of the recipient to produce pathological antibodies against donor tissues. Several non-HLA donor-specific antibodies (DSA) have been identified, such as angiotensin II type 1 receptor (AT1R)- antibodies [9, 10], endothelial cell antibodies [11], and others [12], but their clinical significance has been somewhat questionable [12]. Not all non-HLA antibodies are in fact allo-antibodies, e.g., AT1R antibodies are classically considered auto-antibodies.

A study by Senev *et al.* studied the clinical significance of 13 different pretransplant non-HLA antibodies and was able to show that only antibodies against Rho GDP-dissociation inhibitor 2 (ARHGDIB), a minor histocompatibility antigen, were associated with graft failure in univariable and multivariable models, with an increased risk among patients with also HLA DSA. In addition, increased intrarenal expression of the *ARHGDIB* gene was seen among patients with AMR, although none of the non-HLA antibodies were independently associated with the risk of ABMR [13]. A recent study by Carapito *et al.* investigated the role of the MICA gene matching, located near the HLA B gene in the major histocompatibility complex (MHC) segment, as a candidate histocompatibility locus. Their study showed that mismatches of MICA alleles were associated with reduced graft survival, and anti-MICA DSA were associated with ABMR [14]. Although the MICA gene is located close to HLA B gene that often is matched in kidney transplantation and there is relatively strong linkage disequilibrium between these two genes, in this study the association of MICA mismatches with worse graft survival was HLA-independent and HLA-B independent. The clinical use of non-HLA antibody analytics is still somewhat controversial and routine monitoring is not recommended. Perhaps the best characterized phenotype is with AT1R-antibodies, and in case HLA-DSA-negative ABMR is detected after kidney transplantation, AT1R-antibodies could be measured and therapy with angiotensin receptor blockade considered in case of detection of antibodies [12]. It should be noted, however, that microvascular inflammation in the kidney transplant can be based on other mechanisms as well, and not only antibody-mediated. In addition to their possible clinical applications, non-HLA antibody targets could potentially be candidates for future genome-level matching studies.

CANDIDATE GENE ANALYSES

Early attempts to identify the association between non-HLA genetic variance and transplant outcomes come from studies that focus on candidate gene analyses, such as known polymorphisms in genes that influence inflammatory responses or drug metabolism [15, 16]. They have mostly focused on recipient genetic variation, as many cohorts may not have donor DNA available for analyses. Many studies are limited by small sample size, focusing on a single polymorphism or gene only, and lack external validation cohorts. **Table 1** summarizes some studies of candidate gene – analyses. One of the best characterized single-gene associations with graft survival is genetic variations in apolipoprotein L1 (APOL1). Risk genotypes in *APOL1* in African Americans have been associated with the increased risk of end-stage kidney disease (ESKD) in native kidneys [30], and *APOL1* risk alleles in deceased donors have similarly been identified as a risk factors for graft loss [26, 27]. The association is thought to be mediated via kidney-related mechanisms of *APOL1* risk genotypes, but a recent study in African American recipients showed that also recipient risk alleles are associated with a higher risk of graft loss and identified immunomodulatory mechanisms behind this association [25]. Another interesting genetic aspect is the genetic findings seen in Natural Killer (NK) cell functionalities, which are involved especially in AMR responses. KLRC2 gene deletion variants, which determine the activating receptor NKG2C expression, were associated with microvascular inflammation and AMR-associated gene expression patterns, but the findings observed in a highly selected cohort of DSA positive patients did not translate into graft survival differences in a large multicenter cohort [28, 31]. In another study, FCGR2C Q^{13} in addition to FCGR3A V^{176} was a significant risk allele that could enhance NK

TABLE 1 | Selected studies of candidate gene analyses and kidney transplant outcomes.

Gene/SNP, year	Clinical outcome	Number of patients analyzed	Main finding
Recipient TNFA, IL-10 [15], 1999	Rejection episodes, rejection severity, steroid responsiveness	100	TNF-alpha and IL-10 genotypes were associated with the risk of multiple rejection episodes or steroid-resistant rejection
Recipient CYP3A5 [16], 2003	Tacrolimus dose	80	CYP3A5 genotype predicted tacrolimus dose
Recipient TCF7L2 [17], 2009	New-onset diabetes after transplantation (NODAT)	1,076	Out of 11 polymorphisms, TCF7L2 was independently associated with NODAT
Recipient Exploratory analysis [18], 2010	Acute rejection	990	15 candidate SNPs identified, which were associated with acute rejection and 15 SNPs identified, which were associated with severity of tubulitis
Recipient IFN-gamma [19], 2010	Acute rejection, chronic allograft nephropathy	74	IFN-gamma genotype was associated with acute rejection and chronic allograft nephropathy
Recipient FOXP3 [20], 2013	Acute rejection, death-censored allograft loss	599	FOXP3 genotype was associated with allograft survival
Donor CAV1 [21], 2010	Death-censored graft failure	785 donors discovery, 697 donors validation	the CAV1 rs4730751 SNP was associated with allograft failure
Donor ABCB1, Donor CAV1 [22], 2015	Death-censored graft survival	682 donors, 1,233 recipients	ABCB1 was associated with shorter graft survival
Recipient LIMS1 [23], 2021	TCMR, ABMR, allograft survival	841	LIMS1 risk genotype associated with increased risk of TCMR
Recipient APOL1 [24], 2021	Long-term allograft outcomes	119	Among African-american recipients no association detected with APOL1 genotype and transplant outcomes
Recipient APOL1 [25], 2021	TCMR, death-censored allograft loss	507	APOL1 risk alleles were associated with death-censored graft- loss and TCMR
Donor APOL1 [26],2011	Graft survival	106 donors, 136 recipients	APOL1 risk alleles in African-American deceased donors were associated with higher risk of graft failure
Donor APOL1 [27], 2015	Graft survival	368 donors, 675 recipients	APOL1 risk alleles in African-American deceased donors were associated with higher risk of graft failure
Recipient KLRC2$^{wt/del}$ variants [28], 2022	MVI, Graft survival	86 DSA+ recipients, and 1860 randomly selected recipients	KLRC2$^{wt/wt}$ was associated with MVI in the smaller cohort, but no association with graft survival
Recipient FCGR3A$^{V/F158}$, KLRC2$^{wt/del}$, KLRK1$^{HNK/LN}$ rs9916629-C/T	MVI, Graft survival	86 DSA+ recipients	Only KLRC2$^{wt/wt}$ was associated with MVI, but not graft survival
Recipient FCGR2C Q^{13}/STP^{13} and FCGR3A V^{176}/F^{176} [29], 2024	MVI, Graft Survival	242 recipients	Q^{13} and V^{176} were associated with worse graft survival, and Q^{13} with MVI

ABCB1, multidrug resistance protein 1; CAV1, Caveolin-1; ATR, serine/threonine kinase; APOL1, apolipoprotein L1; FOXP3, forkhead box P3; IFNG, interferon gamma; IL15RA; LIMS1, LIM and senescent cell antigen-like-containing domain protein 1; SNP, single nucleotide polymorphism; TNFA, tumor necrosis factor alpha or TNF-α; TCF7L2, transcription factor 7-like 2; MVI, microvascular inflammation.

cell-mediated antibody-dependent cellular cytotoxicity and contribute to allograft injury and poor survival [29]. Unfortunately, replication studies with large cohorts are missing for most of the reported associations. As positive candidate gene results have not been confirmed in the genome wide associations studies (below), there may also be publication bias toward positive findings. A systematic multi-cohort replication study of the reported associations is clearly warranted for critical evaluation of these results. If validated and proven predictive of higher risk of graft loss, identifying risk genotypes would possibly allow closer follow-up of risk patients, or help in improving organ allocation.

GENOME-WIDE ASSOCIATION STUDIES (GWASS)

In contrast to single or candidate gene analyses, genome-wide association studies (GWAS) present an alternative approach to identify genetic variations associated with the outcome of interest [32]. With GWAS, millions of SNPs scattered across the genome can be analyzed. GWAS studies in other diseases have been useful in identifying novel risk factors and new mechanistic pathways [33–36], but the success of GWAS studies in the field of transplantation has been quite limited, mostly due to lack of adequately powered cohorts and lack of external validation and replication of the findings. As millions of SNPs are studied and as the study cohorts have heterogeneity in diagnoses and transplant procedures and treatments, thousands of recipient-donor pairs are needed for a statistically sufficient power. Usually associations with p-values clearly below 10exp-8 are regarded as indicative. **Table 2** summarizes some GWAS studies within the field of kidney transplantation.

The first recipient-only GWAS for kidney allograft survival included 326 European transplants [37], and the study found two variants of interest showing genome-wide significance. The identified SNPs were independently associated with long-term

TABLE 2 | Selected genome-wide association studies in the field of kidney transplantations.

Associated gene/SNP	Clinical association	Sample size and cohort type
TRA, ZNF516 [37]	Long-term graft function and survival	326 recipients, discovery
Validation of TRA and ZNF516 (see above), no association confirmed [38]	Death-censored graft loss, all-cause mortality	1,638 recipients, discovery
ATP5F1P6 [39]	New-onset diabetes after transplantation	256 recipients, discovery and 441 validation
LINC00882, CACNA1D, CSMD1 [40]	Cutaneous squamous cell carcinoma after transplantation	388 recipients, discovery
CYP3A5*6, CYP3A5*7 [41]	Tacrolimus pharmacokinetics	197 recipients, discovery and 160 validation
PTPRO, CCDC67 [42]	Acute rejection	778 recipients, discovery and 844 validation
No association detected outside HLA loci [43]	Long- and short-term allograft survival	2094 recipients and donors, discovery and 5,866 validation
41 donor SNPs found that contributed independently or interacted with APOL1 [44]	Death-censored graft survival	532 AA donors (978 recipients) discovery, 250 AA donors (465 recipients) validation

ATP5F1P6, ATP, synthase, H+ transporting, Mitochondrial Fo Complex, Subunit B1 Pseudogene 6; CACNA1D, Calcium Voltage-Gated Channel Subunit Alpha1 D; CCDC67, Coiled-Coil Domain-Containing Protein 67; CSMD1, CUB And Sushi Multiple Domains 1; CYP3A5, cytochrome P450; LIMS1, LIM and senescent cell antigen-like-containing domain protein 1; LINC00882, Long Intergenic Non-Protein Coding RNA 882, PTPRO, Protein Tyrosine Phosphatase Receptor Type O; TRA, T-cell receptor alpha; ZNF516, Zinc Finger Protein 516; ZSCAN25, Zinc Finger And SCAN, Domain Containing 25.

graft function and survival. A larger study tried to confirm these findings in a multicenter cohort but failed to show any association of the identified SNPs with graft survival [38].

Another recipient GWAS analyzed 275 European cases of T-cell-mediated rejection and 503 controls, identifying five candidate loci. In a validation cohort of 313 cases and 521 controls, two loci remained significantly independently associated with acute rejection [42]. One locus encompassed *PTPRO* gene, coding for a receptor-type tyrosine kinase essential for B cell receptor signaling, the other ciliary gene *CCDC67*, essential in the functions of immune synapse and primary cilium. These functionally interesting findings could, however, not be validated in a large external cohort [45]. The largest genome-wide association study published to date to our knowledge involved 2,094 kidney transplant recipients and their donors, and a validation cohort of 5,866 pairs. This study could not find any strong donor or recipient genetic associations outside HLA region contributing to long- or short-term allograft survival [43].

These discrepancies in findings highlight the importance of adequate validation of exploratory findings in different cohorts, preferably with a different genetic background. Both kidney transplant rejection and graft failure are highly multifactorial; both recipient and donor characteristics, many perioperative events in both donor and recipient, and many posttransplant events modify the alloimmune risk, graft function, and survival prognosis, and it would require very extensive multicontinental cohorts to show the association of any weak genetic signals. Based on the published evidence so far, it seems unlikely that single genetic polymorphism loci could be identified that would largely explain variation in graft outcomes.

POLYGENIC RISK SCORES

As the effect of any individual single gene variation on transplantation outcome can be assumed to be very small, polygenic risk score (PRS) type of summary statistics may also prove to be informative in risk assessment. Polygenic risk scores (PRS) utilize and combine the existing GWAS findings to determine disease risk based on genetic variance [46]. With PRS, genome-wide genotype data are computed into a single variable of individual-level risk score. PRS approach has already been applied to variety of traits for common diseases. For example, a study by Khera et al [47] developed and validated PRS for five common diseases, showing that highest PRSs for complex multifactorial diseases identify risk levels close to those typically seen in single-gene Mendelian diseases. Their approach identified 8.0%, 6.1%, 3.5%, 3.2%, and 1.5% of the population at greater than three-fold increased risk for coronary artery disease, atrial fibrillation, type 2 diabetes, inflammatory bowel disease, and breast cancer, respectively.

PRS approach has also been studied for the risk of chronic kidney disease (CKD). Khan et el. combined *APOL1* risk genotypes with GWAS data of kidney function, and designed, optimized, and validated a PRS for CKD. The PRS was then tested in 15 independent cohorts, including 3 cohorts of European ancestry (n = 97,050), 6 cohorts of African ancestry (n = 14,544), 4 cohorts of Asian ancestry (n = 8,625) and 2 admixed Latin American cohorts (n = 3,625). The top 2% of the PRS was associated with nearly threefold increased risk of CKD across ancestries [48]. Attempts have also been made to predict native kidney function employing PRS approach. However, the ability of these scores to explain phenotypic variance in kidney function in native kidneys has been limited, e.g., below <4% in a study by Gorski et al [49].

Application of polygenic risk scores could be of potential value when evaluating the association of genetic variance with kidney transplant function or survival. To date, relatively few PRS studies in the field of kidney transplantation have been performed to our knowledge (**Table 3**).

The largest PRS study in the field of kidney transplantations analyzed the association of PRS, calculated using genetic variants associated with non-transplant eGFR, with posttransplant eGFR in a cohort of 10,844 donor-recipient pairs [51]. The polygenic

TABLE 3 | Selected polygenic risk score (PRS) studies in the field of kidney transplantations.

PRS trait	Clinical outcome	Sample size and cohort type	Main finding
Non-transplant NMSC, SCC and BCC [50]	Time to post-transplant NMSC	899 kidney recipients	PRS for non-transplant NMSC was predictive of case/control status and time to post-transplant NMSC
Posttransplant eGFR [51]	1-year and 5-years after transplantation, and change between 1 and 5 years eGFR	10,844 kidney recipients and donors	32% of the variability in eGFR at 1-year was explained by the model, with only 0.3% contributed by the PRS
Non-transplant BCC and SCC [52]	Post-transplant BCC and SCC	1,272 kidney recipients	PRS improves prediction over traditional skin cancer risk factors by 3% for BCC, but not for SCC
Non-transplant type 2 diabetes [53]	New-onset diabetes after transplantation	2,062 kidney recipients and 533 donors; 1,581 liver recipients and 1,555 donors	Recipient T2D PRS was associated with pre-transplant T2D and the development of PTDM. Combined liver donor and recipient T2D PRS improved PTDM prediction > 5% compared to a model with only clinical characteristic
Hypertension, stroke, and intracranial aneurysms (IA) [54]	Deceased donor age of death, graft function after transplantation	6,666 donor-recipient pairs	Donor PRS for hypertension was associated with reduced long term graft survival, and donor PRSs for hypertension and intracranial aneurysm were associated with reduced recipient eGFR at 1 year

BCC, basal cell carcinoma; eGFR, estimated glomerular filtration rate; NMSC, non-melanoma skin cancer; SCC, squamous cell carcinoma; T2D, type 2 diabetes; PTDM, posttransplant diabetes mellitus.

risk score was applied on both donors and recipients to predict kidney transplant function at 1 and 5 ears, in addition to change in eGFR post-transplantation. In this study, PRS calculated using the recipient's genotype alone, as well as combined donor and recipient genotypes were significantly associated with eGFR at 1-year posttransplant. When the donor-recipient PRS was combined with clinical predictors of graft function and principal components, 32% of the variability in 1-year estimated GFR could be explained by the model. However, only 0.3% of the variation was contributed by the PRS. None of the PRS were significant predictors of graft function at 5 years.

In another study, Stapleton *et al.* examined the risk of non-melanoma skin cancer (NMSC) among 889 European ancestry kidney transplant recipients [50]. Genetic variants from previously published squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) non-transplant GWAS was used for PRS, which was shown to be predictive of NMSC status and time to NMSC post-transplant.

Seviiri *et al.* generated PRSs from the general population, and studied whether these PRSs could predict and stratify the risks of BCC and SCC in a cohort of 1,272 solid-organ transplant recipients [52]. In this study, PRS was independently associated with both BCC and SCC. However, when combined with traditional skin cancer risk factors, no additive predictive value of PRS was seen in SCC, and in BCC the prediction was improved only with 3%.

A study by Shaked and colleagues looked for the association of post-transplant diabetes mellitus (PTDM) with type 2 diabetes (T2D) PRS in 1,581 liver recipients and their 1,555 donors, and 2,062 kidney recipients and 533 donors [53]. They examined whether recipient genomics contribute to PTDM development and were able to demonstrate that recipient T2D PRS is a predictor of PTDM, independently of known clinical predictors of PTDM.

A recent study by Collins *et al.* employed a multicenter dataset of 6,666 deceased and living kidney donors from 7 different European ancestry transplant cohorts, and investigated the association of polygenic risk scores for cerebrovascular disease risk factors [hypertension, stroke, and intracranial aneurysm (IA)] on deceased donor age of death, and kidney graft survival, and graft function [54]. Deceased kidney donors had an elevated genetic burden for hypertension and IA compared to living donors and healthy controls, and this burden was associated with donor age of death among donors who died of stroke. In addition, increased donor polygenic risk for hypertension and IA was associated with reduced graft function at 1 year [54].

As shown above, polygenic risk score approaches can be useful in defining the burden of genetic risk also after transplantation, but as expected by the various possible clinical scenarios and posttransplant events, the contribution of genetic risk in predicting posttransplant events remains limited and is not yet ready for clinical use. PRS approaches for predicting higher risk for cancer or acute rejection could be applied in the clinical setting for example for mandating closer follow-up (e.g., for the risk of skin malignancies), or tailoring individualized immunosuppression. One of the main problems with the PRS approaches is, however, that although they can possibly identify effectively the small cohort of patients at highest risk, they usually fail to identify the majority of the patients who experience the particular event of interest.

GENOME-WIDE MISMATCH STUDIES

Matching of HLA alleles is the golden standard in kidney transplantation. However, any genetic differences, or mismatches, in proteins expressed in the transplanted kidney can be recognized as immunologically foreign, leading to immune activation and increased risk of rejection. With the emergence of powerful genome analysis tools, these differences can now be identified. Mismatches in these so-called minor

TABLE 4 | Donor-recipient genetic mismatch studies in the field of kidney transplantations.

Mismatches studied (found gene)	Clinical outcome	Sample size and cohort type	Main findings
Amino-acid mismatches in transmembrane proteins [56]	Long-term allograft outcome measured by eGFR	53 donor-recipient pairs	Allogenic mismatch score was independently associated with posttransplant eGFR
All genome-wide mismatches [57]	ABMR, TCMR	28 donor-recipient pairs	non-HLA mismatch variants were associated with AMR
nsSNPs mismatches in transmembrane and secretory proteins [58]	Graft loss	477 donor-recipient pairs	The degree of nsSNP mismatch was associated with graft loss, independently of HLA incompatibility
Mismatches in genome-shared identity-by-descent SNPs [59]	Death-censored allograft loss	385 donor-recipient pairs	Genome-shared identity-by-descent was associated with graft survival, independent of HLA mismatches, and with early vascular intimal fibrosis
50 deletion-tagging SNPs (LIMS1) [61]	Kidney allograft rejection	705 kidney transplant recipients and 2,004 donor-recipient pairs	Genomic collision at LIMS1 locus was associated with rejection and with production of anti-LIMS1 IgG2 and IgG3
Non-HLA mismatches at variant-, gene-, and genome-wide scales (LIMS1) [62]	Death-censored graft loss	385 and 146 donor-recipient pairs	Mismatch at the LIMS1 locus was associated with graft loss. The deletion resulted in changes of expressions of other genes with functional effects
Mismatches in kidney-related proteins, CFHR- deletion [63]	Acute rejection	1,025 recipient-donor pairs	Sums of kidney-related proteins were associated with acute rejection in unadjusted analyses. A mismatch in CFHR deletion was associated with acute rejection

ABMR, antibody-mediated rejection; eGFR, estimated glomerular filtration rate; nsSNP, nonsynonymous single nucleotide polymorphism; TCMR, t-cell mediated rejection.

histocompatibility antigens have also been hypothesized to increase the risk of graft rejection and failure. In fact, cumulatively the minor histocompatibility antigens constitute a much larger pool of genetic differences between the donor and the recipient. Martin *et al.* were one of the first to use genome-wide single nucleotide polymorphism (SNP) arrays to predict amino acid differences between hematopoietic stem cell transplantation (HSCT) donors and recipients based on 19,104 coding SNPs. In HLA-matched sibling transplants, mismatches in coding SNPs were associated with an increased risk of graft-versus-host disease [55].

The role genome-wide non-HLA mismatches between kidney donor and recipient has also been studied after kidney transplantation. The study by Mesnard *et al.* was one of the first to analyze the importance of non-HLA donor-recipient mismatch in a cohort of 53 kidney transplantation donor-recipient pairs. They performed exome-sequencing for kidney transplant recipients and their living donors and estimated all the cell surface protein mismatches for each donor-recipient pair by calculating the number of amino acid mismatches in transmembrane proteins. This allogenomics mismatch score was predictive of long-term graft function, independent of HLA-A, -B, and -DR matching [56]. Pineda *et al.* tested the role of non-HLA donor-recipient mismatches in rejection in a cohort of 28 pairs, using exome-sequencing and gene expression data. They identified 123 non-HLA variants associated with mainly antibody-mediated rejection processes [57].

In a study by Reindl-Schwaighofer *et al.*, genome-wide mismatches in nonsynonymous (amino acid changing) SNPs (nsSNPs) were evaluated among 477 genotyped kidney transplant recipients and their deceased donors. The degree of nsSNP mismatch in transmembrane and secretory proteins, adjusted for HLA eplet mismatch, was independently associated with graft loss. Furthermore, customized peptide arrays were used to verify a donor-specific alloimmune responses to genetically predicted mismatched epitopes in a subset of 25 patients [58]. In a cohort of 385 donor-recipient pairs of multiethnic origins, Zhang *et al.* analyzed genetic differences between the donor and the recipient using genome-wide SNP array data, excluding the HLA region. They estimated the ancestry in each donor-recipient pair and proportion of genome-shared identity-by-descent (pIBD) between donor-recipient pairs. In donor-recipient pairs of similar ancestry, pIBD was significantly associated with graft survival, independent of HLA mismatches. In addition, pIBD was significantly associated with early vascular intimal fibrosis, which was an independent predictor of graft survival [59].

In addition to studying mismatches at SNP level, one interesting approach is to study gene deletions and their associations to kidney transplantation outcomes. It is shown that some gene deletions are common among population [60]. Individuals who inherit both deleted alleles from their parents lack the functional gene and the protein product. When such an individual receives a graft from a donor who carries at least one functional copy of that gene, recipient's immune system may recognize the protein as foreign. A study by Steers *et al.* tested this hypothesis in a discovery cohort of 705 kidney transplant recipients, and validated findings in a genomic collision model of 2004 donor-recipient pairs from three independent cohorts. Genomic collision was defined as a specific donor-recipient genotype combination, in which a recipient who was homozygous for a gene-intersecting deletion received a transplant from a nonhomozygous donor. They found that a homozygous variant rs893403, a marker for the deletion of LIMS1 gene, was associated with rejection independently of the HLA mismatch and other clinical factors. In addition, a specific antibody response against LIMS1 was identified [61].

In a recent study, Sun *et al.* performed a genome-wide SNP array for two prospective kidney transplant donor-recipient

cohorts (385 and 146 pairs) with the goal of identifying mismatches within non-HLA loci that associate with long-term death-censored graft loss (DCGL). After first confirming that donor-recipient differences resulting from SNP mismatches associate with DCGL, they searched for the mismatches across all annotated gene loci, in order to identify individual gene-level mismatches that significantly associated with increased risk of graft loss. The screening confirmed *LIMS1* as a top-ranked gene locus associated with DCGL, independent of genome-wide mismatches. Interestingly, they demonstrated that rather than leading to alloimmune response against the missing LIMS1 protein, the deletion resulted in changes of expressions of certain other genes with functional effects related to the outcome. Hence, it is not clear whether the LIMS1 gene deletion leads to alloimmune response or other downstream functional effects [62].

In our own study, we analyzed the association of the sums of whole-genome missense variant mismatches and missense mismatches in transmembrane, secretory, and kidney-related proteins, with acute rejection in a single center cohort of 1,025 kidney transplant recipients and their deceased donors. We found that sums of kidney-related proteins were associated with the risk of acute rejection in unadjusted analyses. In deletion analysis, the previously detected association with *LIMS1* deletion and acute rejection could not be confirmed in our cohort. However, a mismatch in rs7542235 genotype GG tagging a homozygous deletion at the complement factor H-related (*CFHR*) proteins locus was independently associated with acute rejection [63]. We have then further characterized 15 patients with *CFHR* deletion of various sizes, and have found that the different deletion types share the complete deletion of the *CFHR1* gene pointing to its primary role. Plasma proteomics studies showed that deletion-tagging allele is associated with altered expression of CFH/CFHR proteins, and some other proteins as well [64]. **Table 4** summarizes donor-recipient mismatch studies in the field of kidney transplantation. These studies very elegantly show that whole genome mismatch concept has proven useful in identifying novel targets of alloimmunity outside the HLA region, and may provide mechanistic targets for future studies and drug development. In addition, these findings could be applied in the clinical setting for individualizing immunosuppression or follow-up for the highest risk groups.

CONCLUSIONS AND FUTURE DIRECTIONS

A common problem with genetic studies in clinical medicine is that the complex interplay of genetic and environmental factors in defining clinical outcomes requires extensive datasets to show any genetic signal to have meaningful association with outcomes. In addition, the genetic factors predisposing to disease states may be different among different populations. Most likely the genetic factors predisposing to disease are different to those regulating the disease progression. Transplantations have some additional challenges. The indications for transplantation can be fundamentally different disease entities from genetic diseases to autoimmune or metabolic diseases. Furthermore, even if PRS models or GWAs studies could identify individuals at higher risk of graft loss or acute rejection, most of these models apply only to the highest risk individuals (top 2%–5%), and fail in sensitivity to identify the large majority of the patients at risk for these events.

Importantly, the transplant outcome always depends on two individuals. The genetic properties of donor and recipient, and their combination or mismatch play a role in predicting outcomes after transplantation, adding further complexity. Therefore, international collaboration and preferably very large multicenter international consortia, such as the iGeneTRAiN[1], are required to validate the findings, and are very important to have any large impact within our field. Although not directly related to genetics of the alloimmune response, the first clinical applications of genetic testing in transplantation come from the field of pharmacogenomics, where genetic variants were identified in thiopurine methyltransferase (TPMT), involved in azathiprione metabolism, predisposing patients to a risk of drug-induced myelosuppression [65, 66]. In addition, genetic variants in the *CYP*-metabolism system can be analyzed and this information can be used to define correct dosing of tacrolimus [67]. Future discoveries and development in genetic testing of alloimmunity could for example, allow identification of high-risk patients for closer clinical monitoring or individualized immunosuppression, or help optimizing organ allocation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CONFLICT OF INTEREST

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[1]http://igenetrain.org/

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REVIEW
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Re-Evaluating the Transplant Glomerulopathy Lesion—Beyond Donor-Specific Antibodies

Arun Chutani[1†‡], *Daniel Guevara-Pineda*[2‡], *Gabriel B. Lerner*[3] *and Madhav C. Menon*[4*]

[1]*Transplant Nephrology, Yale University of School of Medicine, New Haven, CT, United States,* [2]*Nephrology, Yale University, New Haven, CT, United States,* [3]*Renal Pathology, Yale University, New Haven, CT, United States,* [4]*Nephrology, Medicine, Research in Kidney Transplantation, Faculty in Human Translational Immunology and Translational Biomedicine, Yale School of Medicine, New Haven, CT, United States*

There have been significant advances in short-term outcomes in renal transplantation. However, longer-term graft survival has improved only minimally. After the first post-transplant year, it has been estimated that chronic allograft damage is responsible for 5% of graft loss per year. Transplant glomerulopathy (TG), a unique morphologic lesion, is reported to accompany progressive chronic allograft dysfunction in many cases. While not constituting a specific etiologic diagnosis, TG is primarily considered as a histologic manifestation of ongoing allo-immune damage from donor-specific anti-HLA alloantibodies (DSA). In this review article, we re-evaluate the existing literature on TG, with particular emphasis on the role of non-HLA-antibodies and complement-mediated injury, cell-mediated immune mechanisms, and early podocyte stress in the pathogenesis of Transplant Glomerulopathy.

Keywords: transplant glomerulopathy (TG), anti HLA antibodies, banff criteria, podocyte, C4d

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***Correspondence**
Madhav C. Menon,
madhav.menon@yale.edu

†Present address:
Arun Chutani,
Medicine at UMass Chan Medical School, Worcester, MA, United States

‡*These authors have contributed equally to this work*

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INTRODUCTION

Transplant Glomerulopathy (herein referred to as "TG"), by definition, is a morphologic description of histologic or ultrastructural alterations and not a specific clinicopathologic entity. The hallmark morphologic manifestation of TG is glomerular basement membrane double contouring, either by light or electron microscopy [1]. The term was initially described in detail by Zollinger *et al* in 1973 [2]. TG is a contributing factor in early (less common) and, particularly, late allograft loss. TG lesions, when encountered in allograft biopsies, have been repeatedly associated with poor graft outcome [3], but studies have so far not revealed any specific therapeutics that reverse or even improve outcomes in TG [3–5].

INCIDENCE AND EPIDEMIOLOGY

Studies from the French-Canadian cohort have informed the community regarding the cumulative incidence of post-transplant TG. In this study, TG was present in 5% of the 1-year protocol biopsies and was associated with poorest survival [6]. A second study reported the progressively increasing prevalence of TG with transplant vintage: 2.8% at 1 year, 6.1% at 2 years, 8.5% at 3 years, and 11.5% at 5 years [7]. This larger study from the Mayo clinic involving 582 surveillance biopsies collected over 5 years found a mean prevalence of TG near 10% with a cumulative incidence of 20% by 5 years. In this study, TG was associated with both acute and chronic Banff lesions, albeit milder TG (Cg = 1)

TABLE 1 | Banff quantitative criteria for transplant glomerulopathy (cg) (Adapted from Roufosse et al [1]).

cg0	No glomerulopathy. Double contours in <10% of peripheral capillary loops in most severely affected glomerulus
cg1	Double contours affecting up to 25% of peripheral capillary loops in the most severely affected non-sclerotic glomerulus. If noted by EM only, *cg1a* is assigned; if by light microscopy, *cg1b* is assigned
cg2	Double contours affecting 26%–50% of peripheral capillary loops in the most affected non-sclerotic glomerulus
cg3	Double contours affecting >50% of peripheral capillary loops in the most affected non-sclerotic glomerulus

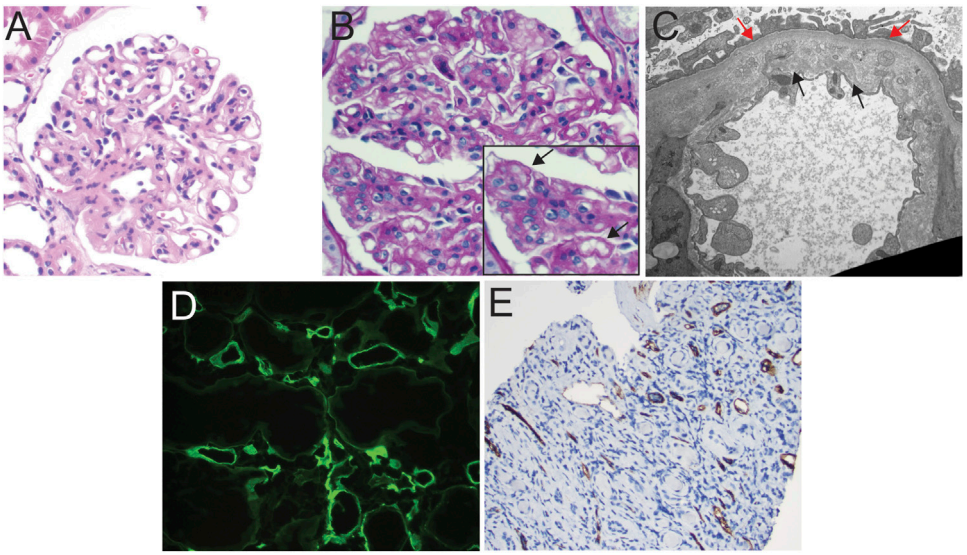

FIGURE 1 | Light microscopic and ultrastructural findings in allograft biopsies demonstrating C4d staining and transplant glomerulopathy. **(A)** Hematoxylin and eosin stain of allograft biopsy with glomerulus showing global wall thickening and increase in mesangial cellularity, suspicious for transplant glomerulopathy, 200x. **(B)** PAS stain of glomerulus demonstrating reduplication of the capillary basement membranes (see inset, black arrows), 200x. **(C)** Transmission electron photomicrograph showing expansion of the lamina rara interna by grungy variably electron-dense material, as well as areas of original (red arrows) and *de novo* (black arrows) basement membrane formation, indicative of transplant glomerulopathy, TEM, 13,900x. **(D)** Immunofluorescent staining for C4d showing diffuse circumferential positive staining in peritubular capillaries in a patient with concurrent transplant glomerulopathy on biopsy (patient developed positive Class II donor-specific antibodies ~2 years after diagnosis; 100x). **(E)** immunohistochemical staining for C4d showing diffuse positive staining in peritubular capillaries in a patient demonstrating early transplant glomerulopathy and findings indicative of chronic antibody-mediated rejection on biopsy, 100x.

was more prevalent in early surveillance biopsies (1 year) and cross-sectionally less associated with Banff chronic lesions, while higher Cg scores were more common in later surveillance biopsies. In general, higher Cg scores in a biopsy was associated with greater numbers of glomeruli samples in the biopsy being involved with TG. In a retrospective review (unpublished) of our institutional database since 2000, 189 cases with TG diagnoses were detected, which provided an incidence of TG at near 3% of allograft biopsies per year.

When TG occurs in biopsies, it has independently been associated with allograft loss [7]. A study examining causes of graft loss censored for death identified TG as the potential driving lesion in ~15% of such cases [8]. Recent meta-analyses, including >5,000 published patients, reaffirmed the increased risk of graft loss with a diagnosis of TG, potentially independent of time from transplantation, showing an estimated difference of median survival of nearly 12 years in patients with TG lesions vs. those without [9]. The prognosis between subclinical and clinical TG has also been reported to be similarly poor. However, as discussed below, within the TG diagnosis, multiple sub phenotypes and clusters may attenuate or aggravate the outcome associated with TG. Hence, together, these studies establish an early prevalence of the TG diagnosis within year-1 of near 5%, followed by a slower increase in the incidence per year of TG related to time from transplantation [7] and an association with increased risk of graft loss with the development of TG.

HISTOLOGY OF TG: CLINICO-PATHOLOGIC CORRELATIONS AND PROGNOSIS

Clinically, patients with biopsy-proven TG tend to show proteinuria, edema, a slowly rising creatinine, and shortened allograft survival. However, TG is a pathologic entity defined primarily as reduplication/multilayering of the glomerular basement membrane (**Figures 1A, B**) as observed by light and/or electron microscopy in the kidney allograft, in the absence of immune deposits. According to current Banff schema, either TG or peritubular capillary multilayering

(*ptcml*) are required features in the diagnosis of chronic active antibody-mediated rejection (cABMR) [10]. TG is defined using the *cg* scoring system, with a lesion Score *cg* > 0 requiring *de novo* basement membrane formation in at least three capillary loops by EM (cg1a), or double contours in only a single capillary loop as the minimal light microscopic finding (cg1b) after the exclusion of chronic thrombotic microangiopathy, recurrent, or *de novo* Glomerulonephritis (**Table 1**; **Figures 1B, C**). The *ptcml* lesion, defined ultrastructurally by seven or more peritubular capillary basement membrane layers in a single capillary, or five or more in at least two peritubular capillaries, has been shown to correlate with worse overall prognosis in cases of TG [11]. An increase in the mesangial matrix may be present but is less specific and is not required for diagnosis. TG can be differentiated from the recurrent and *de novo* glomerulonephritis by negative Immunofluorescence for IgG, IgA, IgM, C1q, C3, κ, and λ and absence of immune complex deposition [12]. Mesangiolysis and glomerulosclerosis may be present, mimicking focal segmental glomerulosclerosis, while microvascular inflammation, including glomerulitis (g) and/or peritubular capillaritis (ptc), may be observed in cases mediated by alloimmune mechanisms [13, 14]. Glomerular C4d is found in lesions associated with TG, and the strength of its association increases when associated with peritubular capillary C4d (**Figures 1D, E**). [15]. Glomerular C4d positivity is not a part of the current Banff criteria, i.e., C4d should be evaluated in peritubular capillaries or vasa recta only. Glomerular C4d staining could be a tool to detect a humoral etiology in cases of DSA-negative microvascular inflammation, if further validated [15–18]. Using electron microscopy (EM), features of TG can be identified as those of endothelial injury, whose definition was refined as endothelial cell enlargement, subendothelial electron-lucent widening, and subendothelial neo-densa glomerular basement membrane formation [16].

It is imperative to consider that TG changes are primarily observed on the lamina rara interna side of the GBM, indicating the role of endothelial side injury. These findings are similar to other entities associated with endothelial injury, such as chronic thrombotic microangiopathy (cTMA). Electron-dense deposits or characteristic immunofluorescence findings are not present in TG and, if present, would suggest a recurrent or *de novo* immune complex glomerulonephritis, especially immunoglobulin-mediated (type 1) membranoproliferative glomerulonephritis or a C3 glomerulopathy. TG is diagnosable in the most recent Banff criteria purely by EM in the absence of double contours by light microscopy. This requires glomerular basement membrane duplication in at least three glomerular capillaries and is designated cg1a by Banff criteria (see **Table 1**).

While the diagnosis of TG relies on a semiquantitative score identified by either light microscopy and/or EM, it oversimplifies a rather complex phenotype resulting from multiple etio-pathogenetic mechanisms. For instance, a retrospective study of 209 allograft biopsies performed for chronic allograft dysfunction identified 25 which met the pathological criteria of TG. Three partially overlapping etiologies accounted for 21 (84%) cases: C4d-positive (48%), hepatitis C-positive (36%), and chronic thrombotic microangiopathy (cTMA)-positive (32%) TG. The majority of patients with confirmed cTMA were also hepatitis C-positive, and the majority of hepatitis C-positive patients had TMA [19]. However, with the success in curing hepatitis C with anti-viral agents, HCV-related TG is expected to be insignificant in the modern context.

Pathologic features encountered in TG have also been associated with prognosis. Using a cohort of 92 patients with TG, of whom 64 developed allograft failure within 5 years of diagnosis, a prognostic index (PI) utilizing component Banff lesion scores was developed and then validated in an independent external of kidney transplant recipients with TG [20]. In this elegant study using principal component analyses, a "chronic-inflammation" score combining Banff ci, ct, and ti scores during TG was associated with time-dependent risk to graft failure. Interstitial fibrosis and tubular atrophy (Ci + Ct) have been consistently associated with poor kidney outcomes. In a recent analysis aimed at evaluating the histological risk factors for kidney allograft loss, the key finding was that, regardless of the specific cause for chronic histological damage, the presence of interstitial fibrosis and tubular atrophy had an additive and independent impact on graft outcomes, including those patients with TG [21]. One study evaluated 147 cases of either active or cABMR using activity and chronicity indices and found that a chronicity index score of 4 or greater (including components of cg score, as well as ci, ct, and cv scores) was associated with worse overall graft survival, independent of other parameters [22]. Others, however, have called into question the reproducibility of such a chronicity index [23]. Indeed, reproducibility and interobserver variability in feature identification, including the cg score, are known issues in the field of allograft pathology [24–27]. Overt evidence of DSA-mediated mechanisms on serology/pathology have shown distinct prognostic associations. In a case series of 71 patients with TG, Lesage *et* al [28] noted that eight were donor-specific antibody positive/C4d negative, six were donor-specific antibody negative/C4d positive, and only 12 were donor-specific antibody positive/C4d positive. The long-term outcomes for all three groups were similar and significantly worse than those with both Cd and donor-specific antibody negativity [28]. Serum creatinine and proteinuria at the time of biopsy have also shown to be independent risk factors for graft survival in TG, signifying co-occurrence of cumulative damage [29, 30].

POTENTIAL PATHOGENETIC MECHANISMS RELATED TO TG

Our understanding of TG continues to evolve. Many active players identified are associated with TG and are potentially incorporated as pathogenetic factors, including HLA and non-HLA antibodies, donor-specific antibodies (DSA), cell-mediated mechanisms, and podocyte stress [summarized in **Figure 2** schema]. In this section, we will discuss in greater detail the role and extent of the scope of each of these entities.

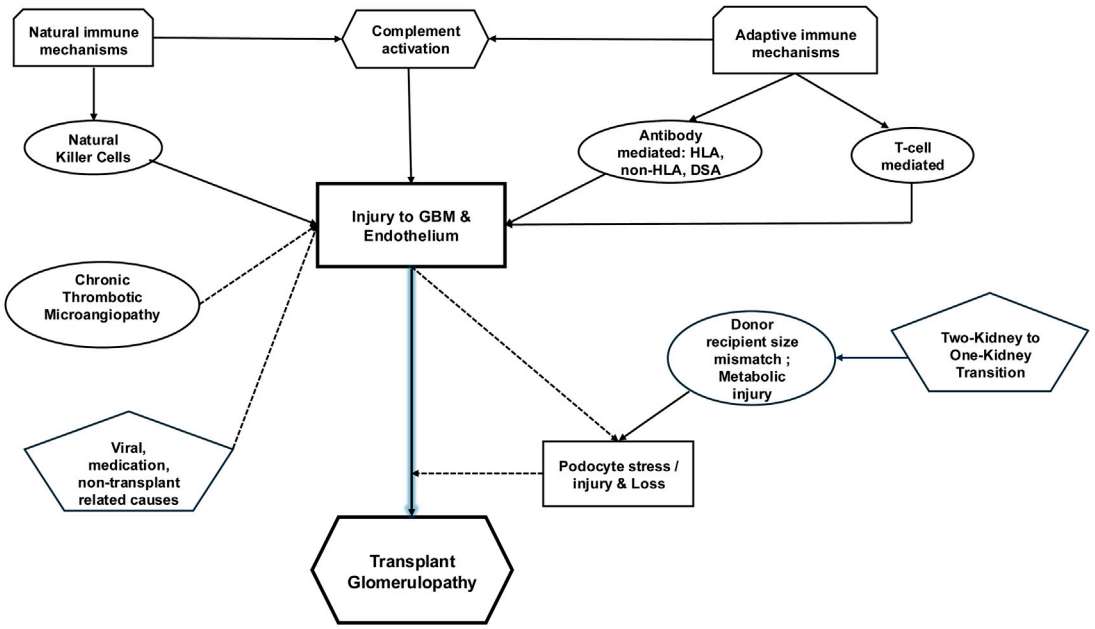

FIGURE 2 | Schematic of potential pathogenetic mechanisms related to TG. TG defined by basement membrane multilayering on the lamina rara densa side is likely initiated by immunologically mediated endothelial injury. While the role of DSA is frequently reported, selected anti-donor antibodies directed against non-HLA proteins have been incriminated. Accompanying complement mediated injury likely plays a role, along with anti-donor antibodies. A proportion of TG is unassociated with DSA and is now attributable to innate and adaptive cell-mediated mechanisms. Activated NK cells seem to be associated with TG in combination with DSA, while T-cell infiltration/activation has been observed without DSA. An understudied contributor to TG is the role played by podocyte stress from obligate glomerular hypertrophy, compounded by donor-recipient weight mismatch and metabolic or immunologic injury. Whether podocyte loss is causal in TG remains a hypothesis in need of testing. Endothelial injury from other sources (cTMA, viral injury, or medications) can cause/compound endothelial injury and multilayering.

Anti-HLA-DSA, Chronic Antibody-Mediated Rejection (cABMR), and TG

Numerous studies identify the role of DSA in TG [31–35], and TG is included in the constellation of findings that constitutes cABMR in Banff schema [16]. In our data, we observed several cases from our institution with concurrent TG and evidence of cABMR (**Figure 1**). While class I [34, 36–41] and Class II DSA-positive cases have been associated with TG [7, 34, 36, 42–44], a role for HLA Class II DSA (whether pre-transplant and *de novo*) with TG pre-eminently emerges from literature [36, 45–47]. For instance, in a study evaluating sub-phenotypes of TG from 1,036 indication biopsies, where TG was diagnosed in 53 (5.1%) cases at a median of 5 years post-transplant, the frequency of circulating anti-HLA alloantibody (both DSA and non-DSA) were connected to peritubular capillary basement membrane multilayering, peritubular capillary deposition of C4d, and double contouring of glomerular basement membranes [48]. Peritubular capillary basement membrane multilayering was present in 48 (91%) and C4d staining was detected in 18 (36%) cases of TG. The presence of anti-HLA antibodies was detected in 33 (70%), among whom 28 (85%) were DSA. Among anti-HLA antibodies, those directed against Class II (13/33) or against class I and II (17/33) were more common than those against class I (3/33) antigens. Thus, from this dataset of biopsies obtained for cause, 70% of TG has potential evidence of antibody-mediated phenomena—especially Class-II DSA. Furthermore, studies also suggest reduced 5-year graft survival with class II sensitization over Class I [36], and the presence of TG at 1 year after transplantation resulted in graft loss of approximately 30% in the former vs. 20% in Class-I-positive patients [36]. Within Class II, anti-DQ DSA has been increasingly associated with the development of TG [46, 49–51]. Coemans et al in 2021 performed a single-center cohort study including 2,761 protocol and 833 indication biopsies. Patients with pretransplant HLA-DSA were more prone to develop histology of acute antibody-mediated rejection, TG histology, or a combination of both. This manuscript provided a detailed statistical analysis of the causal relationship between HLA-DSA and TG and showed that the deleterious graft outcomes of HLA-DSA are mediated by the occurrence of AMR and transplant glomerulopathy [52].

Together, these data point to the concept that, while DSA are associated with TG, nearly 30%–40% of TG lesions must develop from different pathogenetic mechanisms [48]. Analogously, not all patients with detectable class II DSA develop TG, suggesting the need for additive/alternate injury mechanisms. Indeed, traditionally, peritubular capillary basement membrane multilayering has been considered a lesion associated with repeated endothelial injury by DSA; however, in our recent work, we reported that these lesions were identifiable at a similar rate in patients with or without DSA but associated with microvascular inflammation (MVI), suggesting that the

presence of endothelial injury may be causative rather than the specific type of injury associated with DSA [53].

C4d Staining in Transplant Glomerulopathy

In the complement cascade, C4d is generated from C4 following activation of C1Q via DSA or polysaccharides in the mannose binding lectin pathway [4, 54]. Complement activation in the presence of DSA (HLA or non-HLA) may play a role in TG. However, DSA- and C4d-negative TG is well recognized [36, 41]. Similarly, studies of TG report C4d-negative but DSA-positive cases [4, 36], suggesting these two features are not always correlated in TG. It must be noted that C4d is covalently bound to the tissues and, while considered a footprint of prior antibody activity, C4d positivity may remain after injury [4, 46, 55] or indeed be evanescent [4]. It is also important to note that the rate of C4d detection of TG in reported studies is also contingent upon the technique used in a particular dataset, whether immunofluorescence or immunohistochemistry [56]. Pathogenetically, the hallmark lesion of TG is likely endothelial cell injury with swelling, loss of fenestrations leading to sub endothelial widening of lamina rara interna and electron lucent or flocculent material, cell debris and reduplication, or multi layering of lamina densa [4]. Electron dense deposits, which are the *sine qua non* of recurrent or *de novo* immune complex glomerulonephritis in the allograft (immunoglobulin mediated or C3 glomerulopathy), are not encountered in TG and show that complement activation within the glomerulus is not sufficient to induce a TG phenotype in these other glomerulitides.

Transplant Glomerulopathy in the Absence of DSA: Adaptive Immune-Cell-Mediated Mechanisms

Several studies have now demonstrated that DSA is neither necessary nor sufficient to induce the lesions of TG. Vongwiwatana *et al* [57], showed that only 25% of biopsies with TG had associated positive C4d deposition. Similarly, in a study by Aly *et al* [58], all 20 patients with TG were C4d negative. It must be noted here that DSA + C4d-negative TG biopsies were similar to cABMR in their gene-expression profile, suggesting that DSA-mediated injury can occur without detectable C4d [59]. In this latter study by Akalin *et al*, a substantial number of patients with TG did not have either positive C4d staining or DSA [60], while another retrospective study of TG showed that only 45% had detectable DSA and only 14% were C4d-positive. Indeed, demonstration of sub-phenotypes within TG using DSA also highlights non-DSA-mediated TG. Lower glomerulitis and peritubular capillaritis scores, less C4d deposition, and less interstitial inflammation have been seen in the absence of DSA compared with DSA–positive biopsies. Analogously, the severity of Banff *g, C4d,* and *i* scores were less pronounced in the absence of HLA-DSA [29], although the distribution of the chronic lesions and the graft function, assessed by serum creatinine, proteinuria, and eGFR, were comparable between the HLA DSA–negative and HLA-DSA–positive TG phenotypes [29]. Hence, when applying conventional markers of DSA-mediated allograft injury (i.e., serologic DSA or C4d), the pathogenesis of many cases of TG remains unexplained. Novel markers have suggested that cell-mediated mechanisms, rather than DSA, underlie TG in a significant proportion of cases. Dean et al. compared intragraft biopsy gene expression profiles between positive cases crossmatch-associated with TG, non-DSA-TG, and 10 conventional DSA-neg controls with stable histology [61]. Despite the antibody involvement, gene expression profiles associated with cell-mediated immunity were significantly enriched, including those for cytotoxic T lymphocytes and Interferon Gamma [61]. Similarly in six TG biopsies, glomerular staining for the costimulatory molecule ICOS and the chemokine receptor CXCR3 with its ligand Mig were present, indicating the presence of activated T cells in DSA-negative TG.

Innate immune-cell-mediated mechanisms: A role for NK cell-mediated recognition of "non-self" donor tissue and consequent injury, independent of development of DSA, has also been shown in MVI cases [62, 63] without demonstrable DSA. Notably, the association of NK cells with the development of TG in this form of injury is not as well established in current human data. Pioneering animal studies of ABMR using CCR5-knockout-B6 mice as kidney recipients show that depletion of NK cells prevented acute ABMR lesions despite the presence of high DSA titers. However, longer follow up of these allo-transplants allowed the development of TG, suggesting that NK cells may mediate the ultimate TG phenotype when combined with DSA. In this model, high levels of IFN-y, perforin, and granzyme B were found 3 days after transplantation, suggesting T cell/NK cell activation in the allograft occurred even before DSA was detectable in recipient mice with MVI [64]. A recent study compared bulk transcriptomics of 15 cABMR biopsies (all with evidence of TG), 17 T-cell mediated rejection (TCMR cases without Cg), and 18 non-rejectors (NR) [65]. The study found marked enrichment of NK-cell activation signatures in cABMR vs. NR biopsies, which were confirmed by deconvolution analyses. While both TCMR and cABMR showed NK cell enrichment, this was highest in the cABMR transcriptomes. Another recent study delineated differential roles of NK- and T-cell mediated injury in subtypes of TG cases [66]. The authors compared the transcriptomic profiles of 14 TG cases without DSA/C4d with 22 cases classified as TG with $DSA^+/C4d^+$ using the NanoString Banff-Human Organ Transplant (B-HOT) panel with subsequent multiplex immunofluorescence validation. $DSA^+/C4d^+$ TG showed a higher glomerular abundance of natural killer cells/macrophages and increased expression of complement-related genes and DSA-related pathways vs. samples $DSA^-/C4d^-$ TG, while the latter showed enrichment of genes related to the activity of T cells ($CD3^+$, $CD8^+$). At the protein level, using multiplex immunofluorescence, they confirmed increased T-cell markers ($CD3^+$, $CD3^+CD8^+$) in $DSA^-/C4d^-$ TG. Together, these data suggest that T-cell- and NK cell-mediated rejection phenomena are independently or co-operatively involved in the pathogenesis of TG.

Role of Non-HLA Antibodies

Non-HLA antibodies against polymorphic donor antigens can result from classical adaptive allo-immune responses initiated by

T-cells culminating in specialized antibody-secreting plasma cells but can occur as part of the autoimmune phenomena or naturally occurring IgG. Ischemia-reperfusion injury and/or episodes of rejection may unmask polymorphic donor antigens causing antibody formation against non-HLA proteins [67]. Non-HLA antibodies clearly contribute to ABMR in HLA identical renal allografts exemplified by anti-donor endothelial reactive antibodies [68]. Recent evidence suggests non-HLA auto- and alloantibodies (through independent cytotoxicity or with concurrent DSA) contribute to TG (reviewed in [3, 69]). Interestingly, the most widely studied non-HLA antigen target implicated in ABMR is the angiotensin receptor (AT1R). However, the association of anti-AT1R antibodies with TG independent of HLA-DSA is still unclear [70, 71].

Dinavahi et al [72] used protein microarrays to compare antibody panels in pre- and post-transplant sera from patients with and without transplant glomerulopathy and saw reactivity against peroxismal-trans-2-enoyl-coA-reductase being associated with development of TG. In mouse models, the development of antibodies to glomerular basement membrane components, such as perlecan or collagens type IV or type VI, has been associated with the development of TG [73]. In clinical data, the presence of antibodies against the glomerular basement membrane components, particularly anti-Agrin antibodies, were identified in 44% of TG cases (16 patients) and associated with rejection episodes prior to the diagnosis of TG [74]. Another group identified that antibodies to renal tissue restricted self-antigens, particularly fibronectin and collagen type IV, increased the odds of TG [75]. Here, non-HLA antibodies were detected irrespective of the presence of anti-HLA antibodies, while MVI features and C4d deposition were equivalent in patients with non-HLA or HLA-antibodies, suggesting an independent role for non-HLA antibodies in the development of TG. In a highly sensitized cohort (75% DSA-positive), anti-endothelial cell antibodies (AECAs) detected by ELISA were demonstrably increased with ABMR along with MVI lesions and TG [76]. This suggests that non-HLA antibodies are associated with TG clinically and experimentally, but a focused approach examining antibodies specifically against polymorphic or self-restricted endothelial or GBM antigens, restricted to patients with suspected antibody-mediated (C4D-positive) TG, may be required to reveal potential culprits. A major limitation for non-HLA antibody detection is the limited utility of commercial panels currently available for this purpose.

Role of Podocyte Stress in TG: Cause, Effect, or Guilt by Association?

The stereotypic lesions of TG do not develop in usual contexts of glomerular injury in binephric individuals, suggesting a unique predilection for the uninephric allograft state. Although endothelial damage likely initiates the cascade of immunologic injury culminating in characteristic TG, a role for increased podocyte stress leading to critical podocyte depletion [77] and TG is evolving. Notably, advanced TG is characterized by significant proteinuria, progressive injury, and graft loss similar to podocytopathies. To study this aspect of the development of TG, Wiggins et al, tested the role of the "two kidneys to one kidney transition" that occurs in all allografts and resultant podocyte stress using urine podocin/creatinine ratio as a marker of podocyte injury/depletion [78]. Surprisingly, while allograft recipients have half the number of nephrons, they observed a significantly increased rate of podocin mRNA excretion (a surrogate of podocyte loss) in urine of all allograft recipients vs. binephric controls and that urinary podocyte loss was markedly higher in patients with TG vs. patients with non-glomerular allograft pathology (tubular injury or IFTA). The steepest decline in podocyte density occurred in the subset of TG patients diagnosed within 2-year of transplant (early TG). Later stage TG (>2 years) was less associated with biopsy-confirmed rejection episodes and had a distinct podometric profile [78]. The same group reported that such early loss of podocytes in urine from any cause was associated with later graft loss [79]. This was exacerbated in context with donor-recipient weight mismatch, which is a surrogate marker for glomerular stress and suggests a greater need for post-transplant compensatory hypertrophy [78, 80, 81]. Another potential extrapolation of this inference is that post-transplant obesity and metabolic syndrome in the context of immunosuppression could be a predisposing factor in exacerbating podocyte loss, leading to accelerated loss of graft function and early onset of TG. Additionally, the same researchers also reported that, even within normal ranges of mean arterial pressures (MAP), increases in MAP were linearly associated with urine markers of podocyte stress [82], bringing out a role for fluid-flow and shear stress [83]. In this pathogenetic chain, the specific role played by immunologically mediated endothelial injury, leading to exacerbation of podocyte stress, still needs to be better defined.

While associative data shows podocyte damage or podocyturia in the context of TG, it must be noted that there is no conclusive evidence directly linking podocyte damage as a causal factor in TG. Most of the current evidence supports the hypothesis that TG is initially characterized by glomerular basement membrane alterations and endothelial injury, which could then lead to podocyte injury with foot process effacement or podocyte loss. The specific causal role of podocyte loss in TG, if any, remains an area in need of mechanistic research.

Non-Transplant-Related Contributors in GBM Multilayering

Role of hepatitis C: Hepatitis C is associated with immune-complex-mediated MPGN, where histological findings may overlap with TG (reviewed in [4]). It is not entirely clear whether alterations in morphological appearance of usual MPGN result from concurrent immunosuppression with altered immune complex deposition or a uninephric state following transplantation. It is postulated that hepatitis C could upregulate alloimmune responses and microvascular inflammation, leading to C4d positivity [4, 84]. HCV-associated injury, especially cryoglobulinemia, could also induce endothelial injury and histologic features of thrombotic microangiopathy (TMA), all contributing to TG-like lesions.

With effective HCV treatment, such co-occurrences will be increasingly infrequent.

Chronic Thrombotic microangiopathy: cTMA with resultant endothelial injury may produce electron and light microscopic lesions, which may be very difficult to distinguish from TG [4, 84]. Clinically, cTMA has characteristic serologic findings, including microangiopathic hemolytic anemia and thrombocytopenia on peripheral blood smear, suggesting intravascular hemolysis and thereby differentiating it from TG.

CONCLUSION

In summary, in this review article we discuss potential patho-mechanistic processes that have been elucidated by recent research to be in the evolution pathway of TG. While primarily understood to be initiated after persistent injury to the allograft glomerular endothelium from serologic- (DSA, non-HLA antibodies, and/or complement-mediated) or cell-mediated mechanisms, subsequent co-incidental podocyte stress and detachment (hypertrophic, hypertensive, and/or immunologic) could contribute as a common pathway leading to critical podocyte loss, often associated with increasing proteinuria over the course of graft life-span and in turn leading to progressive graft damage. Since podocytes have recently been ascribed a buttressing function in homeostatic and injured glomeruli [85–87], their contributory vs. potential causative role in TG needs to be better defined. A current problem in the field is the limited availability of long-term allo-transplantation models in transgenic rodents that also develop TG lesions. Rodent models of TG could help investigate cell-type- and gene-specific- modulations to differentiate causation vs. association as it relates to podocytes. On the other hand, non-human primate allo-models which do develop TG-like lesions in the context of DSA-mediated injury have not yet specifically focused on podocyte injury—again a deficiency in the field. Hence, mechanistically focused research is needed to unravel molecular targets that could be utilized to arrest the progression of TG. Ultimately, a multi-pronged approach targeting both initiators and effectors of TG will be needed to combat this important contributor to delayed graft loss.

AUTHOR CONTRIBUTIONS

MCM conceptualized the manuscript. AC, DG-P, and GL wrote first draft. AC and MCM made edits. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
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Angiotensin II Type-1 Receptor Antibody in Solid Organ Transplantation – Is It Time to Test?

Paul James Patrick Martin[1,2]*, *Michelle Willicombe*[1,2] *and Candice Roufosse*[1,3]

[1]*Department of Immunology and Inflammation, Imperial College London, Hammersmith Campus, London, United Kingdom,* [2]*Imperial College Renal and Transplant Centre, Imperial College Healthcare NHS Trust, Hammersmith Hospital, London, United Kingdom,* [3]*Department of Histopathology, Northwest London Pathology NHS Trust, Charing Cross Hospital, London, United Kingdom*

Angiotensin II type-1 receptor antibody (AT1R-Ab) has been mooted as a potential effector of both acute and chronic antibody mediated rejection (AMR). A growing body of literature on the topic is now coming under scrutiny in the context of the evolving Banff AMR diagnostic classification system and refinement of recommendations for histocompatibility testing by the Sensitization in Transplantation Assessment of Risk (STAR) workgroup. This mini-review discusses the latest understanding of pathophysiological mechanisms, clinical evidence for the pathogenicity of AT1R-Ab, and methods of laboratory testing.

Keywords: AT1R antibody, AT1R, non-HLA antibody, rejection, antibody mediated rejection

INTRODUCTION

Criteria for the diagnosis of antibody mediated rejection (AMR) in kidney allografts were first incorporated into the Banff Classification in 2001 and comprise both active and chronic components [1]. Classification criteria for AMR in other solid organ transplants have evolved at different rates [2–5]. Conventionally, donor human leucocyte antigen (HLA) proteins have been understood as the primary target of recipient alloimmune response, and are a major driver of late allograft rejection and loss [6]. However, as has become apparent in recent years, accounting for the HLA system alone is not the panacea for all immune-mediated transplant injury [7]. The latest Banff iteration has considered this gap in immunological understanding with the creation of a subcategory of C4d-negative microvascular inflammation/injury with absence of detectable circulating HLA donor-specific antibodies (HLA-DSA) [8].

In 2005, two significant papers on the concept of immune response in kidney transplantation were published. Opelz et al., in an international study of over 4,000 kidney transplant recipients from HLA-identical sibling donors, demonstrated that the presence of panel reactive antibodies >50% was associated with long-term allograft loss, suggesting that non-HLA antibodies may play a role in chronic rejection [9]. Separately, Dragun et al. reported the presence of agonistic IgG1 and IgG3 antibodies to angiotensin II type-1 receptor (AT1R) in the sera of kidney transplant recipients who had vascular rejection refractory to steroid treatment [10].

Since then, specific and sensitive tests for HLA-DSA have been developed and AT1R antibodies (AT1R-Ab) have become the most widely studied non-HLA antibody in transplantation, with conflicting reports on their association with allograft outcomes [11–14]. A comprehensive review of Dragun et al.'s contribution to our understanding of AT1R in transplantation was recently published [15]. This mini-review aims to highlight the latest research on pathophysiological mechanisms; to discuss methods of laboratory testing; and to outline current gaps in knowledge and potential for future research (**Table 1**).

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***Correspondence**
Paul James Patrick Martin,
paul.martin9@nhs.net

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CURRENT UNDERSTANDING OF PATHOPHYSIOLOGY OF AT1R-AB MEDIATED REJECTION AND ENDOTHELIAL INJURY

Angiotensin II, a potent vasoconstrictor that influences endothelial function, inflammation and fibrosis, primarily mediates its effect through AT1R, a G-protein coupled receptor (GPCR) [16–18]. Expression of AT1R is widespread but not ubiquitous, and concentrations on cell membranes fluctuate dependent on genetic and environmental factors [19]. AT1R-Ab function as receptor agonists [19]. They may be present at the time of organ transplantation, or develop *de novo* after transplantation.

Kidney transplant histological features in the context of AT1R-Ab positivity have been reported. Min et al. report that glomerulitis and peritubular capillaritis were the commonest biopsy findings amongst AT1R-Ab positive recipients [20]. In another cohort of 65 paediatric recipients, AT1R-Ab were associated with the presence of glomerulitis or arteritis [21]. In a prospective study, Lefaucheur et al. contemporaneously assessed AT1R-Ab and HLA-DSA serostatus at the time of indication and surveillance biopsies at or within 1 year of transplantation in 1,845 people. Recipients positive for HLA-DSA plus AT1R-Ab had the lowest allograft survival. Higher levels of circulating AT1R-Ab were associated with glomerultis, peritubular capillaritis, and intimal arteritis. Among recipients with histological rejection, AT1R-Ab positivity was associated with lower prevalence of complement deposition in peritubular capillaries ($p < 0.001$) [22]. This circumstantial evidence that AT1R-Ab can mediate vascular injury in a manner independent of complement corroborates the index cohort of Dragun et al., but is not a histological finding borne out uniformly in all studies.

Mechanistic studies have highlighted cellular signalling mechanisms influenced by AT1R-Ab. Catar et al. treated human microvascular cells with AT1R-Ab that had been isolated from seropositive patients with transplant vasculopathy. AT1R receptor signalling was sustained via beta2-arrestin recruitment to the cell membrane and mTOR complexes were activated with consequent impairment of endothelial repair capability [23]. These effects were terminated with pharmacological mTOR inhibition. Moll et al. determined that IgG derived from sera of kidney transplant recipients with vasculopathy stimulated secretion of tumour necrosis factor alpha from human microvascular endothelial cells with subsequent THP-1 monocyte activation [24]. The same effect was not demonstrated using IgG derived from the sera of a control cohort. Although the investigators do not explicitly state that AT1R-ab are implicated, this pro-inflammatory mechanism is proposed to act via GPCR-directed PAR1 signalling [24]. These *in vitro* models offer potential targets for therapeutic intervention.

CLINICAL STUDIES OF AT1R ANTIBODIES IN SOLID ORGAN TRANSPLANTATION

Most studies of AT1R-Ab have been undertaken in kidney transplant recipients (**Table 2**). In 2022, a meta-analysis by Kang et al of 21 studies concluded that recipients with AT1R-Ab were at greater risk of AMR (RR 1.96, 95% CI 1.61–2.33) and allograft failure (RR 2.37, 95% CI 1.50–3.75) [25]. The studies varied in size, but three, one of which has already been discussed [22], were notably larger. In a longitudinal study of 351 recipients, using positivity threshold >15 u/mL, Taniguchi reported that *de novo* AT1R-Ab and dual AT1R-Ab plus HLA-DSA positivity were associated with allograft loss [12]. Giral et al., in a cohort of 599 at a threshold of >10 U/mL, reported AT1R-Ab positivity in 47.2% of participants at time of transplantation, who had a 2.6 fold greater risk of allograft failure beyond 3 years [11]. Not included in the meta-analysis, Deltombe et al., using a positivity threshold of >10 u/mL, found no association between AT1R-Ab status and transplant outcomes in a cohort of 387 patients [13]. In 62 paediatric recipients, AT1R-Ab was associated with AMR using a positivity threshold of 9.5 u/mL [26]. More recently, two observational studies using positivity threshold of >17 u/mL did not show clear association with AMR [27, 28].

In heart transplantation, a prospective study of 30 recipients demonstrated that persistently elevated AT1R-Ab levels were associated with AMR and microvasculopathy [29]. Elevated AT1R-Ab were reported in a cohort of 21 recipients with allograft dysfunction in the absence of AMR [30]. In a cohort of 200 recipients, concomitant *de novo* HLA-DSA and AT1R-Ab were associated with diminished freedom from AMR, and recipients with rising AT1R-Ab titres were more likely to be diagnosed with cardiac allograft vasculopathy (CAV) [31]. Characteristically, CAV does not often respond to conventional treatment of coronary artery disease and is a common cause of cardiac allograft failure [32]. In two studies of patients who underwent bridging to transplantation with a left ventricular assist device (LVAD), one reported that 63.8% of participants tested negative for AT1R-Ab initially and subsequently became positive post-LVAD, and another reported that higher titres of AT1R-Ab at time of transplant had worse outcomes [33, 34]. Vascular injury related to LVAD complements the auto-antigen exposure and auto-antibody recruitment theory previously described [35]. A number of illustrative case reports have been published in the literature: a fatal case of hyperacute AMR in a recipient with no HLA-DSA but high AT1R-Ab levels on the day of transplantation [36]; a paediatric case whereby a second allograft was lost in context of high AT1R-Ab [37]; and a case of AMR, CAV and persistently elevated AT1R-Ab levels despite repeated plasmapheresis, intravenous immunoglobulin and steroids [38]. Conversely, when testing for an array of 44 non-HLA antibodies in a cohort of 64 heart transplant recipients, 67% of whom had AMR, no association with AT1R-Ab was found [39]. In light of these findings, a recent Banff report specifically discussed the role of non-HLA antibodies such as AT1R-Ab in heart transplantation and advocated for development of standardized diagnostic tests, and prospective clinical trials to explore role in rejection and assess efficacy of treatments [3].

Regarding lung transplantation, in a multi-centre study of 162 recipients, 46% were positive for AT1R-Ab prior to transplantation, and frequency of *de novo* HLA-DSA and AMR was greater in those positive for AT1R-Ab [40]. In a

TABLE 1 | Future directions for research.

1. Development of a standardized, validated, high throughput, affordable method of testing
2. Collaboration across transplant disciplines to confirm or refute the association of AT1R-Ab with acute and/or chronic AMR and/or microvascular inflammation/injury, in retrospective and prospective observational cohorts, with an eye to determining a clinically meaningful threshold of AT1R-Ab positivity
3. Further investigate causality by reproducing and validating the mechanistic studies reported to date, and including specific investigation of the role that complement does or does not play in AT1R-Ab mediated pathology
4. Randomised controlled trials of therapeutic interventions that have thus far only been reported in case series

cystic fibrosis patient, rapid onset AMR ensued in the absence of HLA-DSA but positive AT1R-Ab serostatus [41]. In 71 recipients, chronic lung allograft dysfunction at 3 years was more common in the AT1R-Ab positive group compared to the negative (58.3% vs. 11.8%, $p < 0.001$) [42].

Conventionally, liver allografts are perceived as lower immunological risk than other solid organ transplants. In 81 paediatric patients who received living donor transplants and subsequently had withdrawal of immunosuppression, AT1R-Ab >17 u/mL was evident in 65% of patients with advanced fibrosis, a greater proportion than those without fibrosis ($p = 0.02$) [43]. Two pertinent findings from a large study of 1,269 liver transplant recipients who had sera tested for AT1R-Ab and HLA-DSA, found that *de novo* AT1R-Ab was associated with increased risk of rejection and fibrosis progression, and histology from the *de novo* AT1R-Ab subgroup showed distinctive sinusoidal C4d staining spatially related to activated stellate cells [44]. In 79 paediatric recipients, those with active allograft dysfunction were more likely to be AT1R-Ab positive compared to those with stable function (89% vs. 29%, $p = 0.001$). Those with both AT1R-Ab and HLA-DSA were more likely to progress to allograft loss [45]. Regarding 94 patients receiving a second liver transplant, 51.1% had AT1R-Ab >17 u/mL at time of second transplant, and those with an AT1R-Ab level >40 u/mL were more likely to experience allograft loss [46].

As regards intestinal transplantation, in 29 recipients AMR was more common in those with positive AT1R-Ab versus those without (55% vs. 11%, $p < 0.01$) [47]. In 25 paediatric recipients, 68% had AT1R-Ab >17 u/mL pre-transplant; these levels did not vary significantly when sera were tested sequentially, and there was no association with allograft dysfunction. No explicit comment was made regarding AMR [48].

CURRENT TECHNIQUES IN TESTING FOR AT1R-AB AND RELATED PROBLEMS

The most commonly used testing platform for AT1R-Ab is the enzyme linked immunosorbent assay (ELISA) available from CellTrend GmbH Luckenwalde Germany which can test 40 serum samples in 1 run. AT1R is pre-coated on the microtiter plate. During the first incubation, AT1R-Ab in samples are immobilized on the plate and detected with labelled anti-human IgG. The intensity of the colour in the subsequent enzymatic substrate reaction correlates with the concentration of AT1R-Ab. Reinsmoen et al. were the first to use this technique in a clinical study [49].

Senev et al. used the solid-phase Luminex assay to retrospectively test pre- and post-transplantation sera of 874 recipients for 82 different non-HLA antibodies including AT1R-Ab. There was an association between the burden of pretransplant non-HLA antibodies and development of AMR without HLA-DSA (HR 1.3 per 10 antibodies $p = 0.02$) and microvascular inflammation (HR 1.13 per 10 antibodies $p = 0.04$). Only four antigens were identified as independent risk factors for AMR histology, AT1R was not among them [50]. Kamburova et al created a solid-phase assay to test sera for 14 specific non-HLA antibodies. Production of AT1R proteins was hampered by protein cleavage before excretion in the culture supernatant, therefore AT1R-Ab was largely excluded from reported results [51]. Nevertheless, in a study by the same group, AT1R was included as an antigen in the same assay screening for non-HLA antibodies in pre-transplant sera of 4,770 recipients; no association between AT1R-Ab status and graft survival was observed [14].

Alternatives to solid-phase assays have been reported. Delville et al. used a cell-based crossmatch assay to identify pre-formed IgG antibodies to glomerular endothelial cells in a small cohort of highly selected transplant recipients. All participants had microvascular inflammation on early biopsies but no circulating HLA-DSA; 26% were positive for AT1R-Ab at threshold 10 U/mL [52]. Lamarthee et al. refined this strategy by using CRISPR/Cas9 to render glomerular endothelial cells devoid of HLA -A-B-C & -DR expression to develop a non-HLA antibody detection immunoassay (NHADIA). In an unselected cohort of 389 recipients, pre-transplant NHADIA values were associated with AMR histology ($p = 0.0082$) and microvascular inflammation (0.0024). However, using a positivity threshold of 10 U/mL and presumably measured using ELISA technique, there was no correlation between AT1R-Ab levels and NHADIA values [53]. In a novel approach, Lammerts et al. isolated endothelial cells from the perfusion fluid of 102 donor kidneys and propagated a biobank of machine perfusion-derived primary renal endothelial cells (MP-PRECs) to primarily study anti-HLA mediated cytotoxicity, but noted the technique could have utility for investigating non-HLA antibody mediated disease also [54]. Of course, in the context of studying AT1R-Ab, this will be contingent on MP-PRECs expressing AT1R.

CURRENT RESEARCH GAPS AND POTENTIAL FUTURE DIRECTIONS

The role of AT1R-Ab in transplantation continues to be investigated. *In vitro* mechanistic studies have outlined how AT1R-Ab influence signalling pathways to cause vascular injury. A steadily growing number of small to medium sized

TABLE 2 | Studies of angiotensin II type 1 receptor antibody (AT1R Ab) in solid organ transplantation.

Year	Authors	Study population	Transplantation period	AT1R Ab measurement technique	Measurement timepoints	ELISA AT1R Ab positivity threshold for analysis	Concurrent assessment of HLA DSA status	Main findings
2010	Reinsmoen et al.	97 adult kidney recipients	2006–2009	ELISA	Pre transplant Post transplant at time of rejection	>17 U/mL	Yes	1. AT1R Ab associated with AMR in the absence of HLA DSA
2012	Hiemann et al.	30 adult heart recipients	2005–2006	ELISA	Post transplant at multiple timepoints between 24 h and 1 year	>15.9 U/mL	Yes	1. AT1R Ab levels were higher in patients with rejection 2. AT1R Ab were associated with microvasculopathy
2013	Giral et al.	599 adult kidney recipients	1998–2007	ELISA	Pre transplant Post transplant	>10 U/mL	Yes	1. Higher pre transplant AT1R Ab correlated with allograft loss after 3 years
2013	Taniguchi et al.	351 adult kidney recipients	1999–2009	ELISA	Pre transplant Post transplant at multiple time points	>15 U/mL	Yes	1. Pathological synergy between AT1R Ab and HLA DSA 2. *de novo* AT1R Ab were associated with allograft failure
2014	Reinsmoen et al.	200 adult heart recipients	2007–2011	ELISA	Pre transplant Post transplant at multiple time points up to 1 year	>12 U/mL and >17 U/mL	Yes	1. Dual HLA DSA and AT1R Ab positivity was associated with lower freedom from rejection
2014	Ohe et al.	81 paediatric liver recipients	1990–2010	ELISA	Post transplant at time of indication or protocol biopsy	>17 U/mL	Yes	1. AT1R Ab were associated with allograft fibrosis in recipients who underwent withdrawal of immunosuppression
2016	Urban et al.	69 adult heart recipients *LVAD prior to transplant	2008–2014	ELISA	Prior to LVAD implantation Pre transplant	>17 U/mL	No	1. There was no difference in survival or freedom from rejection according to AT1R Ab status
2017	Deltombe et al.	940 adult kidney recipients	2008–2012	ELISA	Pre transplant only	>10 U/mL and >17 U/mL	Yes	1. AT1R Ab were not associated with higher risk of acute rejection episodes or allograft failure
2017	Reinsmoen et al.	162 adult lung recipients	2011–2013	ELISA	Pre transplant Post transplant at 3- and 6-months, and at time of allograft dysfunction	>11 U/mL and >17 U/mL	Yes	1. AT1R Ab were associated with lower freedom from rejection 2. AT1R Ab were associated with lower freedom from *de novo* HLA DSA
2017	Cozzi et al.	1 adult lung recipient	2015	ELISA	Pre transplant Day 4 post transplant	>10 U/mL	Yes	1. Case report highlighting possible implications of AT1R Ab
2017	O'Leary et al.	1,269 adult liver recipients	2000–2009	ELISA	Pre transplant 1 year post transplant	>17 U/mL	Yes	1. AT1R Ab and preformed HLA DSA were associated with increased mortality 2. *de novo* AT1R Ab were associated with rejection and fibrosis
2017	Gerlach et al.	29 adult intestine or multi-visceral recipients	2000–2015	ELISA	Pre transplant at time of listing and 3 monthly thereafter Post transplant weekly until hospital discharge	>12 U/mL	Yes	1. AT1R Ab were associated with rejection

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TABLE 2 | (*Continued*) Studies of angiotensin II type 1 receptor antibody (AT1R Ab) in solid organ transplantation.

Year	Authors	Study population	Transplantation period	AT1R Ab measurement technique	Measurement timepoints	ELISA AT1R Ab positivity threshold for analysis	Concurrent assessment of HLA DSA status	Main findings
					and at time of allograft dysfunction			
2018	Min et al.	359 adult kidney recipients	2010–2014	ELISA	Pre transplant only	>10 U/mL	Yes	1. HLA DSA and AT1R Ab were associated with microvascular inflammation on biopsy 2. Positive AT1R-Ab status was associated with lower allograft survival
2018	Pearl et al.	65 paediatric kidney recipients	2005–2014	ELISA	Pre transplant Post transplant at multiple time points up to 2 years	>17 U/mL	Yes	AT1R Ab positivity is associated with: 1. Allograft loss 2. Lower eGFR over 2 years 3. Higher levels of TNF-alpha, IL-1beta, IL-8
2018	Fichtner et al.	62 paediatric kidney recipients	1999–2010	ELISA	Post transplant at time of indication biopsy	>9.5 U/mL	Yes	1. AT1R Ab positivity in context of indication biopsy was associated with AMR and reduced allograft function
2018	Kamburova et al.	87 healthy controls, 40 patients with miscellaneous kidney disease	Not applicable or not reported	Bead-based immunoassay	not reported	Not applicable	No	1. In house production of AT1R was not achieved 2. Testing for non-HLA Abs using multiplex platform is achievable
2019	Kamburova et al.	4,770 adult kidney recipients	1995–2006	Bead-based immunoassay	Pre transplant only	Not applicable	No	1. Main findings pertain to other non-HLA antibodies
2019	Lefaucheur et al.	1845 adult kidney recipients	2008–2012	ELISA	Post transplant within 1 year	>10 U/mL	Yes	AT1R Ab were associated with: 1. AMR at 1 year 2. diminished allograft survival 3. Microvascular inflammation on biopsy
2019	Delville et al.	48 adult kidney recipients	Not reported	ELISA and endothelial cell flow cytometry	Pre transplant only	>10 U/mL and >17 U/mL	Yes	1. AT1R Ab status failed to differentiate patients with microvascular inflammation in the absence of HLA DSA from stable patients 2. Cell based assays could improve risk assessment pre transplantation
2020	Villa et al.	1 adult heart recipient	Not reported	ELISA and endothelial cell flow cytometry	56 days pre transplant and day of transplant 27 days post transplant	Not applicable	Yes	1. Hyperacute fulminant allograft dysfunction in a recipient with markedly elevated AT1R Ab
2020	See et al.	64 adult heart recipients	1994–2014	ELISA	Post transplant	Not reported	Yes	1. There was no difference in AT1R Ab levels between two groups with and without AMR
2020	Wozniak et al.	79 paediatric liver recipients	2010–2017	ELISA	Post transplant at routine clinical visits	>17 U/mL	Yes	1. AT1R Ab prevalence was high in cases of allograft dysfunction

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TABLE 2 | (*Continued*) Studies of angiotensin II type 1 receptor antibody (AT1R Ab) in solid organ transplantation.

Year	Authors	Study population	Transplantation period	AT1R Ab measurement technique	Measurement timepoints	ELISA AT1R Ab positivity threshold for analysis	Concurrent assessment of HLA DSA status	Main findings
					or during allograft dysfunction			
2020	Xu et al.	94 adult liver recipients	1991–2018	ELISA	Prior to second transplant	>17 U/mL and >40 U/mL	Yes	1. AT1R Ab were common in recipients of a second transplant 2. AT1R Ab were associated with inferior long-term outcomes
2021	Chan et al.	25 paediatric intestine recipients	2000–2016	ELISA	Post transplant at routine clinical visits or during allograft dysfunction	>17 U/mL	Yes	1. AT1R Ab were common post transplant 2. AT1R Ab were not associated with allograft dysfunction or survival
2021	Lamarthee et al.	389 adult kidney recipients	2012–2017	ELISA and endothelial cell flow cytometry	Pre transplant only	>10 U/mL	Yes	1. There was no correlation between immunoassay results and AT1R Ab levels 2. Pre transplant AT1R Ab did not correlate with increased risk for AMR
2022	Kang et al. *Systematic review of 21 studies	4,023 adult and paediatric kidney recipients	1998–2019	ELISA	Various	Various	Various	AT1R Ab positivity is associated with: 1. AMR (RR 1.96 CI 1.61–2.33) 2. Allograft loss (RR 2.37, CI 1.50–3.75)
2022	Pizzo et al.	36 paediatric kidney recipients	2011–2019	ELISA	Pre transplant Post transplant at multiple timepoints	>17 U/mL	Yes	1. AT1R Ab positivity was not associated with rejection or reduced allograft function
2022	Liu et al.	79 adult kidney recipients	2016–2019	ELISA	Pre transplant Post transplant within 1 year	>17 U/mL	Yes	1. AT1R Ab positivity was associated with AMR and lower EGFR
2022	Moreno et al.	21 adult heart recipients	2017–2019	ELISA	Post transplant at time of allograft dysfunction	>10 U/mL		1. AT1R Ab were associated with allograft dysfunction in the absence of rejection
2022	Senev et al.	874 adult kidney recipients	2004–2013	Bead-based immunoassay	Pre transplant Post transplant at 3 months, 1 year and 5 years	Not applicable	Yes	1. There was no association between pre-transplant AT1R Ab and occurrence of AMR
2023	Chou-Wu et al.	1 paediatric heart recipient	2007	ELISA	At time of first and second transplant, and post second transplant	Not applicable	Yes	1. Case report highlighting possible implications of AT1R Ab
2023	Son et al.	71 adult lung recipients	2016–2020	ELISA	Pre transplant Post transplant within 3 months	>17 U/mL	Yes	High AT1R Ab levels were associated with: 1. de novo HLA DSA 2. Acute cellular rejection 3. Early chronic lung allograft dysfunction 4. Lower recipient survival time
2024	Jung et al.	1 adult heart recipient	Not reported	ELISA	Pre transplant Post transplant at multiple time points	>17 U/mL	No	1. Case report highlighting possible implications of AT1R Ab

AMR, antibody mediated rejection; AT1R, Ab Angiotensin II type 1 receptor antibody; CI, 95% confidence interval; eGFR, estimated glomerular filtration rate; ELISA, enzyme linked immunosorbent assay; HLA DSA, human leucocyte antigen donor specific antibody; LVAD, left ventricular assist device; RR, relative risk.

clinical studies highlight a link between AT1R-Ab status, both pre-transplant and *de novo*, and development of AMR, with possible pathological synergy in cases of concomitant HLA-DSA positivity. These studies are hampered by heterogeneous study design and inconsistent outcome reporting with variable thresholds of antibody positivity. A large, prospective two-centre study using solid-phase ELISA assay firmly established a link between AT1-Ab and a phenotype of AMR, but this link has not been borne out in similarly large retrospective studies using cell-based assays. There is no explanation for this discordance at present. The Bradford Hill criteria should be borne in mind when considering these and future studies investigating the role of non-HLA antibodies in causality of AMR [55].

Regarding ELISA specifically, a particular advantage is the capacity to bulk test; for instance, screening the stored sera of a transplant wait-list population. There are a number of disadvantages. For interpolation of results, a standard curve must be created for each ELISA kit. This introduces a degree of inter-assay variability making comparison of results between kits difficult. If antibody titres in a sample are high and the result is beyond the upper limit of the standard curve, the test needs to be performed again at greater dilution factor to obtain a discrete concentration value. Furthermore, as highlighted by Kamburova et al., details of the manufacturing processes of commercially available ELISA assays are obscure, preventing in-house replication of these assays and necessary reagents [51].

In heart transplantation, CAV is considered a manifestation of chronic rejection and is associated with both non-HLA antibodies and HLA-DSA [56]. As a medium-sized vasculopathy, it is possible that CAV is analogous with some types of the morphologically heterogenous radiologic abnormality of transplant renal artery stenosis (TRAS). Kidney recipients with a post-anastomotic TRAS lesion are more likely to have *de novo* class II HLA-DSA, suggesting a possible immune-mediated pathological process for some [57]. No studies assessing the relationship between AT1R-Ab and TRAS have been performed. In liver transplantation, reports of a distinctive histological pattern of sinusoidal C4d staining are in contrast to the reports of C4d negativity in many kidney studies. Mechanistic studies to date have elucidated injurious aberrations to cell-signalling pathways, but perhaps it is too soon to discount a potential complement-mediated mechanism also.

The creation in the Banff 2022 report of the descriptive phenotype microvascular injury, C4d-negative, anti-HLA DSA-negative has focussed the minds of researchers on finding a cause. The burgeoning number of non-HLA antibodies that have been studied and subsequently implicated in allograft injury represents both an opportunity and a challenge for transplantation medicine. Given the broad array of potential antigenic targets, there has been no recommendation to test for specific non-HLA antibodies as part of a transplant recipient's immunological evaluation [58]. The Sensitization in Transplantation: Assessment of Risk (STAR) workgroup, initially established to aggregate cross-discipline knowledge on HLA histocompatabilty, has turned its attention to non-HLA antibodies in its latest report [59]. Acknowledging that studies to date have not firmly established temporal causality between AT1R-Ab status and development of AMR, the report nevertheless advocates for the development of standardized high-throughput testing for non-HLA antibodies [59]. Indeed, given that treatment options such as pharmacological receptor blockade and plasmapheresis have been reported as beneficial, it could be argued that access to validated testing for AT1R-Ab is an unmet clinical need for transplant recipients. For strategies to mitigate the potential pathologic effects of non-HLA antibodies in transplantation, readers are referred to the review by Kardol-Hoefnagel and Otten [60].

Large prospective studies are likely to be required to determine the clinically significant threshold of AT1R-Ab positivity. To take a pragmatic approach, expanding testing capabilities for patients on the transplant wait list first could be prioritised to allow us to better understand the epidemiological burden of AT1R-Ab positivity prior to transplantation, stratify patients at risk of developing chronic rejection related to AT1R-Ab, and identify recruits to prospective randomised controlled trials of therapeutic intervention.

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PM wrote the article. MW and CR reviewed and edited the article. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13280/full#supplementary-material

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REVIEW
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Allorecognition Unveiled: Integrating Recent Breakthroughs Into the Current Paradigm

Xavier Charmetant[1,2,3], *Gavin J. Pettigrew*[4] *and Olivier Thaunat*[1,2,3]*

[1]*Centre International de Recherche en Infectiologie, INSERM U1111, Université Claude Bernard Lyon I, CNRS UMR5308, Ecole Normale Supérieure de Lyon, University Lyon, Lyon, France,* [2]*Department of Transplantation, Nephrology and Clinical Immunology, Edouard Herriot Hospital, Hospices Civils de Lyon, Lyon, France,* [3]*Lyon-Est Faculty of Medicine, Claude Bernard University (Lyon 1), Villeurbanne, France,* [4]*Department of Surgery, University of Cambridge, Cambridge, United Kingdom*

In transplantation, genetic differences between donor and recipient trigger immune responses that cause graft rejection. Allorecognition, the process by which the immune system discriminates allogeneic grafts, targets major histocompatibility complex (MHC) and minor histocompatibility antigens. Historically, it was believed that allorecognition was solely mediated by the recipient's adaptive immune system recognizing donor-specific alloantigens. However, recent research has shown significant roles for innate immune components, such as lymphoid and myeloid cells, which are sometimes triggered by the mere absence of a self-protein in the graft. This review integrates recent breakthroughs into the current allorecognition paradigm based on the well-established direct and indirect pathways, emphasizing the semi-direct pathway where recipient antigen-presenting cells (APCs) acquire donor MHC molecules, and the inverted direct pathway where donor $CD4^+$ T cells within the graft activate recipient B cells to produce donor-specific antibodies (DSAs). The review also explores the role of natural killer (NK) cells in both promoting and inhibiting graft rejection, highlighting their dual role in innate allorecognition. Additionally, it discusses the emerging understanding of myeloid cell-mediated allorecognition and its implications for initiating adaptive immune responses. These insights aim to provide a more comprehensive understanding of allorecognition, potentially leading to improved transplant outcomes.

Keywords: adaptive immunity, innate immunity, allorecognition pathways, graft rejection, pathophysiology

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***Correspondence**
Olivier Thaunat,
✉ *olivier.thaunat@chu-lyon.fr*

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INTRODUCTION

The current dogma in transplant immunology defends that the genetic differences between the donor and recipient play a crucial role in shaping the outcomes of organ and tissue transplants. These differences lead to the expression of alloantigens, which serve as markers for the immune system to distinguish between self and non-self-tissues.

Alloantigens are categorized into two main types: major histocompatibility complex (MHC) and minor histocompatibility glycoprotein antigens. The latter include all the molecules other than the MHC which are polymorphic and therefore differ between the donor and the recipient. These two categories of alloantigens differ in size—six molecules of MHC (three of class I and three of class II) versus a myriad of minor histocompatibility antigens—and level of polymorphism (i.e., the variability of the amino-acid composition, which is very high for MHC antigens and more limited for minor

histocompatibility antigens) [1]. Furthermore, these two categories of alloantigens are recognized by the recipient's immune system through distinct mechanisms (grouped under the umbrella term allorecognition). The complex immune responses that are triggered lead to the various types of graft rejections commonly diagnosed in transplant patients.

Despite extensive studies over the past four decades, allorecognition remains a critical area of interest for transplant physicians. These professionals frequently encounter clinical situations that are not adequately explained by the current conceptual framework of transplant immunology. For instance, some episodes of cellular rejection occur late after transplantation, long after the disappearance of donor-derived passenger antigen-presenting cells. In contrast, very early flares of donor-specific antibodies (frequently observed after lung or intestinal transplantation) arise too quickly to result from a classical humoral response. Finally, while it is still largely believed that allorecognition is solely the domain of the recipient's adaptive immune system, recent publications have revealed a significant role for both the lymphoid and myeloid components of the innate immune system. Furthermore, the latter sometimes discriminate the graft not because it expresses donor-specific alloantigens, but because of the lack of expression of self-proteins.

In this review, we aim to provide a concise overview of the established theories that form the basis of the current understanding of allorecognition. Building on this foundation, we incorporate recent immunological discoveries that address existing theoretical gaps, with the goal of offering a more comprehensive understanding of allorecognition.

A BRIEF HISTORY OF ALLORECOGNITION

George Snell's groundbreaking work in the mid-20th century led to the discovery that graft rejection was primarily driven by genetic differences in the MHC.

The discovery that intact donor-specific MHC molecules on the surface of passenger APCs could be "directly" recognised by a large proportion of recipient's T cell clones introduced the concept of direct allorecognition, which was considered the initial step of the pathophysiological cascade of T-cell mediated rejection (TCMR). However, this model did not fully explain certain clinical observations, such as late TCMR episodes occurring long after the disappearance of donor-derived passenger APCs. In 2004, Herrera et al. proposed the concept of semi-direct allorecognition, in which the recipient's APCs present intact, unprocessed donor MHC molecules on their surface, following the capture of extracellular vesicles. Although this was a significant advance, it took considerable time to fully elucidate the details of this pathway, up to the description of the "three-cell cluster" model, where a single recipient APC presents both intact donor MHC and processed peptides, facilitating interactions between $CD4^+$ and $CD8^+$ T cells. This mechanistic understanding was critical in refining our view of how T cell-mediated alloimmune responses are sustained over time following transplantation. In fact some works suggest that even early episode of TCMR rely rather on the semi-direct pathway than the direct pathway [2].

Meanwhile, in the 2000s, Paul Terasaki proposed the humoral theory of organ rejection, in which the generation of donor-specific antibodies results on an antigenic recognition mechanism described years earlier by Lanzavecchia (1985), and which has come to be known as "indirect allorecognition" in the field of transplantation. This model helped explain antibody-mediated damages to graft endothelium and their role in long-term outcomes.

Parallel to these developments, the concept of "missing-self" was introduced in 1986, whereby NK cells can detect and destroy cells that lack self-MHC molecules. However, it was not until 2019 that this principle was fully applied to the field of transplantation, where NK cells were found to mediate allorecognition and directly contribute to graft rejection, particularly in the context of chronic microvascular damage.

Most recently, in the late 2010s, the concept of innate myeloid allorecognition has emerged. Fadi Lakkis's group has demonstrated that monocytes can directly recognize allogeneic non-self in a MHC-independent manner. This discovery adds another layer of complexity to allorecognition. However, whether this mechanism functions in clinical transplantation remains to be proven, highlighting the long path from fundamental immunological discoveries to their practical application in transplantation.

ALLORECOGNITION PATHWAYS LEADING TO T CELL-MEDIATED REJECTION

MHC molecules allow certain immune cells (such as dendritic cells and B cells) to present antigens (in the form of peptides) to T cells. They are made of a framework region and a binding groove. The amino acids that make up the latter define the peptide to which it will bind with the greatest affinity. The T-cell receptor (TCR) interacts with a complex zone, comprising both framework parts of the MHC and the antigen itself.

In a given individual, the process of thymic education of T lymphocytes shapes TCR repertoire. The clones whose TCR do not recognise self MHC are eliminated ("positive selection"), while self-reactive clones are either eliminated ("negative selection") or selected to differentiate into natural T reg [3]. In this manner, the TCR specificity of peripheral T cells is theoretically limited to recognizing self-MHC molecules presenting non-self-peptides, a concept for which Doherty and Zinkernagel were awarded the Nobel Prize [4]. This immunological dogma however does not explain why transplanted organs are targeted with such intensity by the recipient's cytotoxic T cells. The occurrence of T cell-mediated rejection (TCMR) after transplantation led to the discovery of direct allorecognition.

Direct T Cell Allorecognition: Molecular Mechanisms of Foreign Cell Communication

Direct T cell allorecognition depends on the recognition by recipient T cells of intact allogeneic MHC molecules expressed

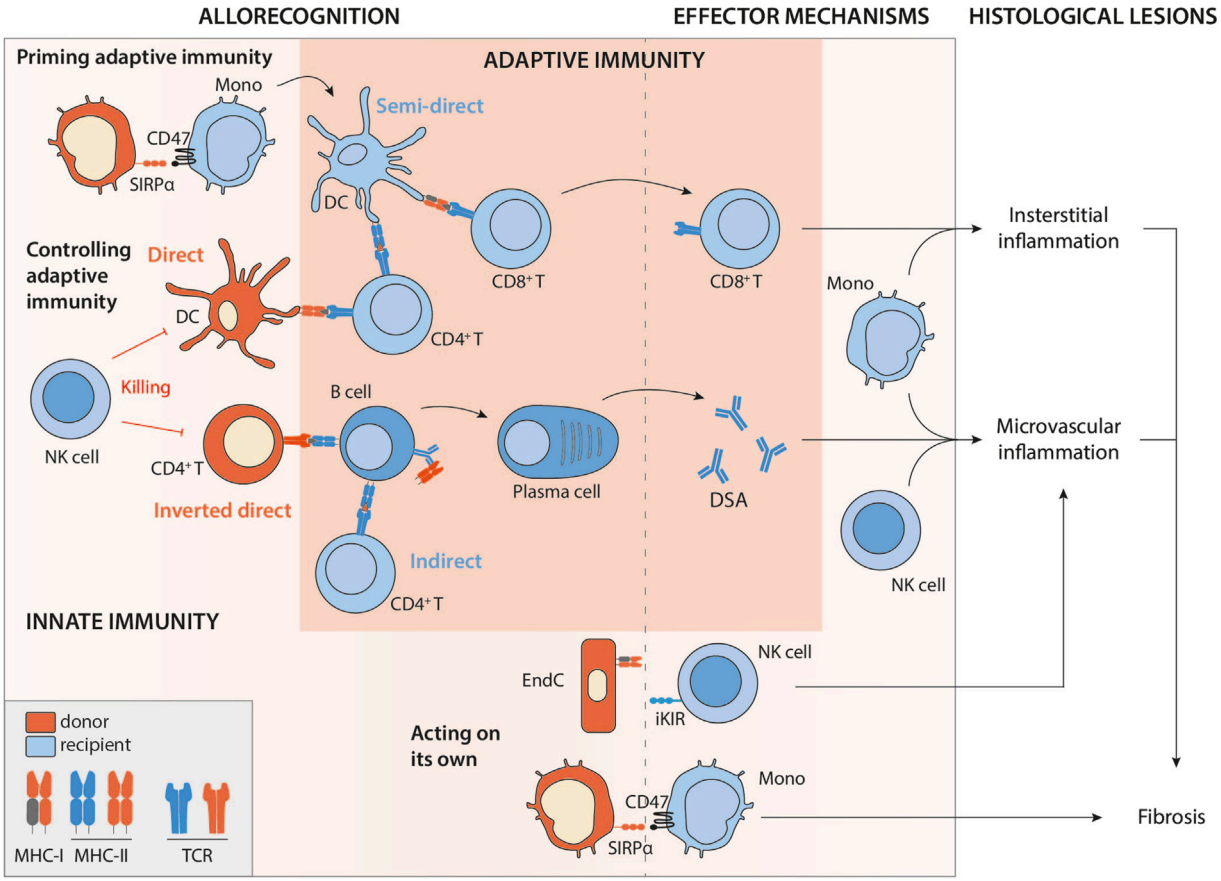

FIGURE 1 | Intricate innate and adaptive allorecognition pathways leading to allograft rejection. Legend: Mono, monocyte; DC, dendritic cell; NK, natural killer; EndC, endothelial cell; MHC, major histocompatibility complex; TCR, T-cell receptor; iKIR, inhibitory killer cell immunoglobulin-like receptor; SIRPα, signal-regulatory protein alpha; DSA, donor specific antibodies.

on graft cells. Left uncontrolled, this phenomenon rapidly leads to TCMR (**Figure 1**), since 1%–10% of a given individual's T cell repertoire is capable of recognising intact allogeneic MHC [5, 6].

There are two theories to explain this phenomenon [7–10], restricted to major alloantigens.

The "multiple binary complex" hypothesis (**Figure 2**) suggests that the principal factor determining the strength of T cell allorecognition is the wide variety of antigenic peptides presented by the donor's MHC molecules. Because differences in the MHC molecules between donor and recipient are concentrated in the peptide-binding grooves, donor and recipient cells present different peptides derived from the exact same protein. Thus, one donor MHC molecule can potentially represent a myriad of different antigenic complexes depending on the particular peptides bound. The "multiple binary complex" hypothesis was first suggested by the observation that T cell clones that responded to allogeneic antigen presenting cells (APCs) presenting peptides derived from human albumin did not respond to the same APCs presenting peptides from bovine albumin [11]. According to this theory, the ability to recognise endogenous peptides, and not the ability to "directly" interact with allogeneic MHC, is the main factor determining alloreactivity [12–14]. Heterologous immunity is a special case, which nevertheless fits into this theory. During an antiviral response, memory T lymphocytes are generated that recognise a self MHC/viral peptide complex. Molecular mimicry between this complex and donor MHC/peptide can divert the antiviral memory response into an allogeneic response [15, 16]. The multiple binary complex theory also explains the occurrence of TCMR in recipients of HLA-identical transplants. In this case, alloreactivity is not driven by polymorphisms in the binding groove (it is identical in donor and recipient), but rather by the fact that intra-familial HLA-identical donors are not genetically identical like monozygotic twins. While they may share the same HLA genes, the rest of their genomes can differ significantly.

Finally, it shall be kept in mind that the expression of MHC-I-peptide complexes on the cell surface depends on the function of various intracellular assembly factors, such as the transporter associated with antigen presentation (TAP), tapasin, calreticulin, ERp57, TAP-binding protein related (TAPBPR), endoplasmic reticulum aminopeptidases (ERAPs), and proteasomes. It is therefore conceivable that polymorphism in these proteins also

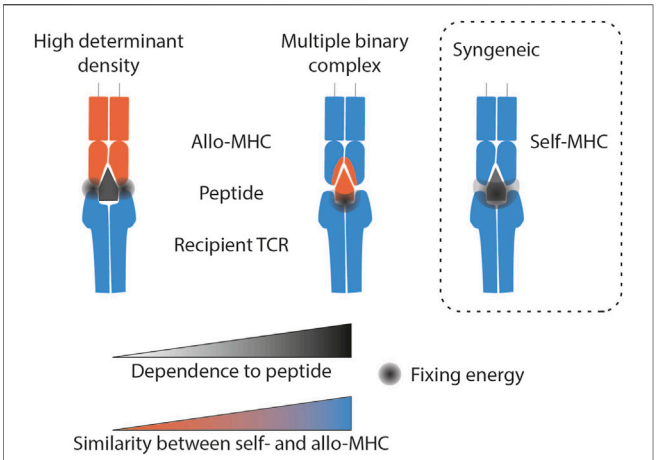

FIGURE 2 | Molecular mechanisms of TCR-mediated direct allorecognition compared with syngeneic recognition. The grey area represents the location where the binding strength of the TCR to the MHC/peptide complex is strongest according to the theory described. Syngeneic recognition implies an interaction force that encompasses the whole complex. The multiple binary complex theory predicts that the ability to recognise endogenous peptides is the main factor determining alloreactivity. Finally, the high determinant density theory proposes that the TCR directly recognises the MHC polymorphism, independently of the peptide antigen. Legend: MHC, Major histocompatibility complex.

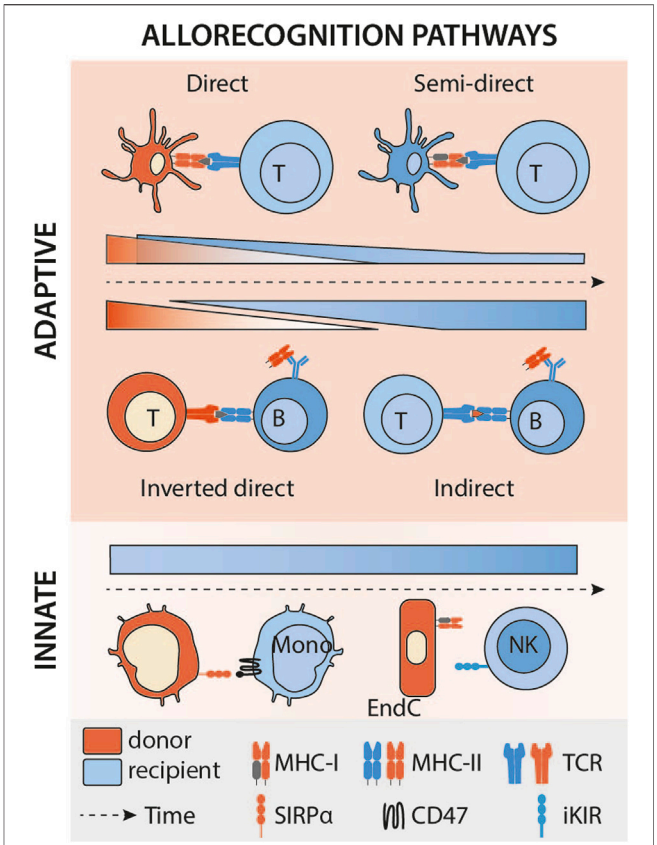

FIGURE 3 | Kinetics of the different allorecognition pathways after transplantation. Legend: Mono, monocyte; DC, dendritic cell; NK, natural killer; EndC, endothelial cell; MHC, major histocompatibility complex; TCR, T-cell receptor; iKIR, inhibitory killer cell immunoglobulin-like receptor; SIRPα, signal-regulatory protein alpha.

contribute to differences in the spectrum of HLA bound peptides (independently of differences in HLA binding groove and polymorphism of minor histocompatibility antigen) [17, 18].

According to the second theory – "the high determinant density theory" - the TCR directly recognises the MHC polymorphism, independently of the peptide antigen (**Figure 2**). Therefore, because potentially every peptide-MHC complex on the surface of the allogeneic APC is recognised as foreign, a strong overall response is triggered, even though the strength of the interaction between the TCR and individual alloMHC/peptide complexes may be very low [19].

These cognitive studies were mainly conducted in mouse models, or on a few specific HLA molecules, which do not encompass the complexity and extreme variability of HLA in humans. It is reasonable to think that for a particular donor/recipient pairing, the modality of interaction between the recipient TCR and the donor MHC lies within a range between the two theories described above.

Direct Allorecognition: The Tree That Hides the Forest

In transplantation, it has long been theorised that donor's leukocytes leave the graft rapidly after transplantation [20]. These passenger leukocytes, in particular the donor's APCs, reach the recipient's secondary lymphoid organs (SLOs), and present intact allogeneic MHC molecules on their surface to recipient's T cells [21]. According to this theory, the incidence of TCMR is directly linked to the presence of donor passenger leukocytes, and it is therefore expected for this incidence to decrease over time as the pool of donor leukocytes fades progressively and cannot be replenished [22] (**Figure 3**). The fact that vascularised composite allografts (VCA), in which the stock of donor APC can self-renew (i.e; Langerhans cells in the skin), are often targeted by late TCMR, can also be seen as an indirect validation of this theory [23, 24].

The development of an effective cytotoxic response requires the provision of $CD4^+$ T cell help to the $CD8^+$ T cell [25]. In the context of direct allorecognition, this help can be provided by $CD4^+$ T cells with direct specificity [26], presumably because within the recipient's SLO, a single donor APC can establish a productive three-cell cluster with $CD4^+$ and $CD8^+$ T cells that directly recognise the MHC class II and class I alloantigens, respectively, on the cell surface (**Figure 1**).

This allorecognition pathway offers the advantage of a treatment that seems readily available, namely, depletion of passenger leukocytes. Thus, several studies have been able to show, in heart, kidney [27] and liver transplantation [28], that depletion of donor APCs induces prolonged graft survival in the absence of immunosuppressants. However, there is a major bias in these studies: depletion of donor APCs not only eliminates the function they carry, but also the stock of antigens (in particular

MHC-II) that they strongly express. Notably, Mandelbrot et al have reported that cardiac allografts from donors whose APCs were unable to deliver costimulatory signals to recipient T cells were still rejected, despite the apparent inability to trigger conventional direct-pathway T cell responses [29].

A Paradigm Shift: Semi-Direct Recognition Takes the Lead

The limitations discussed above and the observation that TCMR can occur late, at a time when the pool of passenger donor leukocytes is expected to be exhausted, led to reconsideration as to how alloimmune responses with T cells with direct specificity are triggered.

A series of publications from the early 2000s have instead suggested that presentation of MHC alloantigen that has been acquired by the recipient APC and represented intact without processing may be key [30] (**Figure 1**). These recipient's APCs could have taken up donor antigens by direct cell-cell contact [31] with either migrating donor leukocytes or graft parenchymal cells (recipient APCs rapidly infiltrate the grafts after transplantation [32]). Alternatively, alloantigen may be acquired from donor-derived exosomes or extracellular vesicles drained from the graft to recipient's SLOs [33, 34]. It should be noted that donor-derived exosomes cannot activate T cells on their own but must be presented by donor APCs. This capture by recipient's APCs and the subsequent presentation to T cells only occurs in an inflammatory context [35], possibly explaining the link sometimes observed between infection and rejection, the causality of which has been difficult to establish [36, 37].

The semi-direct pathway has become gradually recognised as an important mechanism leading to TCMR, since studies (almost exclusively on murine models) by independent groups suggest that it elicits much stronger $CD8^+$ T cell immunity than interaction of the $CD8^+$ T cell with a donor leukocyte, principally because alloantigen from a single donor APC can be presented intact by many-more fold recipient APCs. The semi-direct pathway can still explain the observed kinetics of TCMR after transplantation (**Figure 3**). Indeed, in animal models of chronic renal graft rejection, cross-dressing, although declining with time, persists for many weeks after transplantation [2]. The initial peak is probably of mixed cause, linked to the donor's dendritic cells, which remain a major source of alloantigens in the early stages of the transplant, but also to ischaemia-reperfusion lesions, responsible for the immunogenic death of graft cells and the release of extracellular vesicles. Finally, although, as discussed above, the default pathway for provision of $CD4^+$ T cell help to alloreactive $CD8^+$ T cells is through direct pathway allorecognition of MHC class II alloantigen on the surface of donor APCs, Lee and Auchnicloss' seminal paper has demonstrated that indirect-pathway $CD4^+$ T cells can also provide effective help for generating effector cytotoxic $CD8^+$ T cell alloimmunity [38]. Explaining this phenomenon through the immunological paradigms prevalent at publication was not straightforward, because it necessitated formation of a cumbersome four cell cluster, comprising a donor dendritic cell presenting intact MHC class I alloantigen to an alloreactive $CD8^+$ T cell, and a recipient dendritic cell presenting processed alloantigen to an indirect-pathway helper $CD4^+$ T cell, with moreover no apparent physical linkage between the former two and the latter two cell types. This risks uncontrolled and damaging bystander T cell activation. Semi-direct recognition provides an elegant solution to obviate these concerns, because, assuming a single recipient dendritic cell presents both intact MHC class I alloantigen and self-MHC class II restricted allopeptide for, respectively, direct-pathway $CD8^+$ and indirect-pathway $CD4^+$ T cell allorecognition, this recreates a 3-cell cluster model (**Figure 1**), with physical linkage possible between the three [39–41].

ALLORECOGNITION PATHWAYS LEADING TO ANTIBODY-MEDIATED REJECTION

Although the development of modern immunosuppressive treatments has not completely eradicated TCMR (particularly late rejection and its complications), it has nevertheless improved short-term graft survival. However, the improvement in the long-term graft outcomes has not been as spectacular, as the rates of graft loss beyond the first year have shown more limited progress over different transplant eras [42–46]. This most likely reflects the contribution of humoral alloimmunity to chronic rejection, with the development of donor specific alloantibody (DSA) increasingly emphasised as an important effector of chronic graft damage [47]. This section considers the different allorecognition pathways responsible for DSA production.

The Critical Role of Indirect Allorecognition Pathway in DSA Generation

Although, as discussed, indirect pathway of allorecognition can provide help for generating cellular cytototoxic alloimmunity, its role in the provision of help to allospecific B cells is also important for determining graft outcomes. Indirect allorecognition is akin to conventional $CD4^+$ T cell recognition of model protein antigen, whereby alloantigen is presented as processed peptide held in the binding groove of the recipient's MHC class II antigen. Essential help for thymo (T) dependent antibody responses is provided by $CD4^+$ T cells that similarly recognise complexes of MHC class II and bound peptide antigen, after that antigen has been internalised and processed via the B cell receptor (BCR). Thus, recipient indirect-pathway $CD4^+$ T cells provide help for generating alloantibody [48–50] (**Figure 1**). The scientific literature on T-dependent humoral immunity has expanded prolifically over the last decade, with several recent publications considering how these advances shape our understanding of the B cell alloresponse.

Unlike cellular alloimmunity, whose functional relevance is confined to the response against donor major histocompatibility alloantigens [51], the humoral alloimmune response can be initiated by all alloantigens, both major and minor [52]. Alloreactive B cell responses are targeted generally against intact, conformational antigens [53, 54], which means that alloantigens must be transported from the graft to the SLOs

for presentation to B cells. This may be achieved by exodus of donor DCs from the graft [20]. The graft may also release extracellular vesicles covered with donor MHC that are then captured by recipient DCs [54] or by subcapsular sinus macrophages in lymph nodes (or their equivalents in the spleen) [55] for presentation to allospecific B cells. In parallel, recipient DCs will acquire alloantigen either within SLOs or the graft itself and present it to indirect-pathway $CD4^+$ T cells [34], which then migrate to the border of B cell zone and provide cognate B cell help for triggering initial production of class-switched alloantibody via short-lived extrafollicular foci. It is, however, the subsequent relocation to the B cell follicle and the formation of the Germinal Centre (GC) reaction, with essential help provided by further differentiation of indirect-pathway $CD4^+$ T follicular helper cells [49], that likely determines transplant outcome. GC responses are uniquely capable of producing somatically-mutated, high-affinity alloantibody and animal models have confirmed the essential role of the GC response in mediating chronic antibody mediated rejection [56, 57]. The GC reaction also produces robust humoral memory, composed of memory B cells and bone-marrow resident, long-lived-plasma cells, with the latter potentially capable of producing alloantibody for the life of the individual, long after the GC response has dissipated. It is this aspect of the GC reaction that makes treatment of AMR and the desensitisation of patients awaiting transplantation so challenging [58, 59].

Direct Allorecognition and DSA Generation: The Inverted Direct Pathway

Clinicians' experience and some studies have reported unusually strong *de novo* DSA responses developing early (within the first week) following lung and intestinal transplantation [60]. This early onset of *de novo* DSA suggests that in addition to the canonical indirect pathways, other mechanisms may be responsible for an alloimmune humoral response. In line with this hypothesis, recent work, published independently by Pettigrew's and our own team [61, 62], has highlighted a major role for passenger donor $CD4^+$ T cells that are contained in vascularised grafts and transferred to the recipient after the transplantation. This passenger $CD4^+$ T cell population would be expected to contain a relatively large proportion (1%–10%) of cells with direct allospecificity for the recipient's MHC class II antigens [what is true for recipient cells is also true for donor-derived cells [5, 6]], and are therefore capable of binding with these antigens on the surface of recipient haematopoietic cells. Thus, during inverted direct allorecognition (**Figure 1**), initial activation of recipient alloreactive B cells is triggered by binding B cell receptor (BCR) to its target alloantigen (as occurs with encounter with any classical antigen). Internalising and processing of the bound alloantigen results in upregulated expression of surface MHC class II presenting bound peptide alloantigen, but rather than cognate help being provided by indirect-pathway $CD4^+$ T cells, these MHC class II complexes are recognised in a peptide-degenerate manner by activated passenger donor $CD4^+$ T cells, which in turn deliver a second (costimulatory) signal to the B cell, enabling it to differentiate into a DSA-producing plasma cell [61, 62].

To date, a definitive analysis of how the kinetics of DSA production via the inverted direct pathway compares to conventional indirect-pathway $CD4^+$ T cell help has not been performed, and it remains unclear whether the very early onset of alloantibody production (as soon as day 7) is mediated exclusively via the inverted direct pathway (**Figure 3**). A study of the precursor frequencies of the relative T cell pools mediating each pathway may provide some insight. In the indirect pathway, the frequency of the T cells involved is very low (clonal frequency of around 1/10,000). As a result, time-consuming clonal expansion is necessary to achieve an effective response. In contrast, the recently described pathway involves a very large pool of T cells (1%–10% of the T cells contained in the graft), capable of immediately providing effective assistance to B cells, without amplification. Moreover, the majority of transferred donor $CD4^+$ T lymphocytes with direct allospecificity for the recipient would likely exhibit a memory phenotype, because of cross-reactive heterologous immunity [15, 16], one would expect that this would provide robust help for even more rapid production of alloantibody [50, 63]. This also explains the seemingly paradoxical finding that heart grafts from donors sensitised to recipients were rejected more rapidly than grafts from naïve donors [61].

In clinical terms, the incidence of early DSAs is immediately correlated with the richness of the graft in lymphoid cells: renal grafts produce very few early DSAs, whereas lung and intestinal grafts (which contain a mucosa-associated lymphoid tissue) induce a massive wave of early *de novo* DSAs, in 25%–80% of patients [62]. It is also worth noting that B cells activated via inverted direct allorecognition should also be capable of 'soliciting' their own help from recipient $CD4^+$ T cells [64]. Our recent work has suggested that whereas passenger donor $CD4^+$ T cells trigger a rapid humoral response in the recipient, maintenance of this response as a GC reaction was dependent upon secondary differentiation of allopeptide-specific T follicular helper cells of recipient origin [57, 65].

One further possible consequence of inverted direct recognition is that, because the help provided by the donor $CD4^+$ T cells is likely to be promiscuous and provided to all B cells in a peptide-degenerate fashion, the limiting factor in antibody production is availability of target antigen to bind the BCR. Consequently, in some murine models, inverted direct recognition was associated with production of class-switched anti-nuclear autoantibody responses [65]. Thus, this phenomenon may be responsible for the various autoantibody responses that have been detailed in human transplant recipients [66].

INNATE ALLORECOGNITION

In the same way that improved prevention of TCMR has lifted the veil on the clinical importance of AMR, dissection of the pathophysiological mechanisms of the latter has revealed other holes in the picture: not all allograft rejection depends on the

adaptive immune system and evidence is increasing for a direct role of innate immunity in allorecognition and graft injury [67].

Innate Lymphoid Cells-Mediated Allorecognition: A Double-Edged Sword

NK cells are prototypical of the paradigm shift from adaptive to innate rejection. Long implicated as secondary effectors of the humoral arm through their ability to mediate antibody-dependent cellular cytotoxicity (ADCC), they may have additional and independent role in allorecognition and innate allograft damage.

NK Cells Control Direct Recognition-Mediated Pathways

The first proof of the involvement of NK-mediated allorecognition was provided for the benefit of graft survival. In fact, NK cells can control the survival of donor cells responsible for direct antigen presentation (**Figure 1**). This phenomenon was first demonstrated in mice by transplanting organs whose survival was threatened solely by the cellular arm of the adaptive alloimmune response (because the vascularisation comes from the recipient and cannot be targeted by DSA, namely skin and pancreatic islets [68]). In these models, recipient alloreactive NK cells can destroy donor APCs and thus significantly prolong graft survival [69, 70]. More recently, the evidence has been extended to a lung transplantation model, in a study demonstrating that when the recipient's NK cells are able to eliminate the donor's DCs, the alloreactive T cell infiltrate is significantly reduced, and the lungs are less rejected [71]. Similarly, donor NK cells are able to eliminate donor $CD4^+$ T cells involved in the inverted direct allorecognition pathway [61]. As a result, the latter cannot interact with the recipient's alloreactive B cells, and the production of early DSAs is avoided (**Figure 1**). In all these studies, it is accepted that a MHC-I mismatch activates the recipient's NK cells [72], because the donor's MHC class I ($H2D^d$) is an activating ligand for the Ly49D NK cell receptor in the recipient mouse.

NK cell alloreactivity is however complex because NKs are constantly integrating numerous activating and inhibiting signals, via various membrane receptors. A lack of inhibition (for example, due to the lack of expression on the surface of a target cell of self-MHC molecules, i.e., "missing-self") or an excess of activation (for example, expression of a stress ligand by the target cell) can lead to activation of the NK cell. In humans, several layers of complexity make difficult the prediction of NK cell behaviour towards allogeneic targets. First, HLA mismatches between donor and recipient, along with the diversity of receptors on the recipient's NK cells, are the primary determinants of this alloreactivity. Second, the signals delivered by the target can be influenced not only by the quantity but also by the quality (affinity) of the ligands for NK cell receptors. Third, the ability to activate NK cells also depends on the condition of the target (whether stressed or not). Consequently, predicting the behaviour of NK cells towards a specific allogeneic target is currently impossible. This unpredictability extends to interactions between recipient NK cells and a self-APC that cross-presents alloantigens (i.e., an APC that expresses both self and non-self MHC). Since we cannot determine which activating and inhibitory signals will dominate, the control of the semi-direct pathway by NK cells has not been formally evaluated. Notwithstanding, boosting allogeneic NK innate responses at an opportune moment, i.e., in the first few days following organ transplantation, may prove to be an effective lever for limiting both cell rejection and early DSA production.

. . . But NK Cells can Also Directly Damage the Graft

It is now widely accepted that NK cell-mediated allorecognition can also lead to chronic allograft vascular rejection, independently of DSAs. Indeed, it has been demonstrated in mouse models [73] and then confirmed in clinical studies [73, 74] that the lack of expression of self MHC-I by graft vasculature can activate NK cells and cause chronic vascular damages (**Figure 1**). Of note, about 30% of patients with microvascular inflammation in absence of DSA have also no missing self to explain the histological lesions [73]. Missing-self is therefore likely not the only molecular mechanism able to trigger NK cell response against a graft endothelium and several situations resulting in either an excess of activating signals or a defect in inhibitory signals (or a mixture of both) involving KIR- or NKG2-family of NK receptors could trigger NK cell response against a graft endothelium (reviewed in [75, 76]).

Innate Myeloid Cells-Mediated Allorecognition: Last Discovery, First to Initiate Rejection

For the sake of clarity, we have separated in this review the mechanisms of allorecognition dependent on innate immunity from those dependent on adaptive immunity, but this last part brings us back to the very beginning of this review: the priming of alloreactive T cells.

The initiation of a T cell response requires the activation and full differentiation of APCs. According to the danger theory, this activation is triggered by danger-associated molecular patterns (DAMPs), molecules of all kinds that can be released by certain types of cell death (necroptosis for example). However, in the context of transplantation, the danger is probably not sufficient to initiate an alloimmune response. Indeed, recent work in mice has unequivocally demonstrated that i) the mouse immune system (particularly monocytes) can distinguish between self and non-self [77] and that ii) this recognition of non-self by monocytes is necessary for the development of an alloimmune T response [78]. Indeed, in syngeneic transplantation, monocytes that differentiate into DCs are incapable of sustaining a T response despite the DAMPs provided by ischaemia-reperfusion inherent in transplantation. In contrast, in an allogeneic context, monocyte-derived DCs induced by allogeneic cells are perfectly mature, express IL-12 and effectively stimulate T cell proliferation. This mechanism of innate myeloid recognition is independent and acts on its own account, since it does not depend on DAMPs receptors [78]. Indeed, the same authors demonstrated that it depends (at least in part) on the

recognition, by the recipient's monocytic CD47, of signal-regulatory protein (SIRP) α polymorphisms in the donor [79] (**Figure 1**).

Myeloid allorecognition can also act on its own behalf. Monocytes carry paired Ig-like receptors (PIR) which can recognise allogeneic MHC-I molecules on the surface of donor cells. Monocytes activated in this way can contribute to allograft rejection, and even establish an innate memory response [80]. The establishment of memory via PIRs requires the SIRPα signal described above at the time of monocyte priming [79, 80].

These recent findings have yet to be formally confirmed in humans. Recent studies have characterised an infiltrate of non-classical monocytes expressing both CD47 and leukocyte immunoglobulin-like receptors (LILRs, orthologs of murine PIRs) in rejection biopsies of kidney grafts [81, 82], and suggest that these monocytes may support the $CD8^+$ T cytotoxic response [82]. Further studies are needed to confirm the role of innate myeloid allorecognition in human transplantation.

CONCLUSION

In conclusion, the mechanisms involved in allorecognition are far more complex than initially understood. The direct allorecognition pathway does not fully account for the persistence and dynamics of TCMR episodes. Recent studies have highlighted the importance of the semi-direct pathway, where recipient APCs acquire and present donor MHC molecules. AMR, which depends on the generation of donor-specific antibodies (DSAs), involves both the indirect and direct allorecognition pathways. The indirect pathway engages recipient APCs to present donor peptides to T cells, which then assist B cells in producing DSAs. In contrast, the recently discovered inverted direct pathway involves donor $CD4^+$ T cells within the graft directly driving recipient B cells to rapidly produce DSAs.

Additionally, innate allorecognition mechanisms have emerged, supplementing the understanding traditionally dominated by adaptive immunity. On the one hand, NK cells, typically associated with antibody-dependent cellular cytotoxicity, have been shown to mediate direct antigen recognition and impact graft survival through mechanisms like "missing-self". This has direct clinical implications, because DSA-independent missing self-induced microvascular inflammation, which was classified as "no rejection" according to Banff 2019 [83], now falls into a subcategory of DSA-negative and C4d-negative microvascular inflammation (Banff 2022, [84]). In addition, these microvascular injuries justify a specific, tailored treatment that does not target DSA but only NK, and which is currently being evaluated in a clinical trial. Still on the subject of NK cells, their involvement in controlling the inverted direct pathway in the clinic has yet to be demonstrated. If this were the case, it would pave the way for treatments to modulate NK alloreactivity according to the time of transplantation, by stimulating it at the start of the graft to prevent the formation of early DSA, and by repressing it thereafter to prevent chronic vascular rejection. On the other hand, the involvement of innate myeloid cells, particularly monocytes, in recognizing non-self and initiating alloimmune responses highlights the variety and complexity of allorecognition. However, evidence in humans is still lacking and requires dedicated studies before monocytes can be considered as a therapeutic target.

This expanded understanding of allorecognition is crucial for developing more effective strategies to manage and prevent graft rejection, ultimately improving the outcomes of organ and tissue transplants. These insights highlight the need for a comprehensive approach in managing transplant rejection, integrating targeted strategies against both adaptive and innate immune mechanisms to improve long-term outcomes.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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NK Cells: Not Just Followers But Also Initiators of Chronic Vascular Rejection

Mathilde Chambon[1] *and Alice Koenig*[1,2,3]*

[1]CIRI, INSERM U1111, Université Claude Bernard Lyon I, CNRS UMR5308, Ecole Normale Supérieure de Lyon, University of Lyon, Lyon, France, [2]Hospices Civils de Lyon, Edouard Herriot Hospital, Department of Transplantation, Nephrology and Clinical Immunology, Lyon, France, [3]Lyon-Est Medical Faculty, Claude Bernard University (Lyon 1), Lyon, France

Chronic graft rejection represents a significant threat to long-term graft survival. Early diagnosis, understanding of the immunological mechanisms and appropriate therapeutic management are essential to improve graft survival and quality of life for transplant patients. Knowing which immune cells are responsible for chronic vascular rejection would allow us to provide effective and appropriate treatment for these patients. It is now widely accepted that natural killer (NK) cells play an important role in chronic vascular rejection. They can either initiate chronic vascular rejection by recognizing missing self on the graft or be recruited by donor-specific antibodies to destroy the graft during antibody-mediated rejection. Whatever the mechanisms of activation of NK cells, they need to be primed to become fully activated and damaging to the graft. A better understanding of the signaling pathways involved in NK cell priming and activation would pave the way for the development of new therapeutic strategies to cure chronic vascular rejection. This review examines the critical role of NK cells in the complex context of chronic vascular rejection.

Keywords: antibody mediated rejection, chronic rejection, natural killer cells, missing self, antibody dependent cellular cytotoxicity

INTRODUCTION

Solid organ transplantation represents the optimal treatment option for patients with end-stage organ failure [1]. Nevertheless, the survival of allografts is constrained by the occurrence of rejection, which is initiated when the recipient's immune system identifies donor determinants [in particular, mismatched Human Leucocyte Antigen (HLA) molecules] and conducts the destruction of the graft. Over the past few decades, the development of immunosuppressants has enabled significant improvements in short-term graft survival, largely by preventing early rejections, which are mainly driven by T cells. However, the percentage of grafts that do experience attrition beyond the first transplant year remains unchanged [2]. Long-term graft survival represents an unmet medical need in solid organ transplantation. In the majority of cases, long-term graft loss is of immunological origin and is referred to as chronic rejection. Although there are discrepancies in the lesions found in cases of chronic rejection in the different organs, it is now clear that vascular lesions are a common feature [3]. It is widely accepted that these lesions may be the result of mechanisms involving donor specific antibodies (DSA) [3]. However, in some cases, antibodies are not present, suggesting that other mechanisms may also be involved in chronic vascular rejection. Recent research has challenged traditional views on chronic vascular rejection, revealing a prominent role for Natural Killer (NK) cells in chronic vascular rejection, regardless of the presence or absence of DSA. This challenges the prevailing view that innate immune cells only play a role in rejection

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***Correspondence**
Alice Koenig,
alice.koenig@inserm.fr

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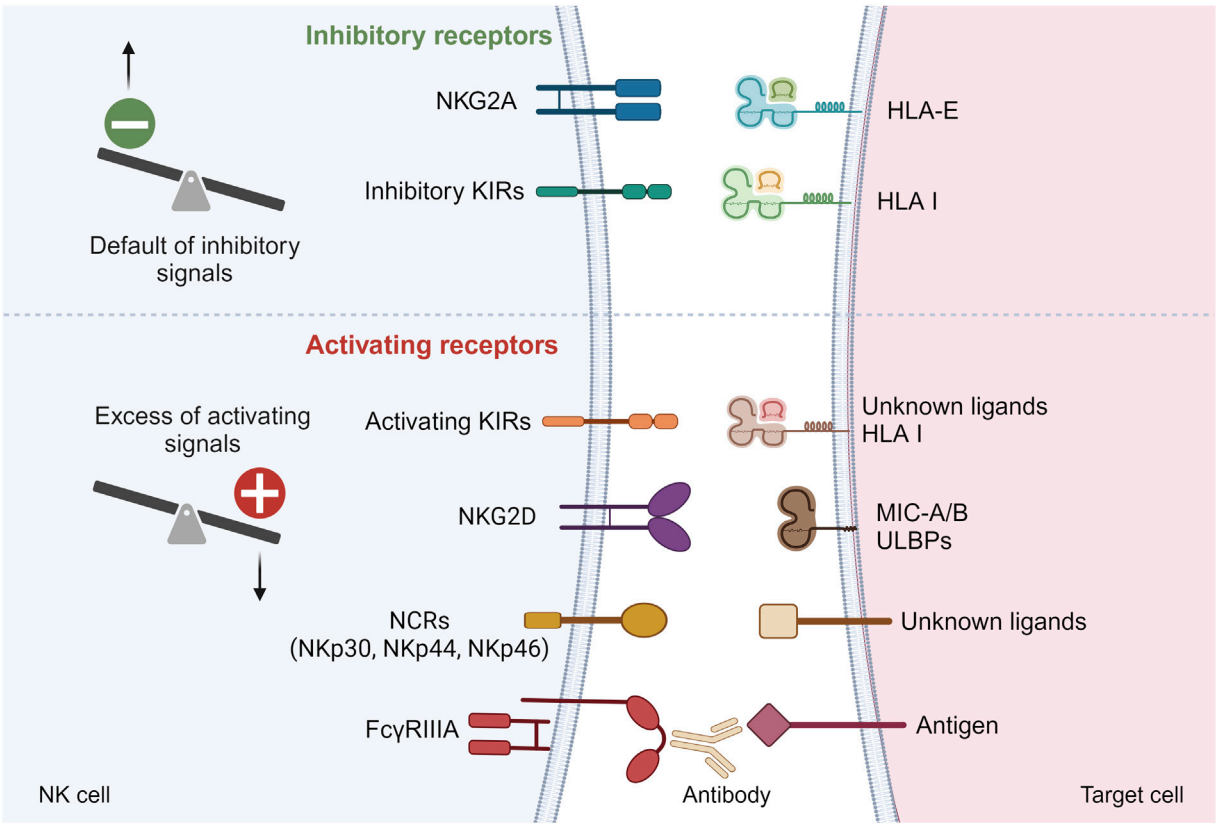

FIGURE 1 | Main inhibitory and activating receptors on NK cell surface. Default of inhibitory signals or excess of activating signals can lead to NK cell activation. KIRs, Killer Immunoglobin-like Receptors; NCRs, Natural Cytotoxicity Receptors; HLA, Human Leucocyte Antigen; MIC-A/B, MHC class I-related chain A/B; ULBPs, UL16-binding proteins.

if they are recruited by the adaptive immune system. The objective of this review is to examine the role of NK cells in chronic vascular rejection, with a particular focus on the mechanisms that can trigger their activation. This will provide insights into the pathways involved and identify potential therapeutic avenues for preventing their activation and, consequently, reducing the occurrence of chronic vascular rejection.

GENERAL OVERVIEW OF NK CELL ACTIVATION

NK cells are a crucial component of the innate immune system, responsible for detecting and eliminating infected, stressed, or transformed cells, such as cancerous cells. Unlike T cells and B cells, which are part of the adaptive immune system and require prior exposure to a specific pathogen for an effective response, NK cells can respond immediately to threats without prior sensitization. To achieve this, NK cells express several germline-encoded activating and inhibitory receptors on their surface (**Figure 1**). The balance between these signals determines whether the NK cell will attack the target cell. NK cells can be activated through three main mechanisms. First, missing-self recognition occurs when a target cell loses or downregulates its HLA class I molecules, often due to viral infection or cancer (**Figure 1**). The lack of inhibitory signals allows NK cells to destroy the target cell. Second, NK cells can be activated by induced-self ligands. Cells under stress or transformation may express specific ligands, such as MICA/B or ULBPs, which are recognized by activating receptors on NK cells, triggering NK cell activation (**Figure 1**). Finally, NK cells can be activated through antibody-dependent cellular cytotoxicity (ADCC). In this process, NK cells recognize antibodies bound to the surface of target cells via their Fcγ receptor (FcγRIIIA) (**Figure 1**). This antibody-mediated recognition leads to NK cell activation and targeted killing of the antibody-coated cell.

In the following sections of the review, we will describe how these mechanisms of NK cell activation might be involved in chronic vascular rejection.

MISSING-SELF INDUCED NK CELL ACTIVATION AND "INNATE" REJECTIONS

Inhibitory Killer Cell Immunoglobulin-Like Receptors (KIR) Bind to HLA Class I

Inhibitory KIRs are highly polymorphic at the genetic level, heterogeneously expressed by NK cells. Inhibitory KIRs possess

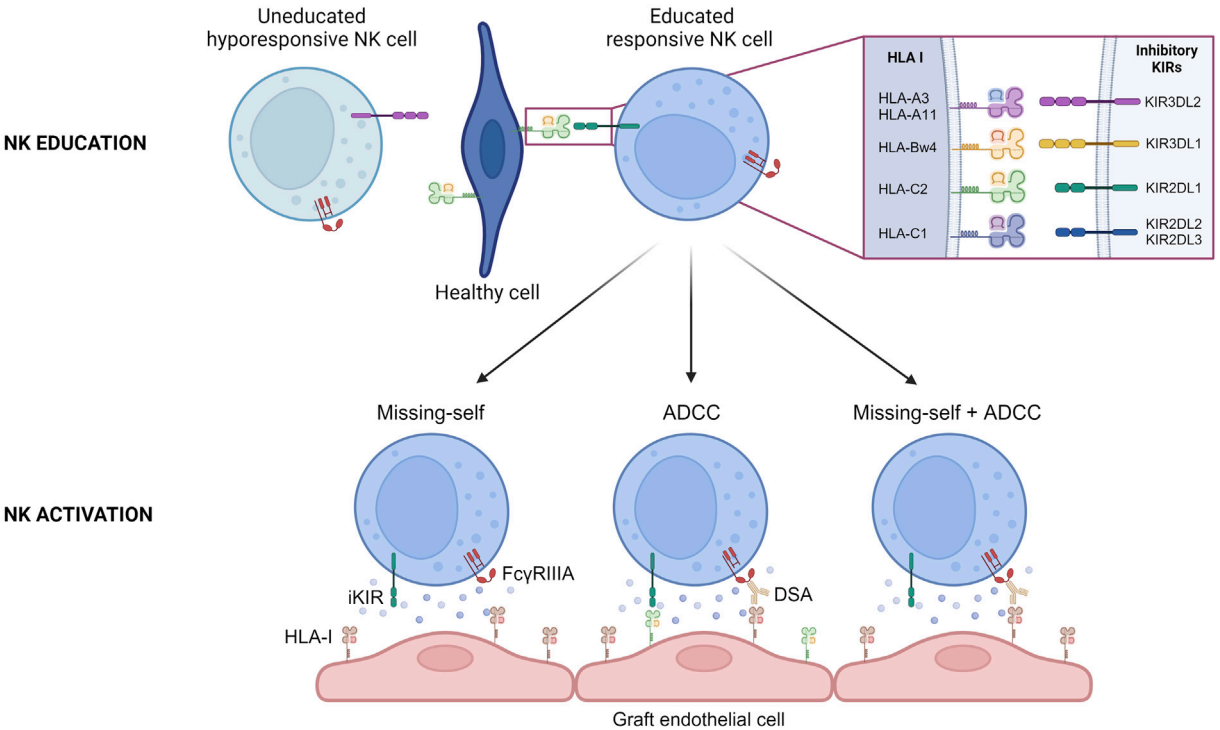

FIGURE 2 | NK cell education and activation during chronic vascular rejection. Schematic representation of the education process of NK cells during their maturation. The interaction between specific inhibitory KIRs on NK cell surface and HLA I on healthy cells allows NK cell to become responsive. Schematic representation of the three situations that can conduct to recipient NK cell activation during chronic vascular rejection. ADCC, Antibody Dependent Cell Cytotoxicity; DSA, Donor Specific Antibody; HLA-I, Human Leucocyte Antigen class I.

two (2D) or three (3D) extracellular immunoglobulin domains, a transmembrane domain and a long (L) cytoplasmic tail containing two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) [4]. Each inhibitory KIR has for ligands a subgroup of HLA class I allotypes. Inhibitory KIRs with 2 extracellular domains recognize HLA-C molecules based on two dimorphisms at position 77 and 80 of the α1 domain of the α chain [4]. KIR2DL1 associates with HLA C2 allotypes [4]. In contrast, KIR2DL2 and three show specificity for HLA C1 allotypes [4]. KIR3DL1 recognizes HLA-A and HLA-B molecules that display the Bw4 motif [5]. Finally, KIR3DL2 solely interacts with HLA-A3 and A11 molecules [4].

NK Cell Education and Activation by Missing-Self

The complex interplay between inhibitory KIRs and HLA class I molecules is of paramount importance in the education and activation of NK cells. As the genes for inhibitory KIRs and HLA class I molecules are on different chromosomes and that NK cells express germline-encoded receptors that do not undergo somatic rearrangement during development, NK cells need to undergo an educational process where they learn to distinguish "self" from "non-self" through interactions between inhibitory KIRs and self HLA class I molecules [6]. NK cells that encounter self-HLA class I molecules during their maturation process receive inhibitory signals, ensuring that they do not attack healthy cells displaying self HLA class I molecules (**Figure 2**). In absence of any interaction between inhibitory KIRs and HLA class I molecules during their development, NK cells become hypo responsive (**Figure 2**). This process helps to refine NK cell self-tolerance and future reactivity as the number of interactions between inhibitory KIRs on NK cells and HLA class I molecules determines the degree of responsiveness of mature NK cells. Conversely, when these educated NK cells encounter virally infected or transformed cells that lack or display altered HLA class I molecules, the lack of inhibitory signals transmitted by inhibitory KIRs allows for the activation of NK cells by the recognition of missing self (**Figure 2**).

Missing-Self Induced NK Cell Activation Triggers Rejection After Organ Transplantation

For a considerable period, the implication of the "missing self" in organ transplantation was overlooked. New advances were made when Colvin and his team observed that when a heart transplant from a parental strain was transplanted to an F1 hybrid strain

(between the parental line and a second strain), the graft presented chronic vascular rejection [7]. It is notable that this model demonstrates that NK cells can initiate chronic vascular rejection, but that they are insufficient to trigger chronic vascular lesions when acting alone. For this to occur, they must recruit T cells that are not specific for donor major histocompatibility complex (MHC) and macrophages in particular, through the secretion of interferon gamma (IFN-γ) [7, 8]. In humans, many studies have looked at this issue for a long time without results, probably because of a lack of data to analyze the presence of missing-self correctly [9–13], or because the chosen read out was not the good one [10], or because of confounding factors [14, 15]. A decade ago, in a cohort of 137 kidney transplant patients compatible for HLA-A, HLA-B and HLA-DR (i.e., in whom minimal T- and B cell alloreactivity was expected), Van Bergen et al. observed that missing self was associated with a worse allograft survival [14]. This was one of the first clinical studies to suggest the involvement of missing self after kidney transplantation, but to go deeper into the demonstration, we have to wait for a study published by our group a few years ago [15]. In a cohort of deeply phenotyped kidney transplant patients, we demonstrated that a greater proportion of patients with microvascular inflammation lesions in the absence of DSA (either HLA or non-HLA DSA) exhibited a missing self on their graft susceptible to being sensed by the recipient's NK cells and this resulted in reduced allograft survival compared to a control cohort (patients with no vascular lesions and no DSA) [15]. This clinical correlation was confirmed in an *in vitro* model in which we demonstrated that the lack of self HLA class I molecules on endothelial cells can activate NK cells, which in turn induce endothelial cell damage (**Figure 2**) [15]. An *in vivo* mouse model of heart transplantation was then developed. Heart transplants were performed from β2 micro KO mice (i.e., mice that do not express MHC class I molecules) to mice of the same genetic background [15]. The grafts developed microvascular inflammation lesions only in the presence of NK cells and a missing self, thereby confirming the existence of missing-self induced NK-mediated rejection [15]. Our results were confirmed in a population-based study which showed that missing self increased microvascular inflammation occurrence after kidney transplantation, independently of DSA [16]. Microvascular inflammation severity increased with the number of missing self [16].

NK CELL-MEDIATED ADCC DURING B CELL-MEDIATED REJECTIONS

Key Role of NK Cells During Chronic Antibody-Mediated Rejection (AMR)

It is widely accepted that NK cells play a pivotal role in the development of chronic vascular rejection in the presence of DSA. Binding of circulating DSA to directly accessible donor HLA molecules expressed by graft endothelial cells can sometimes trigger the classical complement pathway, which accelerates the rejection process [17, 18]. However, this is not a mandatory requirement for the development of chronic AMR [19]. It has been demonstrated that the recruitment of innate immune cells by DSA is sufficient to trigger endothelial cell damage through an ADCC mechanism (**Figure 2**). In 2012, Colvin and his team have shown that when $RAG^{-/-}C3^{-/-}$ mice were transplanted with an allogeneic heart and injected regularly with DSA, they developed chronic vascular lesions in their graft and that these lesions were completely abrogated in the absence of NK cells [20]. In the clinical setting, the key role of NK cells in AMR is supported by transcriptomic analyses of renal graft biopsies [21–23]. Recent data suggesting that afucosylation of HLA-specific IgG1 is directly related to antibody pathogenicity in kidney transplantation also provides indirect evidence for the key role of NK cells in AMR, as fucosylation is known to strongly influence the affinity of IgG for FcγRIIIA, which is expressed by NK cells [24, 25]. Furthermore, the intensity of NK cell infiltration within the graft correlates with graft survival after kidney transplantation [26].

NK cell activation during AMR is thought to be triggered by the interaction between NK's unique Fcγ receptor, FcγRIIIA and DSA (**Figure 2**). In the murine heart transplantation model of AMR described above, mice injected with F $(ab')_2$ DSA fragments failed to develop chronic vascular lesions in comparison to mice injected with intact DSA, suggesting that the interaction of NK cells with the Fc fragment of DSA is necessary [20]. Transcriptomic analyses conducted on sets of renal allograft biopsies from patients with AMR identified NK cell signaling, including evidence for the FcγRIIIA signaling elements [27, 28] suggestive of an NK cell activation through FcγRIIIA in AMR. In a recent study, thanks to single cell RNA sequencing analyses and multiplexed immunofluorescence, Lamarthée and colleagues have shown an association between FcγRIIIA+ NK cells and the severity of intragraft inflammation in the context of AMR [22]. Like in missing-self induced NK-mediated rejections, NK cells do not act alone and seem to interact in particular with FcγRIIIA+ non-classical monocytes via LGALS9-HAVCR2 to trigger allograft destruction [22]. They also interact to a lesser extent with T cells expressing CD74CXCR4 via the secretion of macrophage migration inhibitory factor (MIF) [22].

Factors Modulating NK Cell Activation During AMR

A single nucleotide polymorphism exists in the FcGR3A gene, resulting in the expression of two co-dominant alleles coding for either a phenylalanine (F) or a valine (V) at amino acid position 158 in the extracellular domain of the receptor [29]. This single nucleotide polymorphism modulates the binding capacity of FcγRIIIA to the Fc fragment of IgG, which may in turn modulate the severity of lesions and the prognosis of patients with AMR. In kidney transplantation, Arnold et al. observed that patients with DSA and presenting at least one high binding allele (V/V or V/F) exhibited more frequent and severe peritubular capillaritis lesions [30]. Furthermore, the researchers hypothesize that this heightened microvascular inflammation may be due to the secretion of IFN-γ, which facilitates the recruitment of other immune cells in the graft [30]. These findings were corroborated in a cohort of kidney transplant recipients with chronic humoral rejection [31]. The patients with two V/V alleles exhibited a higher glomerulitis

score and a reduced graft survival compared to patients of other genotypes [31]. The authors propose that the V-allele is associated with an increased expression of FcγRIIIA by NK cells, which translates into a greater propensity for NK cells to degranulate when they interact with their targets [31].

In addition, a recent study has investigated the impact of different genetic variations present in the genes of different NK cells in a cohort of patients with *de novo* DSA [32]. In particular, they got interested in a polymorphism in *KLRC2* gene which encodes NKG2C, an activating NK cell receptor, which binds to HLA-E and is known to be present on memory like NK cells with more potent effector functions [29]. Heterozygous or homozygous *KLRC2* deletion ($KLRC2^{del}$) is associated with a significantly lower or absent NKG2C expression level. Here, they showed that patients with a $KLRC2^{wt/wt}$ genotype presented more microvascular lesions than $KLRC2^{wt/del}$ patients but failed to show any impact on allograft survival [32].

Finally, our group has recently shown that missing self and DSA can combine to induce chronic vascular rejection in a cohort of kidney transplant patients with AMR (**Figure 2**) [33]. Notably, the additive effect of missing self on allograft survival was only observed in patients with ADCC-dependent AMR [33]. This may be explained by the fact that allograft loss is too rapid in patients with complement-dependent AMR.

Whether the NK cell initiates rejection in missing self-induced rejection or is recruited by the adaptive immune system in AMR, it seems that a final common pathway involving NK cell is triggered during chronic vascular rejection. This was suggested in particular by a study which showed that the transcriptomic signatures were similar in biopsies of patients with DSA-positive and DSA-negative microvascular inflammation lesions [34]. These patients showed similar upregulation of pathways such as IFNγ-induced pathways and NK cell activation, and similar enrichment of infiltrating leukocytes [34]. A better understanding of the pathways leading to NK activation may therefore be useful in the development of treatments targeting NK cells in chronic vascular rejection, whether or not DSA are present.

SIGNALING PATHWAYS INVOLVED IN NK CELL PRIMING AND ACTIVATION

To become fully activated, NK cells need to receive a priming signal from cytokines and a second signal that results from the balance of inhibitory and activating signals transmitted by their many inhibitory and activating receptors (**Figure 1**).

NK Cell Priming

Among the various cytokines that may play a role in NK cell priming (IL-2, IL-12, IL-15, IL-18 and IL-21), IL-15 is of particular importance in regulating NK cell homeostasis and activation [35–37]. IL-15 binds to IL-15Rα expressed on antigen presenting cells and is then presented in trans to the IL-2/IL-15Rβγ heterodimer on NK cells. IL-15 induces the phosphorylation of JAK1/JAK3, allowing the recruitment and activation of the transcription factor STAT5 (**Figure 3**), which then translocates to the nucleus to support NK cell survival [38]. In parallel, it induces the activation of the PI3K-AKT-mTOR signaling pathway which promotes the acquisition of cytolytic potential by NK cells when they will encounter abnormal cells (**Figure 3**) [38].

In the context of transplantation, we and others have shown that NK cells need to undergo priming to get activated against the graft [15, 39, 40]. In particular, we have shown that prolonged cold ischemia (suggesting significant ischemia-reperfusion injury) and viral infections such as cytomegalovirus can lead to NK cell priming after transplantation, a prerequisite for NK cell activation and the development of chronic vascular rejection in the presence of a missing self [15]. These two types of events favor the induction of IL-15 and its trans presentation by dendritic cells, thereby promoting NK cell cycle entry, proliferation, survival and cytotoxicity [35, 41–44]. In addition, cytomegalovirus also induces a remodeling of the NK phenotype with an increase in NK cell populations expressing educated inhibitory KIRs and expression of markers such as NKG2C and CD57 [45, 46]. This subpopulation of NK cells is considered to be a memory like population and is known to be better at exhibiting cytotoxicity and secreting cytokines such as IFN-γ [46].

Finally, a recent study also showed that IL-21 may help NK cells to differentiate into polyfunctional type 1 activated cytotoxic effectors with a potentially deleterious ability to infiltrate the kidney allograft during AMR and damage the vascular endothelium [47].

NK Cell Activation

Activating NK cell receptors are associated with different adaptor molecules which contain immunoreceptor tyrosine-based activation motifs (ITAMs) on their cytoplasmic domains. FcγRIIIa receptor which is a low affinity receptor for IgG forms a complex with 2 adaptor molecules FcRγ and CD3ζ. Upon ligation of the Fc fragment of IgG by FcγRIIIa, the ITAMs are phosphorylated. This triggers a cascade of downstream events involving the PI3K pathway, the mitogen-activated protein kinase (MAPK) pathway and calcium release conducting to the activation of nuclear factor of activated T-cells (NFAT) (**Figure 1**) [48–50]. Together, these pathways contribute to the cellular response leading to NK cell activation [51–53]. Unlike activating receptors, inhibitory receptors and in particular inhibitory KIRs have 2 ITIMs in their cytoplasmic tails. When inhibitory KIRs interact with their self-MHC class I ligands, the ITIMs are phosphorylated and recruit the tyrosine phosphatases such as SHP-1 (Src homology containing tyrosine phosphatase-1), which remove phosphate groups from several proteins downstream of activating receptors, thereby preventing NK cell activation (**Figure 1**) [54]. In the absence of ligation of their self-MHC class I ligands, inhibitory KIRs do not interfere with activating signals, allowing NK cell activation. We have shown that missing cell induced-NK cell activation triggers the mTOR pathway [15]. Whether this is also the case for FcγRIIIa-induced NK cell activation is not clear, but experimental data investigating NK cell activation by other activating receptors suggest that this is certainly the case [55].

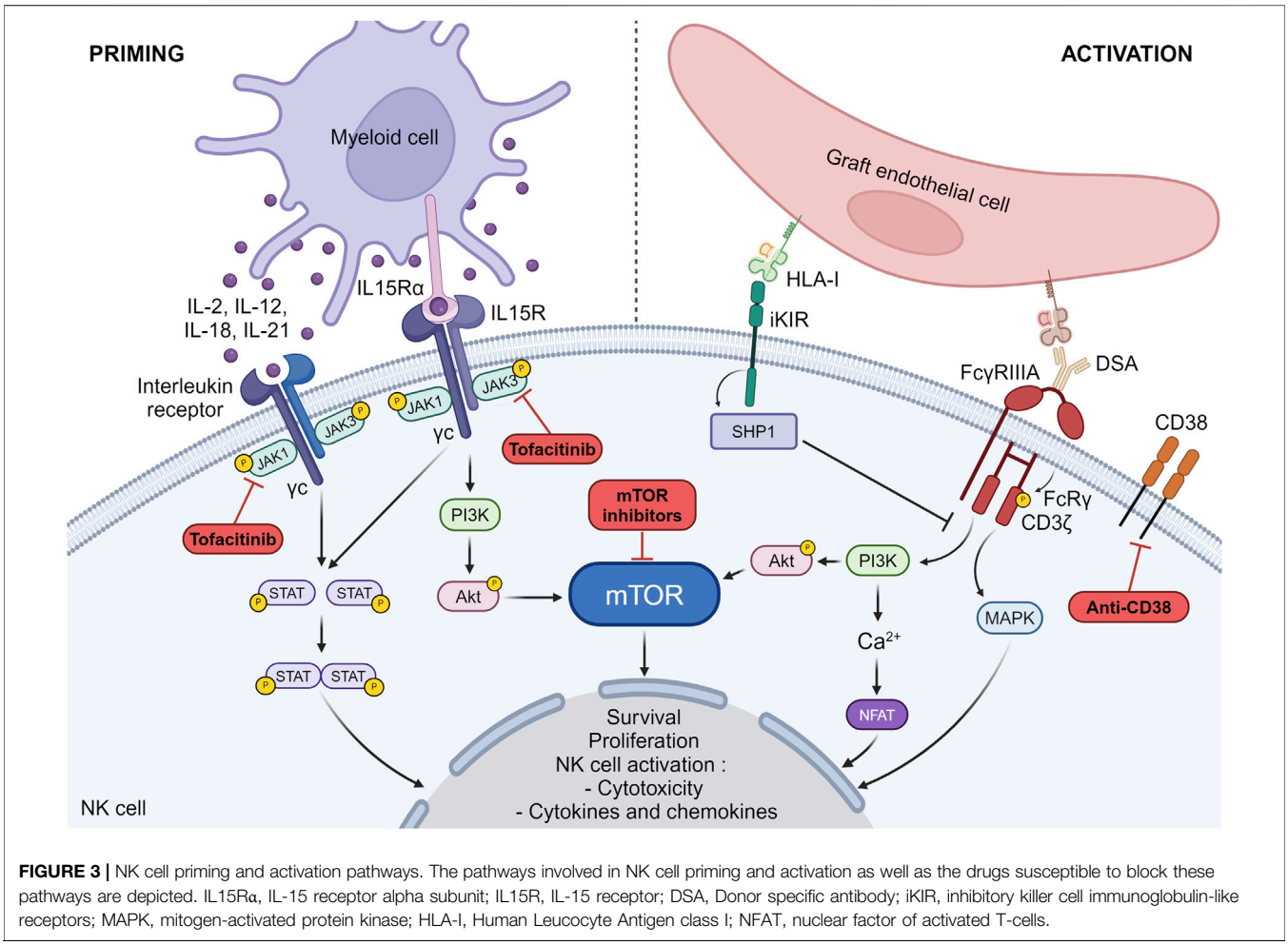

FIGURE 3 | NK cell priming and activation pathways. The pathways involved in NK cell priming and activation as well as the drugs susceptible to block these pathways are depicted. IL15Rα, IL-15 receptor alpha subunit; IL15R, IL-15 receptor; DSA, Donor specific antibody; iKIR, inhibitory killer cell immunoglobulin-like receptors; MAPK, mitogen-activated protein kinase; HLA-I, Human Leucocyte Antigen class I; NFAT, nuclear factor of activated T-cells.

Activation of NK cells can lead to killing of the target cell. NK cells exert their cytotoxic activity through two main pathways: the exocytosis of lytic granules containing perforin and granzymes A and B; and the expression of ligands (FAS ligand, TRAIL) of death receptors (FAS, DR4 and DR5) expressed on target cells. In addition, NK cells can secrete pro-inflammatory cytokines (TNF-α and INF-γ) that favor the activation of dendritic cells, T cells and monocytes [56]. They also secrete chemokines such as CCL-2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), which attract effector lymphocytes and myeloid cells to inflamed tissues [57, 58]. In a murine heart transplantation model of AMR, Lin and colleagues showed that both IFN-γ and contact-dependent cytotoxicity are necessary for NK cells to induce chronic vascular lesions [59]. The same observations have been made in the hybrid resistance model regarding the need for IFN-γ to help NK cells recruit T cells and monocytes to induce the vascular lesions [7, 8]. Several clinical studies have shown that the transcripts involved in IFN-γ pathway and NK cell-mediated cytotoxicity are upregulated in biopsies of patients with HLA positive or negative microvascular inflammation lesions [28, 34].

TARGETING NK CELLS: AN INTERESTING LEAD TO PREVENT AND TREAT CHRONIC VASCULAR REJECTION?

Prevention of NK-Driven Chronic Rejection

Transplant patients classically received a two- or three-drug regimen consisting of a calcineurin inhibitor, an antiproliferative agent, and corticosteroids. The aim of these treatments is to inhibit T-cell activation. Theoretically, calcineurin inhibitors could have an effect on NK cell activation because NFAT is expressed by NK cells [50]. In a first *in vitro* study, human NK cells were treated with cyclosporine and different cytokines (IL-2 or IL-15) for 7 days. This study confirmed that the NFAT pathway was blocked, especially in $CD56^{dim}$CD16+inhKIR+ NK cells, and that this resulted in a decrease in NK cell proliferation but an increase in cytotoxicity against different target cells and an increased ability to secrete IFN-γ after a new stimulation with IL-12 and IL-18 [60]. Two more recent studies confirm the conserved cytotoxicity of NK cells exposed to cyclosporine or tacrolimus and activated by the two mechanisms involved in chronic rejection (MS and ADCC) [61, 62]. *In vivo*, we confirm

that cyclosporine does not prevent missing-self-induced NK-mediated rejection in our murine heart transplant model, suggesting that this immunosuppressant may have a limited effect in preventing chronic vascular rejection in patients [15]. The effect of antiproliferative drugs such as azathioprine and mycophenolate mofetil and low-dose corticosteroids on NK cells has been less studied. Some *in vitro* studies suggest that these drugs may prevent NK cell activation, but these data should be treated with caution due to the lack of *in vivo* data [61, 62].

Alternatively, transplant recipients may receive mTOR inhibitors (mammalian target of rapamycin) as part of their maintenance regimen. As previously depicted, mTOR pathway plays an important role in NK cell education, priming and activation [38, 55]. *In vitro* studies have shown that mTOR inhibitors can prevent NK cell cytotoxicity [55, 61]. *In vivo*, we confirm that rapamycin can efficiently prevent missing-self induced NK-mediated rejection in our murine heart transplantation model [33]. Finally, a recent clinical study in a cohort of lung transplant recipients treated with rapamycin showed that their NK cells had reduced mTOR activity, which was associated with decreased cell proliferation and lower levels of FcRγ, the adaptor molecule of FcγRIIIa, suggesting that they may be less efficient at performing ADCC [63]. A pilot clinical trial (NCT03955172) is currently underway to assess the efficacy of mTOR inhibitors in preventing the formation of chronic lesions in kidney transplant patients presenting a missing-self induced NK-mediated rejection. Although promising, the universality of mTOR inhibitors could be questioned, as this treatment is associated with many side effects, leading to its discontinuation in 30%–50% of patients, and cannot be used late in the course of chronic rejection, as mTOR inhibitors are poorly tolerated in patients with chronic glomerular lesions, as they prevent the adaptation of podocytes to stress [64].

As the cytokine priming process is necessary for all NK cell mechanisms involved in chronic vascular rejection, blocking it offers a unique opportunity to prevent the deleterious effects of NK cells during chronic vascular rejection. As NK cell priming may be the result of multiple cytokines, rather than trying to block one cytokine, blocking the pathways which result from the binding of cytokines on their receptors which share the common γc chain may be more appropriate. Tofacitinib is an oral JAK inhibitor that selectively inhibit intracellular cytokine signaling mediated by JAK3 and/or JAK1. A recent study in a rat model of mixed cellular and antibody-mediated rejection indicates that tofacitinib effectively reduces the infiltration of T cells and NK cells into the graft, thereby limiting the progression of lesions and improving graft and recipient survival [65]. Its use in renal transplant patients has been tested in a clinical randomized phase 2b trial [66]. Patients received either tofacitinib (low or high dose) or cyclosporine in combination with mycophenolic acid and corticosteroids [66]. Tofacitinib was equivalent to cyclosporine in preventing acute rejection and was associated with an improved renal function and less tubular atrophy and interstitial fibrosis [66]. However, it was also responsible for more cancerous and infectious complications in the high dose tofacitinib regimen [66]. This side effect may be the reflect of the efficient blocking of NK cells which are well known for their role in anti-viral and anti-tumoral immunity.

Treatment of NK-Driven Chronic Rejection

The current consensus for the treatment of AMR associates rapid depletion of circulating DSA with plasmapheresis with a combination of corticosteroids and high-dose intravenous immunoglobulins (IVIg) [67]. This costly and prolonged therapeutic approach has a reported 3-year graft survival of <50% [67]. By preventing complement activation, these treatments slow down the course of rejection and convert it from acute to chronic rejection in which ADCC is the main mechanism of graft destruction.

Few studies have investigated the effect of high-dose corticosteroids on NK cells. In one *in vitro* study, human purified NK cells and T cells were incubated with elevated doses of hydrocortisone, comparable to drug levels achieved in patients receiving 1 mg/kg to 1 g of methylprednisolone. In contrast to T cells, NK cells were resistant to steroid-induced apoptosis and their cytolytic capacity against MHC class I deficient tumoral cell was not affected [68]. In the same line, although methylprednisolone seems to affect NK cell activation through some activating receptors such as NKp46, NKG2D or 2B4, it does not seem to prevent FcγRIIIA-mediated NK cell cytotoxicity [69]. High dose IVIg may also prevent NK cell activation by saturating all FcγRIIIa receptors present on NK cells, rendering them blind to DSA. However, their impact on NK cells is still debated [67].

To achieve a sustained therapeutic effect in AMR, attempts have been made to target DSA-producing cells with either anti-CD20 monoclonal depleting antibody (rituximab) or a proteasome inhibitor (bortezomib) without efficacy [70, 71]. Recently, an antibody targeting CD38 (felzartamab), a receptor present on plasma cells and NK cells, has been tested in a randomized phase 2 trial study for the treatment of renal transplant recipients diagnosed with late active or chronic-active antibody-mediated rejection after kidney transplantation [72]. This treatment efficiently reduced rejection lesions. This improvement seems to be due to NK cell depletion rather than plasma cells depletion as DSA MFI barely decreased with the treatment. These data need to be confirmed in a larger cohort of patients. A further clinical trial is currently underway to test an alternative anti-CD38 (daratumumab) in the same indication (NCT05913596). If effective, its use can be extended to missing self-induced NK-mediated rejection.

CONCLUSION

In conclusion, this review has explored the role of NK cells in chronic vascular rejection. They appear to be the masters of chronic vascular rejection, regardless of whether DSA are present or not. This makes them a prime target for attempts to prevent and/or treat chronic vascular rejection, which is currently the leading cause of allograft loss. Inhibition of NK cell priming and activation represent interesting avenues for preventing NK-

mediated chronic vascular rejection, while drugs that deplete NK cells may be of interest for the treatment of chronic vascular rejection. By reducing chronic vascular rejection, these strategies may lead to prolonged allograft survival.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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REVIEW
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Activation and Regulation of Indirect Alloresponses in Transplanted Patients With Donor Specific Antibodies and Chronic Rejection

Sumoyee Basu[1,2], *Caroline Dudreuilh*[1,3], *Sapna Shah*[1,4], *Alberto Sanchez-Fueyo*[2,5], *Giovanna Lombardi*[1,2] *and Anthony Dorling*[1,2]*

[1]*Centre for Nephrology, Urology and Transplantation, King's College London, London, United Kingdom,* [2]*Department of Inflammation Biology, King's College London, London, United Kingdom,* [3]*Transplantation, Renal and Urology Directorate, Guy's and St Thomas' NHS Foundation Trust, Guy's Hospital, London, United Kingdom,* [4]*Renal Unit, King's College Hospital NHS Foundation Trust, London, United Kingdom,* [5]*Liver Sciences, King's College London, London, United Kingdom*

Following transplantation, human CD4+T cells can respond to alloantigen using three distinct pathways. Direct and semi-direct responses are considered potent, but brief, so contribute mostly to acute rejection. Indirect responses are persistent and prolonged, involve B cells as critical antigen presenting cells, and are an absolute requirement for development of donor specific antibody, so more often mediate chronic rejection. Novel *in vitro* techniques have furthered our understanding by mimicking *in vivo* germinal centre processes, including B cell antigen presentation to $CD4^+$ T cells and effector cytokine responses following challenge with donor specific peptides. In this review we outline recent data detailing the contribution of $CD4^+$ T follicular helper cells and antigen presenting B cells to donor specific antibody formation and antibody mediated rejection. Furthermore, multi-parametric flow cytometry analyses have revealed specific endogenous regulatory T and B subsets each capable of suppressing distinct aspects of the indirect response, including $CD4^+$ T cell cytokine production, B cell maturation into plasmablasts and antibody production, and germinal centre maturation. These data underpin novel opportunities to control these aberrant processes either by targeting molecules critical to indirect alloresponses or potentiating suppression via exogenous regulatory cell therapy.

Keywords: indirect alloresponse, chronic rejection, immune regulation, donor specific antibody (DSA), T follicular helper cells, B lymphocytes

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Correspondence
Anthony Dorling,
anthony.dorling@kcl.ac.uk

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INTRODUCTION

There are three pathways by which transplantation antigens are recognized by $CD4^+$ T cells [1–3]. In the "direct" and "semi-direct" pathways, intact donor major histocompatibility complex (MHC) proteins are recognized on the surface of either donor antigen presenting cells (APC) or, in the semi-direct pathway, recipient APC, after MHC transference from donor cells via various routes, including exosome transfer [4]. For detailed description of these pathways, their role and importance in rejection, the reader is referred to several recent reviews [5, 6].

Evidence that a third pathway, called indirect could initiate graft rejection originally came from congenic animal models in which donor and recipient differed only at minor antigenic loci [7–9], and after transplantation of grafts from MHC-deficient rodents [10, 11]. In both, grafts were rejected

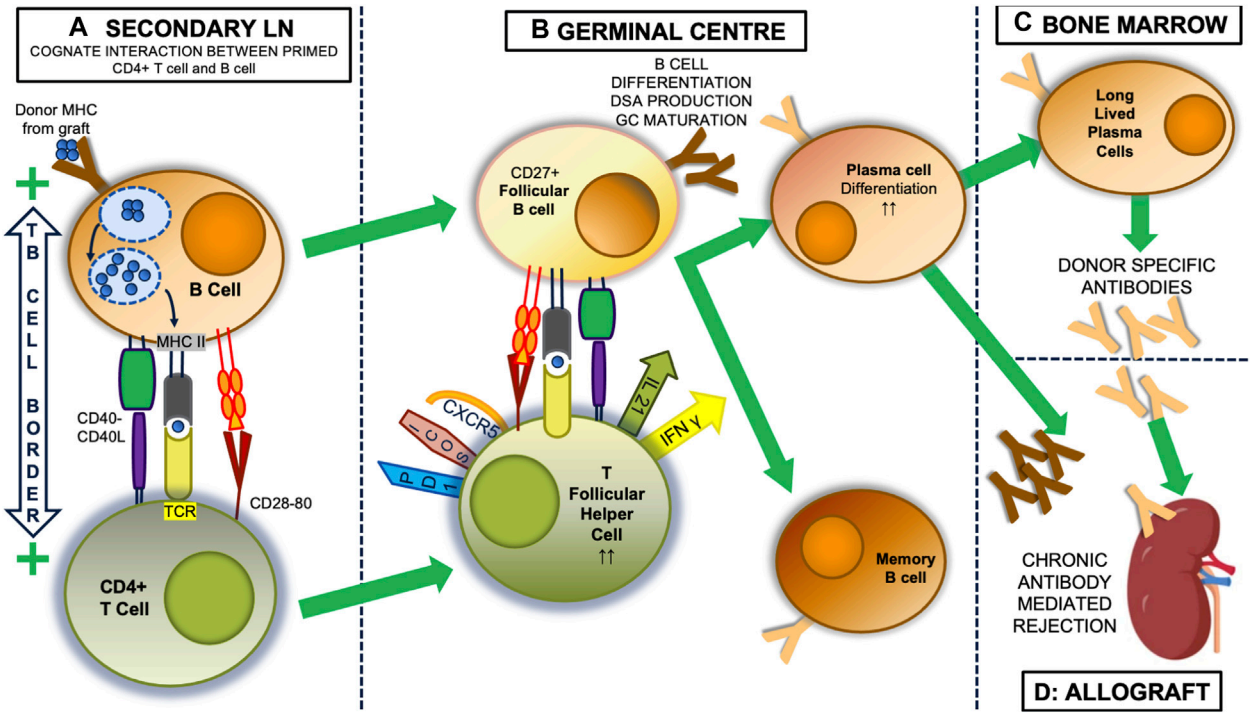

FIGURE 1 | The indirect alloresponse and GC reaction. Within secondary lymph nodes (LN), self MHC-restricted CD4$^+$ lymphocytes with indirect allospecificity are primed by dendritic cells that have picked up donor alloantigen, most usually donor proteins encoded by the major histocompatibility complex (MHC), from the allograft and transported it back to the lymph node. (not shown). Once primed, donor specific B cells become the predominant antigen presenting cell **(A)**. These bind donor antigen via their surface immunoglobulin, initially IgM, after which it is internalised and processed into antigenic peptide that then a presented on the B cell surface in the antigen binding groove of MHC class II molecules. At the T: B border in LN, the T cell receptor (TCR) of CD4$^+$ T cells can bind this processed peptide, and along with critical interactions between CD40:CD40 ligand, and CD28 and CD80, this interaction activates both the T cell and B cell and the two can enter the germinal centre (GC) response **(B)**. Here, CD27$^+$ follicular B cells continue to present antigen to CD4$^+$ T cells that have developed a T follicular helper (TFH) phenotype, with expression of CXCR5, ICOS and PD1. Through expression of IL-21 and IFNγ, they drive the production of donor specific antibodies (DSA) initially from plasmablasts, which appear in the circulation and can initiate graft injury, and later from plasma cells, which can be long-lived, after migration to the bone marrow **(C)**. In the process of this happening, the follicular B cells undergo a series of T cell-dependent processes resulting in changes to the structure of their surface immunoglobulin, including isotype and subclass switching (to IgG3), and importantly increasing affinity for alloantigen, which means the DSA also change (in the figure from dark to light brown), being able to bind antigen more avidly and developing ability to activate complement, for instance. These changes are associated with expression of T-bet, IRF4 and Blimp-1 [27]. Memory B cells also emerge from the GC. Thus the unregulated GC reaction results in secretion of DSA with characteristics that can induce acute or chronic rejection in the allograft (D).

quickly after activation of self-MHC-restricted CD4+T cells recognising alloantigen presented by recipient APC [12, 13]. The extensive pre-clinical data relating to the role of indirect alloresponses in animal models of transplantation will be briefly reviewed in this introductory section.

Thus, indirectly alloreactive CD4$^+$ T lymphocytes exist in the normal repertoire [14, 15], at precursor frequencies lower than T cells activated by direct allorecognition [15, 16], though these frequencies increase after immunisation with soluble MHC [17]. After transplantation, indirectly alloreactive CD4$^+$ T cells appear in regional lymph nodes [18, 19], indicating this pathway is activated physiologically. These cells are important, as pre-transplant immunisation with donor MHC causes accelerated rejection [17, 20]. Once activated, indirectly alloreactive CD4$^+$ T cells can promote the generation of CD8$^+$ cytotoxic T lymphocytes [12], delayed type-hypersensitivity (DTH) responses within the graft [8], and the generation of donor specific antibody (DSA) [8]. DSA are ***only*** generated after indirectly alloreactive CD4$^+$ T cells cognately interact with donor-specific B lymphocytes [21–23]. This involves specific differentiation of T follicular helper (TFH) lymphocytes [24] in germinal centres (GC) of secondary lymphoid organs [25, 26] (**Figure 1**).

Consistent with the crucial role B cells play in the T cell responses to infection [28], there is substantial evidence from animal models that B cells play a central role in indirect alloresponses, especially for the development of chronic rejection (CR). This is likely due to the fact they can undergo clonal expansion through proliferation, and possess specific antigen receptors capable of increasing affinity during an ongoing immune response. For example, when indirect pathway CD4$^+$ T cells can only be stimulated by non-B cell APC (such as dendritic or myeloid cells), after mature B cells are prevented from developing [29], or when MHC-deficiency is confined to B cells [30], graft survival is markedly prolonged. In both cases, no DSA develop. In elegant experiments, Zeng et al

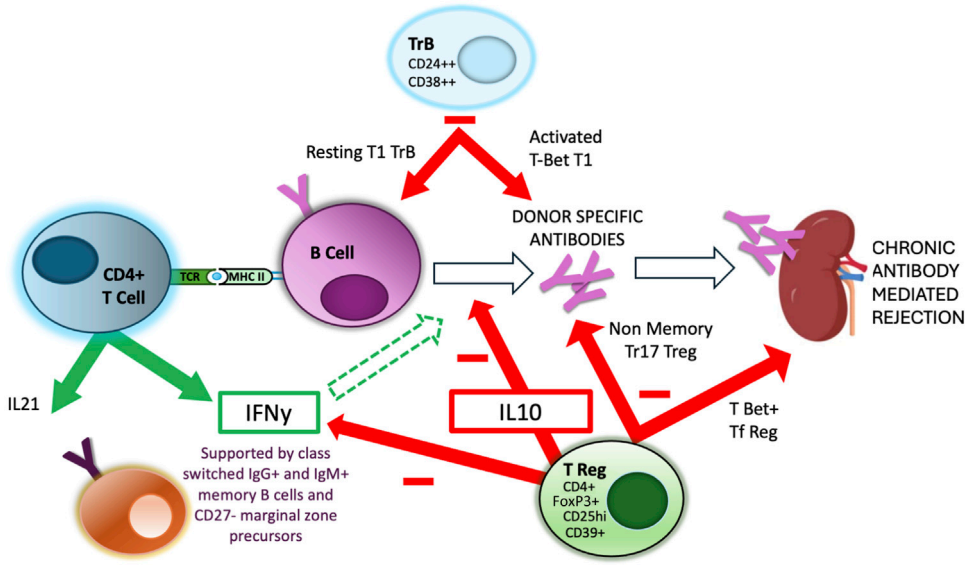

FIGURE 2 | Regulation of Indirect alloreactivity and GC reaction. Aspects of the interaction between $CD4^+$ TFH and GC B cells are regulated by specialised regulatory populations of both B cells, expressing high levels of CD24 and CD38 (TrB) and T cells, expressing high levels of CD25, FoxP3 and IL-10. There is evidence that separate and distinct subpopulations of these regulatory cell populations are responsible for regulating different aspects of the GC reaction, including DSA production and GC maturation [67].

[31] addressed whether the importance of B cells was to produce DSA, as the prime effector of rejection, or to present antigen to $CD4^+$ T cells. They transplanted cardiac allografts into recipients in which B cells were prevented from secreting DSA via the simultaneous knockout of activation-induced deaminase and secretory IgM, but the cells could present antigen. In this model CR lesions developed at the same tempo as in wild-type mice, despite the absence of DSA. The same investigators showed, using MHC-deficient bone marrow chimeras, that self-MHC restricted presentation of allopeptides was needed for CR, as when B cells were present but unable to stimulate the indirect pathway, splenic architecture was maintained but CR was inhibited. These data are consistent with more recent data from Pettigrew's group, who, working in a similar model nevertheless demonstrated that the ability to generate DSA via a GC response markedly enhanced the speed and severity of the developing CR [26]. Thus, at least in these rodent models, stimulation of $CD4^+$ T cells by B cells via the indirect pathway, to generate antibody-independent effector mechanisms, can itself drive the development of CR, but this is significantly enhanced by the presence of class-switched high affinity DSA generated via GC responses. It is being increasingly recognised that the alloantigens that drive indirect allorecognition and maintain B-cell receptor stimulation are transferred to APC via the semidirect-pathway [32].

For the remainder of this review, we will explore clinical data to assess the importance of the indirect pathway for human allograft rejection, particularly CR, review the evidence that this pathway can be suppressed by endogenous regulatory cell populations, and discuss whether this has any potential translational relevance.

SENSITISATION OF THE INDIRECT PATHWAY IN HUMANS ASSOCIATES WITH GRAFT REJECTION

Multiple studies have reported an association between pre-transplant donor-specific IFNγ production in enzyme-linked immunosorbent spot assay (ELISPOT) and risk of post-transplant rejection, as analysed in a recent meta-analysis [33]. ELISPOT is a sensitive assay that measures the frequency of cytokine-producing $CD4^+$ T cells that are responding to a particular antigen stimulus and IFNγ pathways are significantly upregulated in both biopsy specimens showing rejection, particularly antibody mediated rejection (AMR), and peripheral blood cells of patients with AMR [34], so measuring IFNγ production is logical.

However, most of the studies assessing pre-transplant status have used irradiated whole donor peripheral blood mononuclear cells (PBMC) or splenocytes as the source of donor material, meaning they are likely detecting cytokine production by directly alloreactive lymphocytes as well as CD4+T cells activated by the indirect pathway.

Assays that assess only indirect pathway sensitisation (see **Table 1**) use donor antigen prepared in ways unable to stimulate direct responses. Saleem et al [35] used synthesised peptides representing donor human leukocyte antigen (HLA) class I to stimulate proliferation of recipient PBMC in 4-day mixed lymphocyte reactions (MLR), and found no responses from 12 kidney transplant recipients (all had had at least 1 episode of rejection) and 3 paediatric heart/lung patients with CR. Iniotaki-Theodoraki et al [36] studied 14 kidney transplant recipients, using APC-depleted donor PBMC in 5-day MLR, and found proliferation in 6 out of 14 patients. Under follow-up, 11 of the 14 maintained stable graft

TABLE 1 | Summary of functional assays evaluating indirect alloreactive donor specific responses in transplant recipients.

Publication	Study Group	Stimulus	Assay used	Response	Responses after B Cell Depletion	Responses after depletion of CD25hi cells
Saleem et al [35]	12 kidney transplant and 3 paediatric heart lung recipients with history of rejection	Synthesised class I peptides matching donor HLA	Recipient PBMC in 4-day MLR	No responses	N/A	N/A
Iniotaki-Theodoraki et al. [36]	14 kidney transplant recipients	APC-depleted donor PBMC	Recipient PBMC in 5-day MLR	Proliferation[a] in 6/14 at baseline. Proliferation in 12/14 on serial testing, but not associated with future graft dysfunction	N/A	N/A
Coelho et al [37]	14 kidney transplant recipients	APC-depleted donor PBMC	Recipient PBMC in 9-day MLR	Proliferation[a] in 8/14. No association with future graft dysfunction	N/A	N/A
Liu et al [38, 39]	32 heart transplant recipients	Synthesised class II peptides matching donor HLA	Serial limiting dilution analyses (detecting proliferating cells) using recipient PBMC *AND* T cells isolated from donor heart	Proliferation[a] in 18/28 who went onto have episode of rejection within 4 weeks. Correlation between responses from cells in circulation and graft. Association with DSA in patients with CR	NA	NA
Crespo et al [40]	101 kidney transplant recipients	CD2 or CD3-depleted donor PBMC	Recipient PBMC in IFNγ ELISPOT at 3 and 6/12 post-Tx	3-month ELISPOT response[a] correlated with protocol biopsy-proven rejection at 6 months, and with 24-month DSA development	N/A	N/A
Najafian et al [41]	Recipients of a) HLA-DR-matched kidney transplants (n = 9), HLA-DR mismatched transplants with b) no rejection (n = 11), or c) history of rejection (n = 15)	Synthesised peptides representing hypervariable regions of 5 commonest HLA-DR	Recipient PBMC in IFNγ ELISPOT	Frequency of responding[a] T cells increased with HLA-DR mismatches and history of rejection	N/A	N/A
Besterd et al [42]	33 kidney transplant recipients	Donor cell membrane preparations	Recipient PBMC in IFNγ ELISPOT	Detectable responses[a] in 20/33 (60%) – strong correlation with time since Tx and presence of proteinuria	N/A	N/A
Hornick et al [43]	10 heart transplant recipients, 6 with CR. 1 kidney transplant recipient with CR	Donor cell membrane preparations or synthesised donor class I peptides	Limiting dilution analyses (detecting IL-2-producing cells) using recipient PBMC	Detectable responses[a] in 5/7 with CR but 0/4 without CR	N/A	N/A
Haynes et al [44]	5 cohorts of kidney transplant recipients; a) identical twin donor organ (n = 2), b) clinically tolerant (n = 11), c) stable monotherapy (n = 7), d) standard therapy (n = 18), e) CR (n = 7)	Donor cell membrane preparations, or HLA coated beads	PBMC in trans-vivo assay	Increasing responsiveness[a] from groups a) – e). Responses reduced in e) with antibodies against IFNγ or IL-17. Responses revealed in a) with antibodies against TGFβ. Responses to HLA coated beads associated with DSA	No impact on responses of two patients	N/A
Vella et al [45]	4 cohorts of kidney transplant recipients; a) HLA-DR MM with CAD (n = 11), b) HLA-DR MM without CAD (n = 10), c) No HLA-DR MM with CAD (n = 5), d) no HLA-DR MM, no CAD (n = 18)	Synthesised peptides representing hypervariable regions of 3 common HLA-DR	Recipient PBMC in 7-day MLR, plus limiting dilution analyses (detecting proliferating cells)	Responses[a] in 9/11 group a), but 0/10 group b) and 2/23 groups c) and d). Highest frequency of responding cells in group a)	N/A	N/A

(Continued on following page)

TABLE 1 | (*Continued*) Summary of functional assays evaluating indirect alloreactive donor specific responses in transplant recipients.

Publication	Study Group	Stimulus	Assay used	Response	Responses after B Cell Depletion	Responses after depletion of CD25hi cells
Baker et al [46]	22 renal transplant recipients, 9 with CAD	Donor cell membrane preparations	Limiting dilution analyses (detecting IL-2-producing cells) using recipient PBMC	Significantly higher frequencies of responding[a] cells in the 9 patients with CAD	N/A	N/A
Shiu et al [56, 57]	65 kidney transplant recipients with 'for cause' or protocol biopsies 52/65 with AMR	Donor cell membrane preparations	CD8 depleted recipient PBMC in IFNγ ELISPOT	Donor specific IFNy production[a] in 45/119 (38%). samples. This correlated with reduction in eGFR over time	29/37 (78%) responsive AMR samples had significant reduction in IFNy production compared to 4/8 (50%) in samples from no AMR. In contrast, 17/69 (25%) samples had significant increase in IFNy production	21/66 (32%) samples had significant increase in IFNy production
Shiu et al [58]	51 kidney transplant patients with cAMR.	Donor cell membrane preparations	CD8 depleted recipient PBMC in IFNγ ELISPOT	Donor specific IFNy production[a] in 58/203 (29%) samples	30/58 (52%) responsive samples had significant reduction in IFNy production	14/30 (46%) samples had significant increase in IFNy production
Burton et al [59]	43 HLA sensitised kidney transplant recipients	PURE HLA proteins matching DSA	CD8 depleted recipient PBMC in IFNγ ELISPOT	Donor specific IFNy production[a] in 19/98 (19%) samples	13/19 (69%) responsive samples had significant reduction in IFNy production, associated with HLA binding by $CD27^+$ B cells. In contrast, 11/98 (11%) samples had significant increase in IFNy production, associated with high proportion of transitional B cells	5/50 (10%) samples had significant increase in IFNy production
Salama et al [68]	23 kidney transplant patients, 8 with previous rejection and CAD.	Donor specific HLA-DR allopeptides	Recipient PBMC in IFNγ ELISPOT	Not reported	N/A	Increased IFNy production in 6/15 (40%) stable patients but only 1/8 (12.5%) with history of rejection. Responses increased in 8/17 (47%) of all non-responsive samples
Tanaka et al [71]	62 kidney or liver transplant recipients. 17 pre sensitised with DSA	Donor Cells	Recipient PBMC in 5-day MLR	N/A	Significant post-rituximab increase in proliferation by $CD4^+$ T cells ONLY in DSA + group	N/A
Schachtner et al [72]	150 blood group compatible (n = 98) or incompatible (n = 52) living donor kidney transplants treated with rituximab induction	Irradiated donor PBMC	Recipient PBMC in IFNγ ELISPOT	Pre-treatment responses[a] seen in 20/98 (20%) ABO compatible and 12/52 (23%) ABO incompatible patients	Rates of 12-month TCMR were 8/20 (40%) in ABO compatible and 7/12 (57%) in ABO incompatible	N/A

[a]*Detectable responses in all these different assays imply the presence of $CD4^+$ T cells that are sensitised to donor antigens.*

Abbreviations: AMR, antibody-mediated rejection; APC, antigen presenting cell. CAD, chronic allograft dysfunction; cAMR, chronic AMR; CD8,25,27, cluster of differentiation 8,25,27 +cells; CR, chronic rejection; DSA, donor specific antibody; ELISPOT, enzyme-linked immunosorbent spot assay; HLA- human leukocyte antigen; IFNγ -interferon gamma; IL-2, 17, interleukin-2, 17; MM, mismatch; MLR, mixed lymphocyte reaction; N/A, not applicable; PBMC, peripheral blood mononuclear cells; TCMR–T, cell-mediated rejection; TGFβ, transforming growth factor-beta; Tx–transplant.

function, 10 of whom showed intermittent indirect alloreactivity. The remaining 3 developed chronic allograft dysfunction (CAD), 2 of whom showed intermittent indirect alloreactivity. Finally, Coelho et al [37] studied 14 living donor kidney transplant recipients using APC-depleted donor PBMC to stimulate proliferation in 9 days MLR. 8 of the 14 showed evidence of indirect alloreactivity, 2 of whom developed CR but in 6, graft function stayed stable. Of the remaining 6 without evidence of indirect alloreactivity, 1 developed CR. The conclusion from these studies, which did not include testing for DSA, was that indirect alloreactivity could be detected in the peripheral blood of long-term renal transplant patients, but this did not seem to predict *future* graft (dys)function. However, none of these studies involved surveillance biopsies.

In contrast, in 32 heart transplant patients studied within 10 weeks of transplantation, all of whom underwent protocol biopsies, Liu et al [38] isolated PBMC from the circulation and T cells from graft biopsy specimens. They performed limiting dilution analyses to calculate the proportion of recipient $CD4^+$ T cells proliferating when stimulated with synthesised peptides representing mismatched donor HLA DR. They detected sensitised T cells in the circulation of 18 of the 28 (64%) patients who went on to have an episode of biopsy proven acute rejection (BPAR) 1–4 weeks later, but only 3/50 samples (6%) when patients had no rejection within the next 1–4 weeks. Moreover, by detecting T cells reacting against the same peptides in the grafts of patients undergoing rejection at up to 10x higher frequencies than in the circulation, they concluded that these T cells play a part in the rejection process. The same group showed, in a separate study [39], that indirect pathway CD4+T cells could be detected in the circulation prior to both episodes of acute and chronic rejection, in the latter case, in association with DSA. Crespo et al [40] performed prospective pre-transplant and 3-month IFNγ ELISPOT analysis in 101 consecutive kidney transplant recipients undergoing a 6-month protocol biopsy. ELISPOT reactivity at 3 months (but not pre-transplant) correlated with sub-clinical BPAR at 6 months, and strongly correlated with DSA development at 24 months. Thus, in contrast to above, these studies involving protocol biopsies in both heart and renal transplant recipients are consistent with the notion that indirect pathway activity is a pre-requisite for both future rejection and DSA development.

Along similar lines, Najafian et al studied indirect alloresponses in several cohorts of renal transplant recipients using recipient PBMC stimulated with synthesised peptides, chosen to represent sequences from the five most frequent donor HLA DR types [41]. They measured ELISPOT IFNγ and found the frequency of cells responding to the allopeptides in healthy controls and DR-matched recipients averaged 4 cells per million CD4+T cells, whereas those in DR mismatched recipients were higher. Frequencies of responding cells were higher still in DR mismatched recipients with a history of rejection, who tended to have frequencies >60 cells per million. Using the same assay, but stimulating PBMC with donor cell membrane antigen preparations, Bestard et al [42] studied 34 renal transplant recipients several years post-transplant, 18 of whom had a history of BPAR. 20 of 33 from whom they had samples had detectable responses to donor antigens, and although there was no correlation between indirect alloreactivity and creatinine there was a strong association with proteinuria and in multivariate analysis, detectable indirect alloreactivity was the only variable associated with proteinuria. These data therefore associate indirect alloreactivity with previous rejection and future graft dysfunction.

Hornick et al [43] found an increased frequency of $CD4^+$ T cells capable of producing IL-2 after stimulation with donor membrane antigen preparations in 5 of 7 heart transplant patients with CR but none of the 4 included with no rejection. In kidney transplant patients, Haynes et al [44] used the trans vivo DTH assay to study indirect pathway activation in patients with CR. This assay involves injecting recipient PBMC with donor cell sonicates (as a source of antigen) into the foot pad of an immunodeficient mouse. The degree of swelling that develops over 24 h is proportional to the number of cells responding to antigen. PBMC from patients with CR had the greatest degree of swelling compared to all other patient groups.

Vella et al [45] compared cohorts of kidney transplant patients with and without CAD. This group used synthesised class II allopeptides representing mismatched donor HLA class II to stimulate recipient PBMC in 7-day MLR. Proliferative responses were seen in 9 of 11 patients with CAD but in none of 10 patients without CAD. Responses were seen in only 2 out of 23 controls (for whom the allopeptides did not represent mismatched donor HLA). Proliferative responses were not always seen in serial samples from the same individuals, but it was noted there was evidence of epitope drift in some patients. These authors performed limiting dilution analyses to calculate the proportion of recipient $CD4^+$ T cells proliferating, and in CAD, 1 in 9,000 to 1 in 660,000 $CD4^+$ cells responded to donor peptides, compared to 1 in 1-2 million cells from controls. Baker et al [46] used a similar approach, using donor cell membrane preparations to stimulate indirect pathway $CD4^+$ T cells in 22 renal transplant recipients, 9 of whom had CAD. They showed that the frequency of IL-2 producing cells were significantly higher in the patients with CAD than the patients with stable function. These studies in heart and renal transplant recipients therefore associate T cells primed by the indirect pathway with CR.

Finally, there are numerous studies linking "predicted indirectly recognizable HLA epitopes" (PIRCHE) scores with development of subsequent DSA [47–49] and long-term graft survival [50–53]. As suggested by the name, PIRCHE is an algorithm that identifies parts of mismatched donor HLA that can be presented by recipient HLA class II after processing by APC, so reflects the capacity of specific donor/recipient mismatches to activate the indirect pathway of alloreactivity.

In combination, all these pieces of evidence link sensitisation of $CD4^+$ T cells recognising donor antigens via the indirect pathway with previous rejection, and strongly associate the indirect pathway with the development of DSA and subsequent graft dysfunction manifesting as CAD/CR.

$CD4^+$ T CELLS WITH A T FOLLICULAR HELPER PHENOTYPE ARE INVOLVED IN INDIRECT ALLOREACTIVITY

Louis et al [54] studied 105 patients, including 20 with DSA and a history of AMR and 31 with DSA but without AMR. In patients with

DSA & AMR, there was an over-representation of a CD4 + CXCR5+ TFH subset compared to patients with DSA but no AMR. These cells expressed activation and memory markers and responded to donor cell lysates by expressing IL-21. They could promote DSA appearance when incubated with autologous B cells and their transcriptional profile suggested they were involved in GC responses, providing help to B cells. The DSA in these patients were skewed towards IgG1, IgG3 and C1q binding and the majority of these TFH were Th-1 and Th-17, consistent with a role in isotype switching.

Kenta et al [55] studied indirect alloreactivity in different cohorts of renal transplant recipients, including 12 with DSA pre-transplant, 13 who had developed *de novo* DSA and 33 who were DSA negative throughout. They purified whole $CD4^+$ cells or CD45RA+ or CD45RA-negative subfractions (representing naïve and memory cells respectively) and stimulated them with autologous monocytes, differentiated into dendritic cells (DC) *in vitro* and pulsed with donor membrane antigen preparations. These DC mediated emergence of proliferating donor-specific $CD4^+$ TFH cells making both IFNγ and IL-21, both key GC cytokines. In the non-sensitised patients, these TFH came mainly from CD45RA+ $CD4^+$ cells, as did the IFNγ and IL-21 production, but in DSA+ patients, the CD45RA-negative fraction also contributed.

Both these studies indicate that the $CD4^+$ T cells involved in indirect alloresponses adopt a TFH phenotype, capable of secreting IFNγ, IL-17 or IL-21.

ROLE AND PHENOTYPE OF B CELLS IN INDIRECT PATHWAY

Shiu et al [56] used donor membrane antigen preparations to stimulate recipient CD8-depleted PBMC in IFNγ ELISPOTs. In a cohort of 65 patients undergoing protocol or "for cause" biopsy, 52 of whom had AMR, there was evidence of donor specific responsiveness in 38% of samples. Depletion of B cells from the PBMC prior to ELISPOT caused a significant reduction in the frequency of cells producing IFNγ in 29 out of 37 samples (78%) from patients with AMR, but only 4 out of 8 (50%) in samples from controls with non-immune or no pathology on biopsy. Serial changes in patterns of ELISPOT reactivity correlated strongly with changes in eGFR over time [57]. In a second cohort of 51 patients with cAMR studied in a similar way [58], 29% of 203 samples showed evidence of donor specific IFNγ production, 52% of which were dependent on the presence of B cells. Burton et al [59] studied 43 HLA-sensitised patients, using synthesised whole HLA proteins (chosen to match DSA) to stimulate CD8-depleted PBMC. IFNγ production was noted in 23% of 84 samples, 69% of which were B-dependent. In all these studies, IFNγ production was prevented by Btk/Syk inhibitors, leupeptin/pepstatin A/E64-d, or anti-HLA class II blocking antibodies, confirming that alloantigen recognition involved antigen processing and presentation via the indirect pathway. By biotinylating the same HLA proteins, Burton et al were also able to define the phenotype of HLA-binding B cells associated with this pattern of IFNγ production: both class-switched (IgG+) and IgM+ memory ($CD27^+$) B cells as well as antigen-experienced (but CD27-negative) marginal-zone precursor B cells appeared to support cytokine production in ELISPOT.

All these data indicate that antigen-experienced, donor antigen specific B cells are present in the circulation of patients with DSA and cAMR and are capable of presenting donor HLA peptides to Th-1, IFNγ secreting $CD4^+$ T cells.

Consistent with these data, Louis et al [27] studied B cell phenotypes in the circulation of 96 kidney transplant recipients, 28 of whom had DSA but no AMR, and 20 of whom had DSA with AMR[1]. They found activated memory B cells present in blood and biopsies of patients with AMR. These cells were less frequent in DSA+ AMR- patients. These cells were T bet+, had restricted IGHV gene expression, and were primed for plasma cell differentiation. Importantly, the authors detected DSA secretion after incubation with autologous TFH and the polyclonal activator staphylococcal enterotoxin B, but only from B cells from AMR+ group. These data indicate that there is a difference in the phenotype of donor specific B cells present in DSA+ patients with AMR compared to those with no AMR, with B cells from the former reflecting more GC differentiation than those from the latter.

INDIRECT ALLOREACTIVITY IS INHERENTLY INVOLVED IN THE GC REACTION

GC formation within secondary lymphoid organs during an immune response has been studied extensively within mouse models (**Figure 1**). Antigen-specific B cells initially undergo clonal expansion within the dark zone of the GC, before moving to the light zone, where continued interaction with TFH recognising the same antigen via the indirect pathway is critical for the GC processes of affinity maturation, driven by somatic hypermutation of immunoglobulin genes, class switching, memory B cell formation and plasma cell differentiation. In humans, it is known that GC TFH cells can be found circulating in the blood [60] and that secretion of IL-21 is a key feature of their GC functionality. Thus there are multiple studies associating the relative proportions of circulating TFH cells post-transplantation with the risk of developing *de novo* DSA [61–63], as discussed by several recent reviews [64–66]. Importantly, many of these GC processes, at least prior to the formation of long-lived plasmas cells, are physiologically regulated as discussed below (**Figure 2**), representing a potential avenue to therapeutically intervene to prevent DSA development and CAD.

PHYSIOLOGICAL SUPPRESSION OF THE INDIRECT PATHWAY AND RELEVANCE *IN VIVO*

By Tregs

Salama et al [68] were the first to show that depletion of CD25hi regulatory T cells (Tregs) significantly increased indirect IFNγ production in response to donor-specific allopeptides. This was seen in 6 of 15 (40%) stable patients with no history of rejection, but only 1 of 8 (12.5%) of patients with a history of rejection. Of all the

[1]In 17 of the 20, the rejection was classified as mixed AMR/TCMR.

non-responsive samples analysed, they found evidence of regulation by Tregs in almost 50%. Interestingly, in a patient in whom serial samples were available, the loss of regulation in the ELISPOT was followed clinically by rejection and graft loss, suggesting that the Tregs were inhibiting anti-donor responses, preventing rejection and that loss of regulation may be a factor in precipitating rejection.

However, Shiu and Burton [56, 58, 59] found that regulation by CD25hi T cells was evident in up to 46% of non-responsive samples from patients with biopsy-proven chronic AMR: in half of these, when Tregs were present, there was complete suppression of IFNγ production and these were the samples that contained the highest proportion of $CD4^+$ CD25hi CD39hi Tregs, as assessed by flow cytometry. This suggested that chronic AMR was not associated with a universal loss of the ability to regulate indirect responses. Additional depletion of $CD19^+$ cells after CD25hi depletion significantly reduced the frequency of IFNγ+ spots in up to 90% of samples [56], suggesting that Tregs were inhibiting B-cell–dependent indirect alloreactivity; this was seen particularly in samples from patients with AMR. Burton et al went onto show that the phenotype of antigen-binding B cells in these samples were predominantly $CD27^-$ naive cells [59], as opposed to the memory phenotypes mentioned above.

Shiu [69] also made interesting observations in a follow-up study of a group of seven highly sensitised kidney transplant recipients, several of whom showed evidence of transplant accommodation [70]. Although 5 of the grafts were lost within 8 years, mostly through CR, two of the accommodated grafts were still functioning 12- and 17-years post-transplantation, both with good transplant function, no proteinuria, but with persisting DSA. ELISPOT analysis showed both had undetectable responses to membrane donor antigen preparations, but responses became evident when CD25hi cells were depleted. Thus, in these highly sensitised individuals with DSA, long term survival associated with complete suppression of indirect anti-donor alloreactivity by Tregs.

By Bregs

With regard to regulation by B cells, it was also clear from some of the studies above that in some samples, initial depletion of B cells was associated with increases in the frequency of IFNγ producing $CD4^+$ cells. This pattern was found in up to 25% of samples [56, 58, 59], and was associated with higher IL-10 production by B cells after polyclonal stimulation [56], lower proportions of HLA binding memory B cells and higher proportions of transitional T1 and T2 B cells (TrB), as assessed by expression of CD38 and CD24 [58, 59]. Importantly, in individuals where serial samples were analysed, two things were apparent. First, both B cell and CD25hi suppression of IFNγ producing cells waxed and waned over time [58]: Second, patients in whom any samples showed IFNγ production in the absence of any regulation appeared to have the worst clinical outcomes [57, 58]. Consistent with this, Haynes et al [44] found no evidence of regulatory B cell activity in the trans vivo DTH assay in patients with CR.

All these data are also consistent with that generated by other groups. Tanaka et al [71] compared pre- and post- rituximab 5-day donor MLRs in 62 kidney or liver transplant recipients, 17 of whom were sensitised with DSA. Pre-rituximab $CD4^+$ T cell proliferation, measured using CFSE dilution was equivalent in sensitised and non-sensitised recipients, but in post-rituximab MLRs, there was significantly increased proliferation of the $CD4^+$ T cells from the DSA+ group. The authors speculated that B cells were suppressing donor specific $CD4^+$ T cells in sensitised recipients.

Consistent with this, Schachtner et al measured IFNγ ELISPOT reactivity in patients receiving either blood group compatible or incompatible kidney transplants [72]. 20% of those receiving ABO compatible kidneys demonstrated pre-transplant anti-donor reactivity, implying prior sensitisation and 40% of these patients had an episode of T cell-mediated rejection in the first year. 23% of those receiving ABO incompatible (ABOi) kidneys had a positive ELISPOT, but the rejection rate in these patients was 57%. Of the various differences between these two populations, one explanation is that rituximab, used exclusively in the ABOi patients, depleted regulatory B cells that spontaneously regulate the indirect alloresponse in some sensitised patients [73], an interpretation consistent with the data generated by Shiu et al [58], who found that rituximab depleted the TrB cells associated with regulation of IFNγ production, but did not deplete the antigen-binding memory B cells responsible for B dependent, non-suppressible IFNγ production.

Mechanistic Insights

Regarding how these different regulatory populations function, Spadafora-Ferreira et al [74, 75] showed that at least some indirectly alloreactive T cells were FoxP3+ Tregs secreting IL-10. Shiu et al [57] found that in samples with evidence of regulation by B cells, the $CD4^+$ T cells making IFNγ were also secreting IL-10, suggesting that regulation involved switching on a well-defined autocrine pathway [76, 77] in Th-1 cells designed to prevent inappropriate IFNγ-driven immunopathology [78]. However, others have defined an important role for B cell-derived IL-10 in regulation of anti-donor alloresponses [79, 80].

Finally, Louis et al, in a second report [67], studied the same patient subgroups (defined above) and found that the numbers and proportions of both Tregs and TrB cells were reduced in DSA+ patients, more so in those who developed AMR. There were significant differences in the qualitative analysis of Treg and TrB cell subsets in the different patient groups. Whereas all DSA+ patients had a deficiency of a non-memory Tr17 Treg subpopulation and a resting T1 TrB subset, only DSA+ AMR+ patients were deficient in both a T-bet+ T follicular regulatory Treg subset and an activated T-bet+ T1 subset of TrB cells. These changes associated with more severe histological features seen in AMR, with the presence of IgG3 and C1q binding DSA and with poorer longer term allograft outcomes. They reported that their regulatory populations limited the APC capacity of B cells, and inhibited TFH proliferation, plasmablast differentiation and IgG secretion. They acted through a combination of direct cell contact (via CTLA4) and IL-10 secretion. The implication of this work was discussed by Basu et al [81]; their data imply separate and distinct tiers of regulation, performed by different subsets of regulatory cells, capable of inhibiting DSA production, but additionally capable of limiting the degree of isotype and subclass switching and affinity maturation within GC. More recently, Dudreuilh et al [82] compared the Treg subpopulations in highly sensitised dialysis patients with those from non-sensitised dialysis patients and

healthy controls. Of various differences described, highly sensitised patients had significantly lower proportions of the same two Treg subpopulations identified by Louis et al [67] as being associated with DSA formation and development of AMR, suggesting these deficiencies exist prior to any subsequent transplant.

All these lines of evidence indicate the existence, in patients, of different regulatory populations of T and B cells capable of suppressing mechanisms involved in different aspects of the indirect alloresponse and GC reaction, including donor antigen-specific T cell cytokine production, DSA development and GC differentiation (**Figure 2**). Importantly, the presence/absence and activity of these populations correlates with graft outcomes. As each layer of regulation may act separately from others, this is one potential reason why the tight links between indirect alloreactivity, DSA formation and clinical phenotype have not always been obvious from the published literature until recently.

THERAPEUTIC MANIPULATION OF INDIRECT ALLOREACTIVITY

Potential molecular targets to disrupt interactions between TFH and B cells involved in indirect alloimmunity, GC formation and DSA generation have been recently reviewed by Louis et al [65]. An alternative strategy would be to enhance regulatory mechanisms involved in limiting DSA formation or GC maturation. Multiple clinical studies have explored the safety and tolerability of *ex vivo* expanded Treg therapy in transplant patients [66, 83, 84], though most to date have had an emphasis on promoting immunosuppression reduction, minimisation or even elimination in the context of induced immunological tolerance. Second generation studies using manipulated Treg populations, including use of CAR-Tregs, are underway [85].

On the back of the evidence presented above, Dudreuilh et al have initiated a phase 2 clinical trial in sensitised dialysis patients awaiting a transplant, to investigate primarily whether adoptive transfer of ex-vivo expanded Tregs can suppress the $CD4^+$ T cell responses to HLA proteins in indirect IFNγ/IL-17 fluorospot assay [86]. Secondary objectives are to determine the proportion of sensitised dialysis patients who may be eligible for a future trial ased on patterns of IFNγ/IL-17A responses to HLA, how long HLA-specific responses remain suppressed, what adverse events associate with Treg therapy, how adoptive Treg therapy changes the number and phenotype of circulating Tregs comparing baseline to post-Treg treatment and finally to determine how adoptive Treg therapy changes HLA Ab profiles. The HLA proteins used are based on the DSA that patients have. The trial is actively recruiting and at the point of writing, has entered the treatment phase, expecting to complete in late 2025. The ambition beyond this is to perform a second trial with clinical endpoints to assess the feasibility of treating highly sensitised patients with Tregs prior to any future transplant. If feasible, this strategy has the potential to improve clinical outcomes in these patients without using significantly enhanced immunotherapy.

SUMMARY

The indirect alloresponse describes a pathway of antigen recognition involving uptake of donor antigens by APC that are processed into peptides then presented in the antigen-binding grooves of recipient HLA class II to $CD4^+$ T cells, following which all immune effector pathways capable of injuring the transplant can be activated. B cells have been shown to be extremely important APC and are necessarily involved in the development of DSA, via a GC reaction and AMR. These B cells have a specific phenotype associated with a GC reaction and can be found in the circulation, particularly in patients with AMR. The indirect pathway also favours the development of $CD4^+$ T cells with a TFH phenotype: these can also be found in the circulation, particularly in patients with a history of AMR.

Although initial evidence supported the idea that rejection mediated via the indirect pathway was associated with a loss of immune regulation, newer data support the idea that different aspects of the indirect alloresponse, including $CD4^+$ T cell cytokine production, B cell differentiation into antibody secreting plasmablasts, and processes involved in GC maturation, can each be regulated separately by different populations of regulatory T and B cells, including in patients with CR. Importantly the proportion and activity of these populations associates with clinical outcomes. This opens the possibility that CR might be managed by targeting specific molecules involved in the indirect alloresponse, but also the possibility that autologous ex-vivo expanded regulatory populations might be used to treat patients to improve outcomes associated with DSA/AMR without the side effects associated with excessive immunosuppression.

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All authors contributed to writing and review of the manuscript. AD conceived and planned the article. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Perspective for Donor-Derived Cell-Free DNA in Antibody-Mediated Rejection After Kidney Transplantation: Defining Context of Use and Clinical Implications

Aylin Akifova[1*†], *Klemens Budde*[1†], *Michael Oellerich*[2†], *Julia Beck*[3], *Kirsten Bornemann-Kolatzki*[3†], *Ekkehard Schütz*[3†] *and Bilgin Osmanodja*[1†]

[1]*Department of Nephrology and Intensive Care, Charité—Universitätsmedizin Berlin, Berlin, Germany,* [2]*Department of Clinical Pharmacology, University Medical Center Göttingen, Göttingen, Germany,* [3]*Chronix Biomedical GmbH, Göttingen, Germany*

Antibody-mediated rejection (AMR) is a major cause of graft failure limiting long-term graft survival after kidney transplantation. Current diagnostic strategy to detect AMR is suboptimal and requires further improvement. Previously suggested treatment regimens for AMR could not demonstrate efficacy, however novel therapeutic agents are currently under investigation. Donor-derived cell-free DNA (dd-cfDNA) is a novel non-invasive biomarker for allograft injury, that has been mainly studied in the context of rejection. Its short-half-life in circulation and injury-dependent release are its key advantages that contribute to its superior diagnostic accuracy, compared to traditional biomarkers. Moreover, previous studies showed that dd-cfDNA-release is well-linked to histological and molecular features of AMR, and thus able to reflect real-time injury. Further observations suggest that dd-cfDNA can be used as a suitable screening tool for early detection of AMR in patients with donor-specific-anti-HLA-antibodies (DSA), as well as for monitoring AMR activity after anti-rejection treatment. The weight of evidence suggests that the integration of dd-cfDNA in the graft surveillance of patients with AMR, or those suspicious of AMR (e.g., due to the presence of donor-specific anti-HLA-antibodies) has an added value and might have a positive impact on outcomes in this specific cohort.

Keywords: kidney transplantation, antibody-mediated rejection, molecular diagnostics, donor-derived cell free DNA, donor-specific antibodies (DSA)

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***Correspondence**
Aylin Akifova,
aylin.akifova@charite.de

†ORCID:
Aylin Akifova
orcid.org/0009-0003-9227-8017
Klemens Budde
orcid.org/0000-0002-7929-5942
Michael Oellerich
orcid.org/0000-0002-9255-2261
Kirsten Bornemann-Kolatzki
orcid.org/0009-0008-0440-5634
Ekkehard Schütz
orcid.org/0000-0002-9639-4870
Bilgin Osmanodja
orcid.org/0000-0002-8660-0722

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Abbreviations: AMR, antibody-mediated rejection; cfDNA, cell-free DNA; ci, interstitial fibrosis; cg, glomerular basement membrane double contours; CNI, calcineurin inhibitor; ct, tubular atrophy; cv, vascular fibrous intimal thickening; dd-cfDNA, donor-derived cell-free DNA; ddPCR, droplet digital PCR; dnDSA, *de novo* donor-specific anti-human leukocyte antigen antibodies; DSA, donor-specific anti-human leukocyte antigen antibodies; eGFR, estimated glomerular filtration rate; g, glomerulitis; HLA, human leukocyte antigen; i, interstitial inflammation; IgG, immunoglobulin G; KTR, kidney transplant recipient; MFI, mean fluorescence intensity; MMDx, Molecular Microscope Diagnostic System; MVI, microvascular inflammation; NGS, next-generation sequencing; NPV, negative predictive value; PPV, positive predictive value; ptc, peritubular capillaritis; SNP, single nucleotide polymorphisms; TCMR, T-cell mediated rejection; uACR, urine albumin-creatinine ratio; v, intimal arteritis.

INTRODUCTION

Antibody-mediated rejection (AMR) is associated with inferior long-term outcomes and is a leading cause of premature graft loss after kidney transplantation [1, 2]. The complex pathogenesis behind AMR, along with its variable clinical course, provides a diagnostic and therapeutic challenge for transplant physicians today and requires novel strategies for the future [3]. The emerging era of personalized immunosuppression demands new advances in monitoring allograft health, especially for kidney transplant recipients (KTR) at high risk for AMR, e.g., sensitized recipients as well as KTR who develop *de novo* donor-specific anti-HLA antibodies (dnDSA). Standard-of-care surveillance biomarkers, such as serum creatinine and urine albumin, are not sufficient for detecting early subclinical AMR and cannot differentiate kidney allograft dysfunction due to AMR from other causes.

According to the Banff classification, serological evidence of DSA is one of the main criteria for the definite diagnosis of AMR [4]. However, despite global assay standardization [5], posttransplant timing and frequency of DSA screening is not uniformly established and there is no robust evidence for the benefit of routine monitoring of pre-formed DSA in the posttransplant course [6]. Furthermore, two recent studies showed no added value of systematic DSA screening for detection of AMR at an earlier stage [7, 8]. While development of dnDSA was predictive of graft failure, optimization of baseline immunosuppression and adherence training in the OuTSMART trial only had a limited intervention effect, such as reduction in biopsy-proven rejection in the treated group, while optimization of baseline immunosuppression and adherence training had no impact on allograft survival in this trial [8].

Besides limited treatment options for AMR, other aspects are challenging for a straightforward implementation of regular screening for dnDSA: DSA can fluctuate, become undetectable, and reappear over time, which does not necessarily reflect the immune-mediated damage or correspond with the clinical course [9, 10]. Some DSA characteristics, such as mean fluorescence intensity (MFI), IgG-subclass, or complement-binding capacity were studied to estimate the clinical impact of DSA and predict AMR outcomes, but the diagnostic value and immediate clinical utility remained uncertain [11–14].

While the first appearance of dnDSA is associated with clinical AMR in only 6.5% of patients, the majority of cases are initially uneventful without injury [10]. Therefore, it is unclear whether occurrence of DSA (i) sets the beginning of a latent alloimmune injury ultimately leading to AMR, (ii) is a first measurable sign of subclinical AMR, (iii) or simply represents a laboratory artifact without any clinical significance. Given the poor prognosis after first detection of dnDSA, this clinical dilemma requires a novel approach for better risk stratification, especially in DSA-positive KTR with stable graft function. Any strategy to prevent the progression of DSA-mediated injury requires an early detection of ongoing injury. The failure of previously investigated treatment regimens [15–17] could be partly explained by a delayed diagnosis and the presence of already irreversible chronic injury at treatment initiation.

Performing kidney allograft biopsy remains the gold standard to verify or rule out suspected rejection. Although it is a well-established low-risk procedure, it is not completely risk-free, being an invasive intervention that usually requires hospitalization and still has its limitations such as sampling errors, inter-observer variability, and the non-specificity of histological lesions [18, 19]. Another crucial aspect is the fact that biopsies triggered by the detection of DSA confirm AMR in less than 50%, whereas indication biopsies triggered by deterioration of the graft function usually describe advanced rejection stages as well as collateral damage that might further limit treatment initiation and efficacy [20–23].

Altogether, this leads to a significant diagnostic gap, and precise and early detection of subclinical AMR is an unmet medical need, that needs to be addressed by the transplant community. As current screening strategies with creatinine, urine protein, and DSA are unable to detect subclinical AMR, a combined screening strategy with an additional biomarker beyond anti-HLA-DSA may be the path forward [6, 24].

Donor-derived cell-free DNA (dd-cfDNA) is a non-invasive biomarker indicating allograft injury that has recently gained attention for the care of patients after solid organ transplantation and was deemed promising in identifying rejection with greater accuracy than traditional parameters [25, 26]. Previous research mainly focused on discriminating rejection from no rejection and demonstrated superior diagnostic performance in discriminating AMR compared to T-cell-mediated rejection (TCMR) and borderline changes [27–32]. However, it is important to keep in mind that dd-cfDNA release is an unspecific marker of graft injury, not limited to rejection phenotypes, and is also observed in microvascular inflammation in the absence of DSA (DSAnegMVI), BK-virus associated nephropathy (BKVAN), other infections and ischemia-reperfusion injury [33–36].

Given these considerations and other relevant aspects such as availability, cost-efficiency, and feasibility, a broad and unselected use of dd-cfDNA may not be the ideal approach to advocate for the integration of this biomarker into clinical routine [37]. Instead, defining a suitable context of use is recommended, where dd-cfDNA could serve a well-defined population (such as DSA-positive patients) as a potentially useful biomarker to facilitate early detection of AMR and to guide treatment for improved outcomes in AMR [24, 37]. Hence, evidence will be reviewed on whether dd-cfDNA surveillance has the potential to be practice-changing in the contemporary management of AMR in KTR with DSA.

METHODS OF QUANTIFICATION AND AVAILABILITY

To date, several methods of detection have been established to assess dd-cfDNA in transplant recipients' blood. All detection methods rely on genetic differences between donor and recipient DNA and share the advantage that no separate genotyping of the donor is needed. Available tests are based on highly abundant genetic polymorphism, such as single nucleotide polymorphisms (SNPs), insertion/deletion polymorphisms (indels) or copy

number variations to distinguish between graft-derived and recipient DNA using new generation sequencing (NGS)-based and polymerase chain reaction (PCR)-based assays [38–43].

Nevertheless, it is important to acknowledge that there are some differences between the quantification methods that should be addressed before adopting dd-cfDNA as a diagnostic tool to guide clinical decisions. Unlike NGS techniques, quantitative PCR-based methods enable the assessment of both absolute and relative levels of dd-cfDNA [43]. Several studies showed that relative quantification is more likely to be error prone, as it can be influenced by variations in the total recipient cfDNA by non-physiological increases in cell turnover, such as cases of severe infection or malignancy where it should be interpreted cautiously. Since cfDNA is primarily released by recipient leucocytes, fluctuations in leucocyte numbers can impact absolute recipient cfDNA and consequently relative content of dd-cfDNA. Absolute values of dd-cfDNA can be affected by pre-analytical changes, such as variation in DNA extraction efficiency and cfDNA degradation in the bloodstream [25]. For values around a predefined threshold, this might lead to false-negative or false positive results, misinterpretation and even triggering unnecessary biopsies.

To overcome these limitations, it has been proposed to add absolute quantification, which demonstrates a greater diagnostic accuracy compared to the dd-cfDNA% fraction [44, 45]. Therefore, some authors support a combination of both relative and absolute quantification in the context of the total cfDNA concentration, which might enhance comprehensiveness in the decision-making process [44–47].

Another practical aspect for clinical decision-making is the turnaround time, defined as the time from blood draw to result. In some cases, it can take up to a week if the sample is shipped to a central laboratory, which not only represents a relevant logistical burden but also has consequences for its clinical implementation. Besides, when comparing the different quantification methods, sequencing in NGS processing alone can take up to 30 h and therefore is more labor-intensive, compared to the droplet digital PCR (ddPCR) approach, which can offer same-day results and is easier in set-up. From a clinical perspective, it would be advantageous if the dd-cfDNA processing could be conducted at a local laboratory with a short turnaround time, which would directly lead to faster treatment decisions while also increasing availability of the test [43].

CORRELATION OF DD-CFDNA WITH HISTOLOGICAL AND MOLECULAR AMR

Overall, most studies demonstrated good-to-very-good test characteristics of dd-cfDNA (sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV)) for detecting rejection with best performance in discriminating AMR and mixed rejection from TCMR and borderline [26–32]. However, positive predictive value (PPV) and negative predictive value (NPV) varies depending on the rejection prevalence in the screening cohort [48]. Most studies reported remarkably high NPV for AMR, which indicates that dd-cfDNA can be used to reliably rule out underlying AMR in DSA-positive KTR with a quiescent clinical course [24].

To gain a more granular understanding of dd-cfDNA release, several studies aimed to examine the association between dd-cfDNA and histological and molecular rejection features in dd-cfDNA-paired kidney allograft biopsies. This was first explored in a small cohort (n = 37) by Zhang et al. who did not demonstrate any statistical differences between the different Banff categories and within Banff lesion scores [29]. In contrast, a subsequent analysis from a larger cohort (n = 106) with a higher prevalence of rejection found a significant correlation between dd-cfDNA and glomerulitis (g) and intimal arteritis (v), respectively, and also observed higher dd-cfDNA levels with greater Banff lesion severity [49]. Furthermore, they described an association of dd-cfDNA with microvascular inflammation score (MVI>2) and severe interstitial inflammation (i3) but not with chronic features of AMR, such as the cg (glomerular basement membrane double contours), interstitial fibrosis (ci), tubular atrophy (ct), and vascular fibrous intimal thickening (cv), which was partially confirmed in subsequent studies [42, 50–52].

Notably, the majority of the following studies demonstrated a distinct correlation of dd-cfDNA with the Banff peritubular capillaritis (ptc) score. This was seen in AMR but also in other circumstances of ptc, irrespective of the underlying diagnosis [50–52]. Interestingly, Whitlam et al. correlated both relative and absolute dd-cfDNA with the Banff scores and found that an increase in absolute dd-cfDNA was linked to active AMR features, such as g, ptc and C4d-staining, whereas dd-cfDNA fraction was also associated with other lesions, but was less specific for AMR and rejection in general [42].

The prospective multicenter Trifecta-Kidney study (NCT04239703) was conducted to calibrate dd-cfDNA against conventional histology and the molecular archetypes in the Molecular Microscope Diagnostic system (MMDx) according to rejection stage, severity, and activity, as previously introduced by the MMDx study group [53, 54]. The study extensively investigated anti-HLA-antibodies and histology, follow-up data, and clinicians' feedback to explore the relationship between these findings in this multi-layered diagnostic approach. Results from 300 matched biopsies were analyzed to correlate dd-cfDNA with histologic and molecular features associated with AMR and overall rejection. Random forest analysis verified ptc as the most important variable associated with increased dd-cfDNA, followed by the much weaker g- and v-scores. Among the molecular variables, the molecular ptc-lesion classifier (ptc_{Prob}) was the top predictor of elevated dd-cfDNA, followed by the all-rejection (Rej_{Prob}) classifier and the ABMR probability classifier ($ABMR_{Prob}$) [52]. Overall, the prediction of increased dd-cfDNA was more accurate with the molecular variables than with the histological variables for AMR, which was confirmed in a real-world prospective study by Gupta et al. [55].

Another key aspect is the relationship between dd-cfDNA and AMR archetypes in MMDx, e.g., dd-cfDNA being the highest in fully developed AMR (FABMR) with a tendency to be lower in late AMR (LABMR), which is usually less active, as described previously [54]. These insights strongly underscore the

hypothesis that dd-cfDNA is linked to the severity of the injury as reflected by the molecular AMR activity. Under this assumption, dd-cfDNA could be an easily obtained non-invasive biomarker that potentially reflects AMR activity and outperforms standard clinical and conventional biomarkers such as DSA [56, 57].

In summary, current evidence strongly suggests that high dd-cfDNA values are associated – but not exclusively - with microvascular inflammation (MVI) in the allograft, which is of high diagnostic significance, as the MVI lesion is a hallmark of AMR, although it is seen in other circumstances as well. Given the relevant discrepancy between histology and MMDx, with a tendency for AMR being underdiagnosed in conventional microscopy [58], high dd-cfDNA levels can provide additional evidence for AMR or make AMR less likely in cases with DSA and ambiguous or insufficient histology.

DD-CFDNA FOR EARLY DIAGNOSIS OF AMR

Early diagnosis of therapy-requiring conditions in general and AMR in particular, is essential and should also be recognized as an important determinant for improved patient and graft survival after kidney transplantation [59, 60]. In the specific context of AMR, early detection is crucial since biopsies presenting with chronic AMR changes at initial diagnosis are associated with more adverse outcomes and could rapidly progress to graft loss [22, 23]. Given its key features, like the short half-life in circulation and injury-dependent release, dd-cfDNA can signalize the onset of antibody-mediated damage much earlier and preceding clinical deterioration in KTR with stable graft function. In the advent of novel therapeutic agents, such as CD38 targeting monoclonal antibodies, the early detection of subclinical AMR may become increasingly important [61].

This is supported by findings of the multicenter, observational ADMIRAL study (NCT04566055), which was the largest study to prospectively follow up KTR with dd-cfDNA surveillance as part of the routine monitoring. In this cohort, high dd-cfDNA values were observed in a significant proportion of AMR biopsies of KTR with no major impairment in kidney allograft function, as defined by a decline in eGFR or proteinuria [62]. Another retrospective observational study, where longitudinal assessment pre-biopsy was available, also showed that the determination of elevated dd-cfDNA levels could have led to an earlier detection of AMR [63].

These observations were supported by a recently completed, single-center, diagnostic randomized clinical trial (NCT04897438). In this trial, patients with a functioning kidney graft and dnDSA without evidence of AMR, were assigned to either intervention (dd-cfDNA-guided biopsy) or a control group (clinician-guided-biopsy), and dd-cfDNA was longitudinally assessed in both groups over 1 year. Increase over the predefined absolute threshold in the intervention group, indicated a diagnostic biopsy, regardless of kidney function. The primary endpoint “time to AMR-diagnosis” was met by a significant 9-month earlier AMR diagnosis in the intervention group, compared to the control group [64]. Again, dd-cfDNA had very good test characteristics (sensitivity 83%, specifity 79%, PPV 0.75, NPV 0.85) in dnDSA-positive KTR, extending previous observations [27–32]. This is the first prospective randomized trial that provides evidence for the potential benefit of dd-cfDNA monitoring in KTR with dnDSA. The data suggest that, the additional determination of dd-cfDNA can reliably identify AMR in an early and potentially reversible stage of rejection and enable timely therapeutic intervention (**Figure 1**).

Contrary to previous approaches, dd-cfDNA was assessed in a well-defined cohort of KTR with DSA, thereby increasing the pre-test probability and, explaining the favourable test characteristics. Within this context of use, a dd-cfDNA-guided biopsy would dramatically reduce unnecessary biopsies by around 50% compared to a general protocol biopsy approach in DSA positive KTR, where only 50% of biopsies reveal AMR (**Figure 2**) [20, 21].

Another randomized controlled experiment addressed the clinicians perspective, namely, how the availability of complementary dd-cfDNA results will affect the decision-making process. Interestingly, the study showed that nephrologists aware of the dd-cfDNA results were more likely to correctly diagnose early and subclinical graft rejection, refer to a transplant center, or appropriately change treatment compared to nephrologists without dd-cfDNA access [70]. Future multicenter prospective outcome studies are warranted to deliver a greater power of these promising findings.

THERAPEUTIC CHALLENGE OF AMR AND THE POTENTIAL OF DD-CFDNA TO MONITOR TREATMENT SUCCESS

Along with the continuous efforts to develop therapy solutions for AMR, a suitable tool to assist in validating the success of investigational or available treatment is also desirable. Traditional parameters may not be ideal for guiding therapeutic response due to their non-specificity and inability to reflect resolving injury. As a result, follow-up biopsies are required to assess whether and to what extent applied anti-rejection treatment was efficacious.

Unlike TCMR or borderline rejection, AMR remains a major therapeutic challenge [3], and without approved treatment, timely diagnosis of AMR may not be ultimately helpful. Better classification and ability to include early subclinical AMR into treatment trials may contribute to successful drug discovery [3]. Although different agents have been investigated in the attempt to target potentially pathogenic pathways, no substance or multimodal regimen led to an evident success so far [15–17, 71]. Several studies suggested some stabilization of the graft function, and novel therapy concepts are currently under investigation in randomized-controlled trials (NCT04561986, NCT05156710, and NCT05021484) [61, 72, 73]. Recently, a phase-3 trial was terminated due to lack of efficacy in the interim analysis (NCT03744910).

There is some anecdotal evidence for the use of dd-cfDNA after anti-rejection treatment. Hinojosa et al. first suggested that dd-cfDNA might confirm real-time response to treatment after

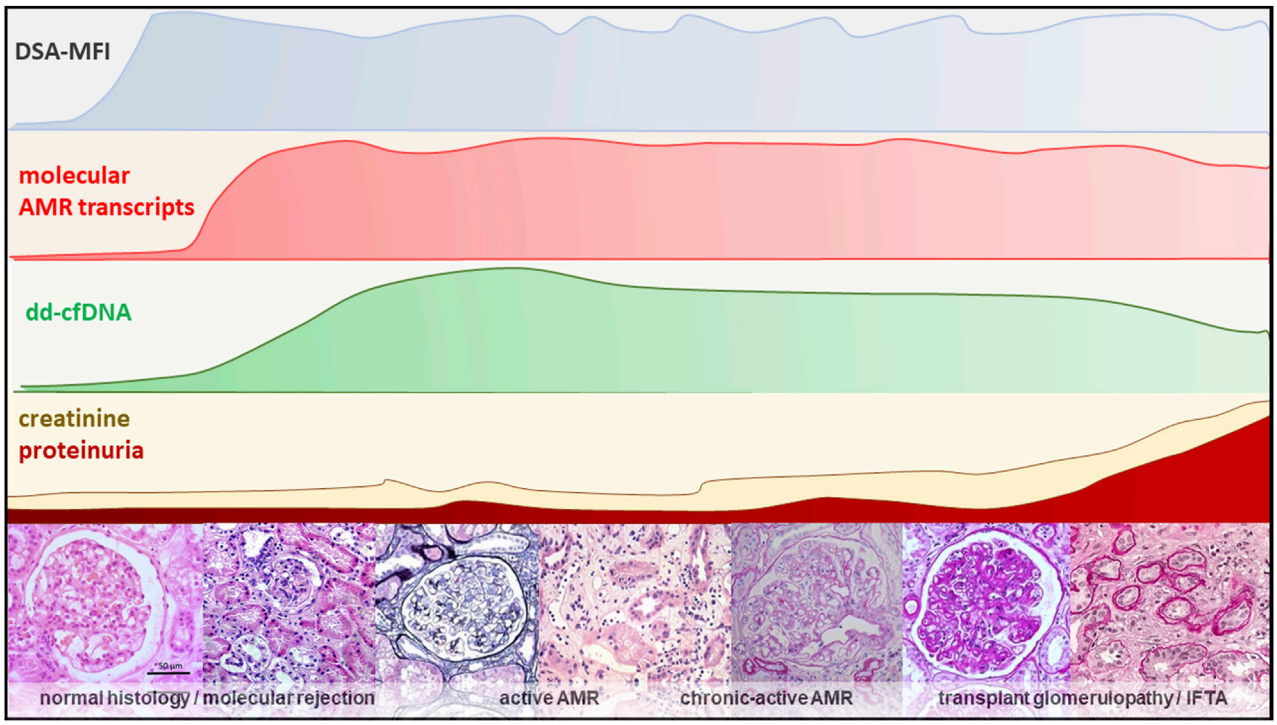

FIGURE 1 | An overview of different diagnostic modalities showing the relationship between dd-cfDNA and the molecular, clinical and histological progression within the natural course of AMR [65–69].

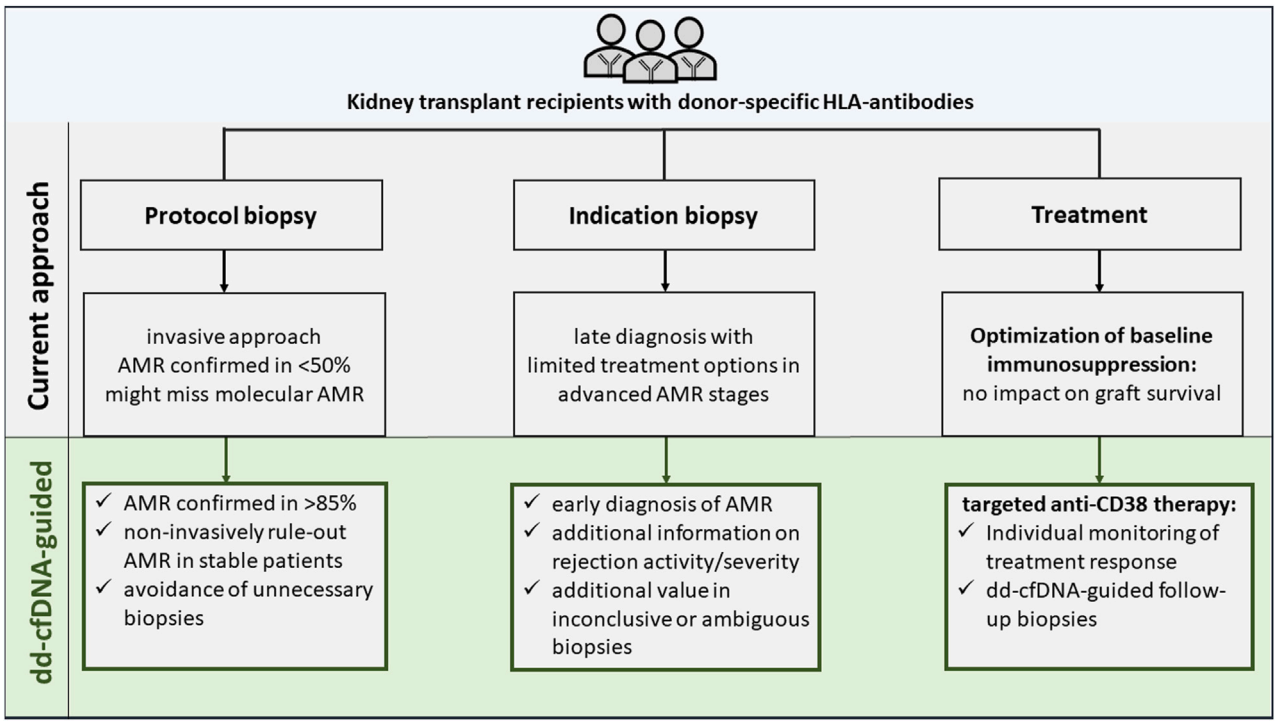

FIGURE 2 | Current versus donor-derived cell-free DNA-guided diagnostic and therapeutic approach in kidney transplant recipients with donor-specific antibodies.

acute rejection [74]. This was confirmed in a larger cohort consisting of the rejecting biopsies identified in the DART study, where dd-cfDNA decrease was observed in both TCMR and AMR biopsies with longitudinal surveillance after treatment [75]. A similar trend towards decreasing dd-cfDNA values in some patients was reported in two studies that performed dd-cfDNA follow-up monitoring after rejection, which were mostly interpreted as therapy response [51, 55]. Similarly, a single-center report described dd-cfDNA to assess treatment response of the monoclonal anti-IL-6-receptor antibody tocilizumab in patients with AMR and observed a significant decrease in dd-cfDNA, potentially reflecting resolving injury, but only a nonsignificant trend in proteinuria reduction [76]. In contrast, Mayer et al. could not confirm changes in dd-cfDNA values after treatment with anti-IL6 antibody clazakizumab in patients with AMR, suggesting ongoing injury [77].

Advances in unveiling the pathogenesis of AMR focused on the role of natural killer-cells (NK-cells) in AMR and microvascular inflammation in kidney allografts [78]. Accordingly, anti-CD38 antibodies are becoming interesting candidates to target antibody-producing plasma cells as well as NK-cells that are described to be key injury mediators in AMR [78–81]. Recently, results from the first randomized-controlled phase-2 trial (NCT05021484) investigating the novel anti-CD38 agent felzartamab (fully human IgG1 monoclonal anti-CD38 antibody) in KTR with late AMR suggest the potential efficacy of this targeted approach [72, 82].

Together with resolution of AMR activity in felzartamab-treated patients, a rapid normalization of dd-cfDNA was seen. Again, dd-cfDNA levels were highly correlated with AMR activity in histology and molecular AMR scores. Six months after treatment discontinuation, recurrence of AMR activity was seen in some patients together with increasing dd-cfDNA levels and molecular AMR scores. Together, these data further support the hypothesis that dd-cfDNA is a reliable biomarker, closely related to AMR activity, that could serve as a companion diagnostic to guide treatment response in patients with AMR (**Figure 2**) [82].

The full utility of dd-cfDNA as a companion biomarker to guide anti-rejection therapy is to be determined in future prospective multicentric trials. It might help monitor individual treatment response, avoid repeat biopsies to control treatment response or perform such with greater precision through dd-cfDNA-triggered indication.

CONCLUSION

Dd-cfDNA is a sensitive biomarker of ongoing cellular injury in the transplanted kidney. Although not specific for AMR, dd-cfDNA has a promising potential to add value for the detection, diagnosis, and monitoring of AMR after kidney transplantation. Most importantly, the context of use must be well defined, as for any other test. It cannot replace DSA screening, but in patients with DSA, it provides additional information that enables earlier detection of AMR and may help to avoid unnecessary biopsies in patients with low dd-cfDNA values due to its high negative predictive value. In addition, dd-cfDNA may help to better judge the severity of ongoing AMR, although more studies are needed to fully establish the relationship of dd-cfDNA with outcomes in AMR patients. The correlation between dd-cfDNA, microvascular inflammation in histology and molecular AMR may also translate into a better understanding of the evolution of subclinical AMR. Finally, evidence is evolving that dd-cfDNA may be a valuable non-invasive tool for monitoring ongoing AMR activity and potential treatment effects.

We believe that dd-cfDNA has an added clinical value for the timely diagnosis of AMR in DSA-positive patients and may also be useful for monitoring AMR treatment response, as the integration of this biomarker into clinical care of DSA-positive patients would reduce the need for biopsies due to high positive and negative predictive values. However there are still some limitations and knowledge gaps that deserve consideration and require additional research, such as the biological background of release, optimal frequency of dd-cfDNA testing, comparability and cost of different assays as well as cost-effectiveness of different approaches [24, 37]. Within the context of DSA-positive patients more data are needed for an optimal clinical implementation and ideally future prospective-randomized clinical trials with robust endpoints and sufficient follow-up will provide more evidence on the clinical impact of complementary dd-cfDNA monitoring on the clinical course of patients with DSA and AMR.

Other aspects that deserve attention are the use in other indications such as DSA-negative-MVI, and BKVAN. Beyond this, other biomarker candidates, such as peripheral blood gene-expression tests, urinary chemokines (e.g., interferon gamma (IFNG) dependent, CXCL9 and CXCL10) and the Torque-Teno-virus (TTV), are also being studied to further improve graft surveillance. However, only a few reports specifically addressed their utility in patients with AMR. Contrary to dd-cfDNA, preliminary results for patients with AMR are conflicting and evidence limited. Additional aspects that limit their transition into clinical routine are reproducibility, assay standardization and availability. More focused research or strategies with a combined approach might help to better define their role for monitoring patients with DSA or AMR in the future [24, 77, 83–85].

In conclusion, dd-cfDNA is currently the best available, non-invasive biomarker, beyond anti-HLA-DSA, to identify patients with AMR. The current evidence suggests that the integration of dd-cfDNA in the graft surveillance of patients with AMR, or those suspicious of AMR (e.g., due to the presence of DSA) has additional value and may help to improve outcomes in this specific cohort. dd-cfDNA could be helpful in personalized post-transplantation therapy with the potential to reduce premature graft loss.

AUTHOR CONTRIBUTIONS

AA, KlB, and BO wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

Authors JB, KB-K, and ES were employed by Chronix Biomedical GmbH.

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BRIEF RESEARCH REPORT
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Donor-Derived Cell-Free DNA as a Companion Biomarker for AMR Treatment With Daratumumab: Case Series

Bilgin Osmanodja[1], Aylin Akifova[1], Klemens Budde[1], Michael Oellerich[2], Julia Beck[3], Kirsten Bornemann-Kolatzki[3], Ekkehard Schütz[3], Joachim Velden[4], Claudia Lehmann[5], Bastian Malte Krüger[6], Anette Bachmann[6] and Jan Kowald[6]*

[1]Department of Nephrology and Intensive Care, Charité—Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Berlin Institute of Health, Humboldt-Universität zu Berlin, Berlin, Germany, [2]Department of Clinical Pharmacology, University Medical Center Göttingen, Göttingen, Germany, [3]Chronix Biomedical GmbH, Göttingen, Germany, [4]Department of Nephropathology, Institute of Pathology, University Hospital Erlangen, Friedrich-Alexander University Erlangen-Nuremberg, Erlangen, Germany, [5]Institute for Transfusion Medicine, Laboratory for Transplantation Immunology, University Hospital Leipzig, Leipzig, Germany, [6]Medical Department III, Division of Nephrology, University of Leipzig Medical Center, Leipzig, Germany

Antibody-mediated rejection (AMR) is among the most frequent causes for graft loss after kidney transplantation. While there are no approved therapies, several case reports with daratumumab and the very recent phase 2 trial of felzartamab in AMR have indicated the potential efficacy of therapeutic interventions targeting CD38. Donor-derived cell-free DNA (dd-cfDNA) is an emerging biomarker with injury-specific release and a short half-life, which could facilitate early diagnosis of AMR and monitoring of treatment response. We describe two cases of patients with chronic active AMR, who were treated with monthly daratumumab infusions, and in whom donor-derived cell-free DNA (dd-cfDNA) was measured longitudinally to monitor treatment response. In both patients, daratumumab treatment led to stabilization of kidney function parameters, a strong decline of dd-cfDNA below the previously established threshold for rejection, and partial or complete histologic resolution of AMR activity. Our case series suggests that dd-cfDNA may be a useful companion biomarker for longitudinal monitoring of anti-CD38 treatment in patients with AMR.

Keywords: kidney transplantation, antibody-mediated rejection, daratumumab, donor-derived cell-free DNA, biomarker

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***Correspondence**
Bilgin Osmanodja,
bilgin.osmanodja@charite.de

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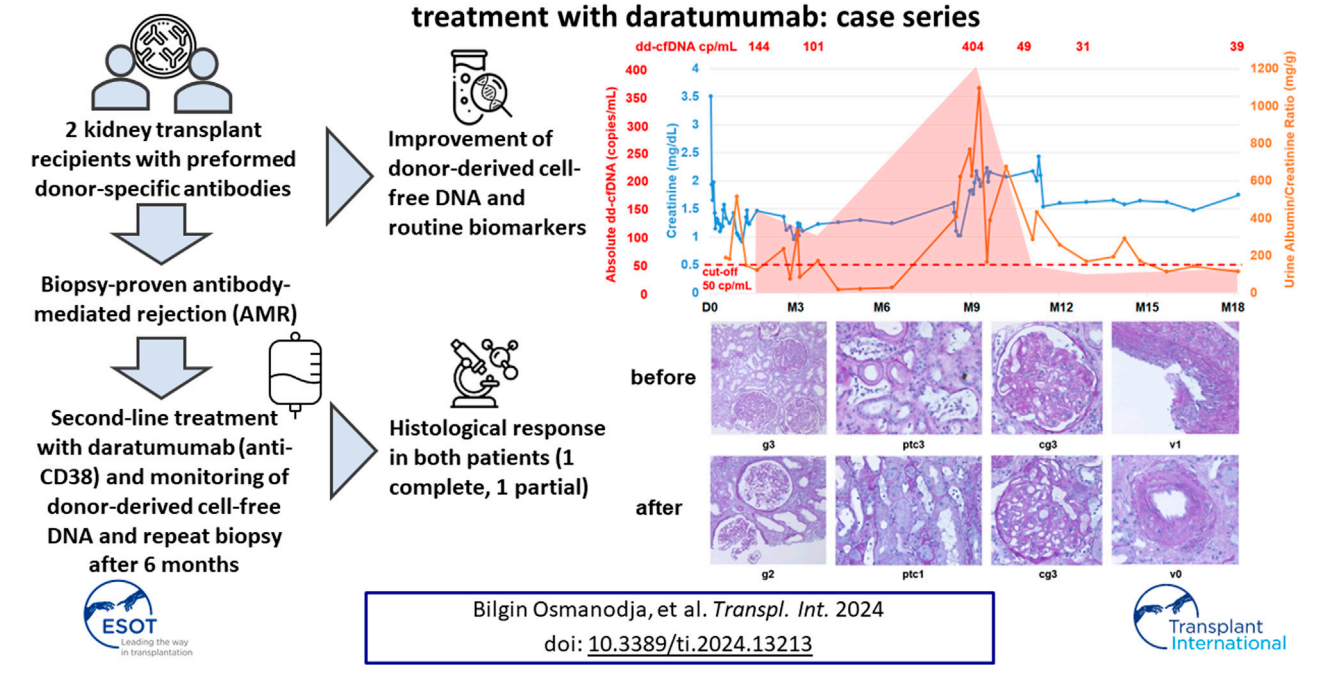
INTRODUCTION

Antibody-mediated rejection (AMR) is among the most frequent causes for graft loss after kidney transplantation [1]. Treatment of AMR remains a challenge, and while there are no approved therapies [2–7], several case reports have indicated the potential efficacy of therapeutic interventions targeting CD38 [8–11]. Recently, a phase 2 trial of felzartamab, an investigational, fully human IgG1 monoclonal anti-CD38 antibody in patients with AMR demonstrated safety and tolerability, and showed resolution of AMR in a majority of patients [12]. The proposed mechanisms of action of anti-CD38 treatment are depletion of alloantibody-producing plasma cells (PC) and natural killer (NK) cells, the latter of which are key effector cells in the pathogenesis of AMR [13].

Meanwhile, off-label use of daratumumab has been performed, which is currently approved for multiple myeloma. Clinical routine parameters such as creatinine or estimated glomerular filtration



GRAPHICAL ABSTRACT

rate (eGFR) and urine albumin-creatinine ratio (uACR) are neither optimal to monitor AMR activity nor treatment response. Donor-derived cell-free DNA (dd-cfDNA) is an emerging biomarker with injury-specific release and a short half-life, which could facilitate early diagnosis of AMR and monitoring of treatment response [14].

In this case series, we demonstrate the use of dd-cfDNA for longitudinal graft monitoring in two patients with AMR that were treated with daratumumab as second line therapy.

METHODS

Daratumumab Treatment

Daratumumab was administered as an intravenous infusion in a dosage of 16 mg/kg body weight, every 4 weeks. The first infusion was started at a rate of 25 mL/h and was subsequently increased by 25 mL/h every 30 min up to a maximum of 200 mL/h. Subsequent infusions were started at 50 mL/h and increased by 50 mL/h every 30 min up to a maximum rate of 200 mL/h.

Premedication included prednisolone (100 mg), dimetindene (4 mg), cimetidine (200 mg), ondansetron (4 mg), paracetamol (1,000 mg) and montelukast (10 mg).

Since daratumumab is associated with increased risk of opportunistic infections, both patients received pneumocystis prophylaxis and, due to intermediate risk of cytomegalovirus infection, antiviral prophylaxis with valganciclovir [15].

Donor-Derived Cell-Free DNA Testing

Measurement of dd-cfDNA was performed as described previously [16, 17]. In brief, for each patient, four informative single-nucleotide polymorphisms (SNPs), defined as an SNP for which the recipient has a homozygous allelic state, and the graft carries at least one heterozygous allele, were selected from a predefined set of 40 SNPs. These four SNPs were used to quantify the dd-cfDNA (%) concentration, which is defined as donor-alleles/(donor-alleles + recipient-alleles). Results for SNPs with heterozygous graft genotypes were corrected by a factor of two. Total cfDNA was extracted from up to 8 mL plasma collected in certified blood collection tubes (Streck Corp., Omaha, NE, United States). The concentration was determined using droplet-digital PCR (ddPCR) and was corrected for extraction loss and cfDNA fragmentation as described previously [16]. The absolute concentration of dd-cfDNA per mL plasma was calculated by multiplying total cfDNA (copies/mL) and dd-cfDNA (%). An abnormal dd-cfDNA result was defined as a value of >50 copies/mL for absolute quantification [16, 17].

Detection and Differentiation of HLA Antibodies With Bead-Based Technique

HLA-antibody differentiation was performed using Luminex Single Antigen Bead assays (One Lambda, West Hills, CA, United States) LSA1A04 and LSA2A01. The assays have been performed according to the manufacturer's instructions. The

antibody determination was performed on the Luminex 200 device using xPONENT® software and was analyzed in the HLA-Fusion software v4.4. MFI values > 1,500 were defined as the cut-off for positive detection of HLA antibodies in the single tests.

Statement of Ethics

Written informed consent was obtained from both patients for publication of the details of their medical case and any accompanying images. The underlying observational study involving human participants were reviewed and approved by the ethics committee of Charité - Universitätsmedizin Berlin (EA2/144/20, date of approval 25.02.2021). The patients provided written informed consent to participate in this study. The clinical activities being reported are consistent with the principles of the Declaration of Istanbul as outlined in the "Declaration of Istanbul on Organ Trafficking and Transplant Tourism".

RESULTS

Case Descriptions

Case 1

The first patient is a 52-year-old female with autosomal dominant polycystic kidney disease (ADPKD), who received a living-donor kidney from her husband despite preformed donor-specific anti-HLA antibodies (DSA) against donor antigen DR7 (mean fluorescence intensity [MFI] 3339) triggered by a previous pregnancy. The donor-recipient HLA-A, -B, -C, -DR, -DQ mismatch grade was 10 (2-2-2-2-2).

Before kidney transplantation, desensitization with 5 cycles of plasma exchange (PLEX) and intravenous immunoglobulins (IVIG) was performed per institutional standard. Preformed DSA declined to an MFI of 1,564, which was deemed sufficiently low to perform transplantation (**Figure 1**). Two weeks after kidney transplantation and induction immunosuppression with anti-thymocyte globulin (ATG; three doses of 1.25 mg/kg each), as well as triple maintenance immunosuppression with methylprednisolone, tacrolimus (through levels 10–12 ng/mL) and mycophenolate mofetil (MMF; 2 g/d), the preformed DSA MFI increased to 5339, and *de novo* DSA (dnDSA) against DQ2 (MFI 2296) and B44 (MFI 5896) occurred.

Six weeks after transplantation, we observed a rise in creatinine from a best value of 1.0 mg/dL to 1.5 mg/dL without a significant increase in urine albumin/creatinine ratio (uACR). At that time, DSA MFI have already declined below 1,500 (DR7 1366, DQ2 18, B44 203). We performed donor-derived cell-free DNA testing, which showed markedly increased levels of 144 copies/mL (2.61%) compared with the prespecified cut-offs of 50 copies/mL and 0.5%. Given the history of the patient, this was highly suggestive of AMR. We performed kidney biopsy, which confirmed active antibody-mediated rejection (aAMR) and showed acute T-cell mediated rejection (TCMR) Banff IIA (g2ptc2v1t2i2tIFTA1iIFTA0cg0mm0cv1aah0ct1ci0).

The patient was treated with 5 PLEX and IVIG (10 g after each PLEX) as well as ATG (three doses of 1.25 mg/kg each) and steroid pulse for TCMR. Tacrolimus levels were maintained between 6–8 ng/mL and MMF dose was 2 g/d. Kidney function parameters stabilized, but dd-cfDNA was still increased with 101 copies/mL (0.84%), suggesting ongoing graft injury. However, due to the clinical improvement of kidney function (serum creatinine 1.1 mg/dL), we chose not to further intensify the immunosuppressive regimen. The day after completion of PLEX, MFI for all DSA were 0; 6 weeks after PLEX, MFI were still low for all DSA (DR7 312, DQ2 56, B44 40). A second biopsy 2 months after the initial biopsy showed complete resolution of TCMR and partial improvement of AMR activity (g1ptc1v0t0i0ti2tIFTA0iIFTA3cg0mm0cv2aah1ct2ci2).

Six months later, the patient had a severe urinary tract infection (UTI) and a subsequent deterioration of kidney function with increases in serum creatinine and uACR. Two weeks after successful treatment of the UTI (no leukocyturia, normal CRP), we performed dd-cfDNA testing, which showed very high levels of 404 copies/mL (0.7%). DSA MFI at this point had been stable since PLEX (DR7 194, DQ2 9, B44 0). A third kidney biopsy confirmed ongoing chronic active AMR (caAMR) with severe transplant glomerulopathy (cg3), severe glomerulitis (g3), severe peritubular capillaritis (ptc3), mild to moderate intimal arteritis (v1), matching the high dd-cfDNA levels (**Figure 2A**) (g3ptc3v1t0i1ti1tIFTA0iIFTA0cg3mm0cv1aah3ct1ci1). Since there was no approved treatment available, repeated DSA elimination was not promising due to low MFI, and some recent case reports suggested potential efficacy, we decided to perform off-label treatment with daratumumab as outlined above with regular monitoring of kidney function parameters, DSA, dd-cfDNA and repeat biopsy after 6 months. Maintenance immunosuppression with tacrolimus (through levels 6–8 ng/mL) and methylprednisolone (4 mg/d) was continued, but MMF dose was reduced (1 g/d) due to leukopenia (2,800/μL) 1 week after first administration of daratumumab.

Kidney function stabilized after initiation of treatment and donor-derived cell-free DNA normalized 8 weeks after starting daratumumab (49 copies/mL; 0.37%) and further decreased at 16 weeks (31 copies/mL; 0.22%) and 9 months (39 copies/mL; 0.25%). Three months after treatment initiation, creatinine was around 1.5 mg/dL and uACR below 300 mg/g and MFI <1,500 (DR7 680 and DQ2 34).

Peripheral blood NK cell count declined shortly after initiation of daratumumab from 120/nL to less than 20/nL and stabilized at 11-12/nL after 4 months of treatment.

A fourth biopsy after 6 months still showed caAMR with unchanged chronic changes (cg3cv1ci1ct1), but improved Banff scores for AMR activity: glomerulitis decreased from severe (g3) to moderate (g2), and peritubular capillaritis improved from severe (ptc3) to mild (ptc1), while the intimal arteritis (v1) was no longer detectable (v0), **Figure 2B** (g2ptc1v0t1i0ti1tIFTA0iIFTA3cg3mm0cv1aah2ct1ci1).

Given the good response, we extended therapy with daratumumab to 9 months, and increased the interval to 6 weeks with repeated monitoring of laboratory values.

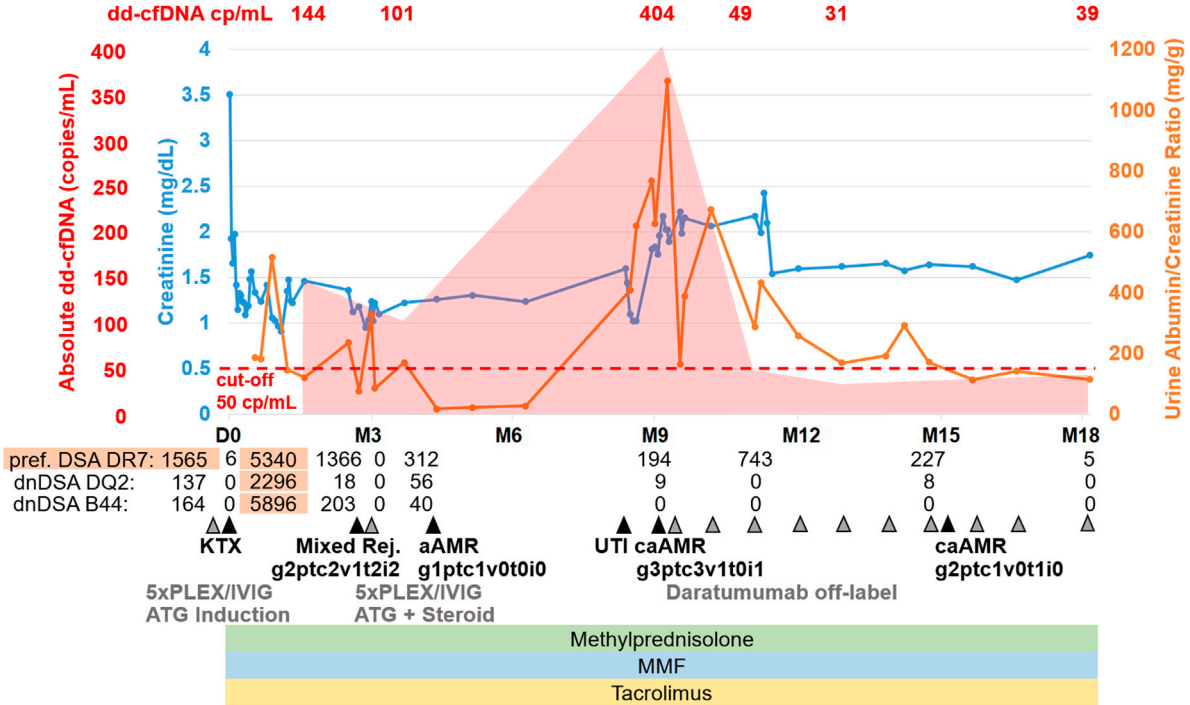


FIGURE 1 | Case summary including key clinical events, allograft biopsy results, therapeutic interventions, routine biomarkers (creatinine, urine albumin/creatinine ratio) as well as donor-derived cell-free DNA (dd-cfDNA). The red shaded area depicts the absolute dd-cfDNA levels and numeric values are provided at the top of the figure. The prespecified cut-off of 50 copies/mL derived from previous validation studies is shown. Grey arrowheads indicate therapeutic interventions, black arrowheads indicate clinical events including kidney allograft biopsies. Orange shape indicate the time periods when DSA MFI were above 1,500. dd-cfDNA, donor-derived cell-free DNA; cp, copies; D0, day of transplantation; M, months after transplantation; DSA, donor-specific anti-HLA antibodies; dnDSA, *de novo* DSA; KTX, kidney transplantation; PLEX, plasma exchange; IVIG, intravenous immunoglobulins; ATG, anti-thymocyte globulin; Mixed Rej., mixed rejection; aAMR, active antibody-mediated rejection; TCMR, T-cell mediated rejection; UTI, urinary tract infection; caAMR, chronic active antibody-mediated rejection; MMF, mycophenolate mofetil.

During the initial administration of daratumumab, nausea and vomiting occurred, which disappeared after administration of antiemetic therapy. Other infusion-related reactions, in particular bronchospasm or shortness of breath, did not occur with the premedication including montelukast.

Two months after initiation of daratumumab, the patient developed SARS-CoV-2-infection. She was admitted to hospital and treated with remdesivir for 3 days, but only mild symptoms were observed. In this context, severe hypogammaglobulinemia (serum-IgG-level <4 g/L) was detected. A single dose of 30 g IVIG was administered. Subsequently, only mild hypogammaglobulinemia was detected (serum-IgG-level >4 g/L). There were no further hospitalizations.

The main complications during daratumumab treatment were recurrent UTIs treated with oral antibiotics and not requiring hospitalization. Eventually, the patient was started on antibiotic prophylaxis with nitroxoline after which no further UTIs were observed. As the UTIs had already occurred before starting daratumumab treatment, they were not necessarily treatment-related.

At the last visit, the patient is in good condition with serum creatinine of 1.5 mg/dL, uACR 114 mg/g, MFI practically undetectable (DR7 5, DQ2 0, and B44 0), and dd-cfDNA of 39 copies/mL (0.25%), while blood pressure medication was partially discontinued.

Case 2

The second patient is a 25-year-old female patient with Mayer-Rokitanski-Küster-Hauser syndrome, who underwent a second kidney transplantation at the age of 24 within the Eurotransplant Acceptable Mismatch program (vPRA 97.2%). After a 1-year period on peritoneal dialysis at the age of two, she underwent first kidney transplantation at the age of three. Due to early urological complications with recurrent UTIs, an ileal conduit urinary diversion was performed. Eventually, the graft was functioning for 18.5 years. Afterwards, the patient was on hemodialysis for almost 4 years before the second transplantation was performed.

Despite well matched graft (mismatch grade HLA-A, -B, -C, -DR, -DQ 1-1-1-0-0) within the Acceptable Mismatch (AM) program, two preformed DSA were present (Cw6; MFI 1743, DP2; MFI 8890) after transplantation (**Figure 3**). Initial immunosuppression consisted of Interleukin-2 receptor antibody (basiliximab), tacrolimus (through levels 10–12 ng/mL), MMF (2 g/d) and methylprednisolone. Due to delayed graft function, a first kidney biopsy was performed on day 9 after transplantation and showed active AMR (g2ptc3v0i0t0ti0tIFTA0iIFTA0cg0mm0cv0aah0ct0ci0). A steroid pulse and 5 courses of PLEX were initiated after

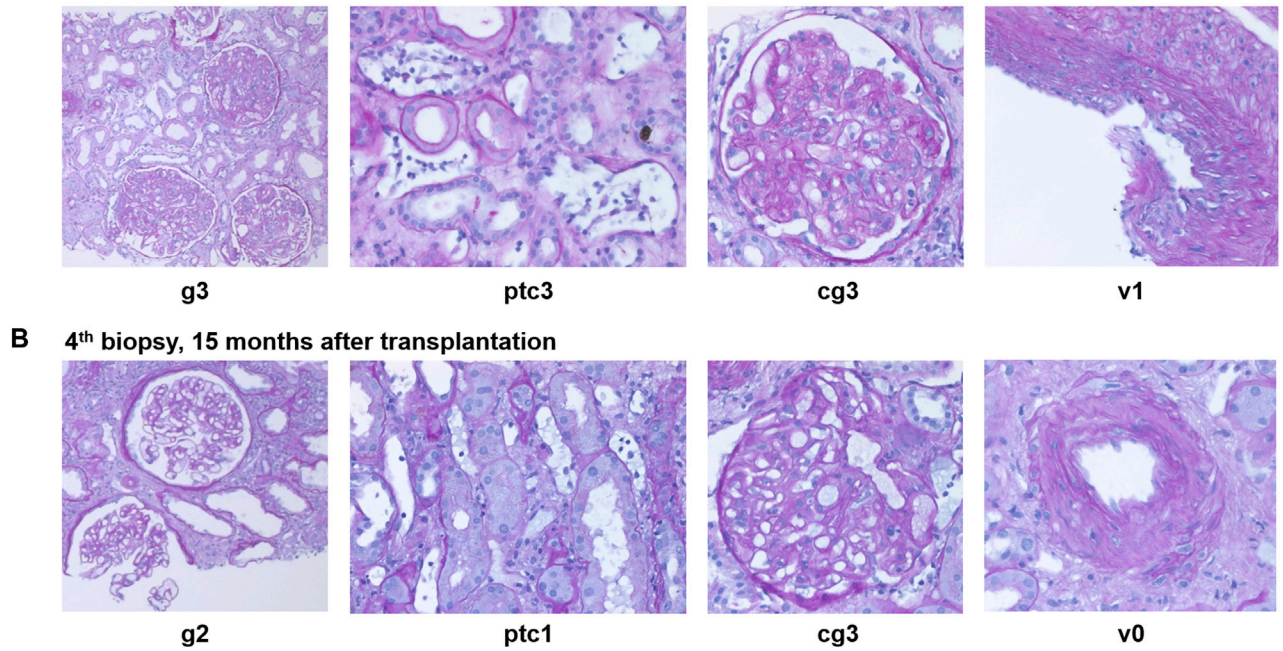


FIGURE 2 | Representative histopathological findings for patient 1 showing glomerulitis (g), peritubular capillaritis (ptc), chronic transplant glomerulopathy (cg), and intimal arteritis (v) at **(A)** 9 months after transplantation (before daratumumab) and **(B)** 15 months after transplantation (6 months after treatment with daratumumab).

which creatinine improved to a baseline of 1.5 mg/dL with low uACR. While the preformed DSA against Cw6 were decreasing to MFI <1,500 (444 after PLEX), the antibodies against DP2 persisted at MFI 1688 after PLEX. Clinical routine parameters remained stable; therefore, no additional anti-rejection therapy was initiated and maintenance immunosuppression with tacrolimus (through levels 10–12 ng/mL), MMF (2 g/d) and methylprednisolone (5 mg/d) was continued. A follow-up biopsy was declined by the patient due to stabilization of kidney function. Seven months after transplantation, DSA MFI of DP2 increased to 4,223, and DSA MFI of Cw6 remained stable below the cutoff at 428.

Eight months after transplantation, a rise in creatinine (1.6–1.9 mg/dL) was observed, while DSA MFI were decreasing (DP2 2040, Cw6 241). A second biopsy showed caAMR (g3ptc2v0t0i1ti1tIFTA0iIFTA0cg3mm0cv0aah0ct1ci1). Due to persistent DSA, a first-line therapy with immunoadsorption (IA), IVIG (10 g after each IA) and rituximab (RTX; 375 mg/m^2 body surface) was initiated and led to a transient decrease in creatinine and effectively lowered DSA for 2 months (DP2 778, Cw6 155). However, 2-3 months later, creatinine rose again (1.9–2.3 mg/dL), and a third biopsy still showed caAMR with high activity (g3ptc2v0t0i0ti0tIFTA0iIFTA0cg2mm0cv0aah0ct1ci1, **Figure 4A**). In line with the histology, dd-cfDNA was elevated at the time of biopsy (77 cp/mL; 0.55%). MFI of DP2 DSA increased to 5849 (Cw6 397).

Again, we decided to perform off-label treatment with daratumumab with regular monitoring of kidney function parameters, dd-cfDNA and repeat biopsy after 6 months. Similar to the first patient, this patient also developed nausea and vomiting during the first administration of daratumumab. No other infusion-related reactions occurred in this patient. Only mild hypogammaglobulinemia was detected (serum-IgG-level 5.3 g/L) after 2 months of treatment. There were no infectious complications and no hospitalizations. During the first 3 months of daratumumab treatment, leukopenia (minimum 2,100/μL) was detected 1 week after daratumumab administration. MMF dose was reduced to 1 g/d, while tacrolimus (through levels 6–8 ng/mL) and methylprednisolone (4 mg/d) remained unchanged.

After 2 months, creatinine stabilized at 1.7 mg/dL and uACR was below 100 mg/g. Dd-cfDNA declined to the lower limit of detection (7 copies/mL; 0.1%) after 2 months of treatment and was still low after 5 months (8 copies/mL; 0.28%).

NK cell count declined after initiation of daratumumab from 53/nL to 20/nL after 3 months of treatment and 18/nL after 4 months of treatment.

A fourth biopsy after 6 months of daratumumab therapy showed chronic (inactive) AMR with AMR activity being almost completely resolved (g0ptc1v0t1i0ti1iIFTA2tIFTA1cg2mm1cv1aah2ct1ci1), which is matching the dd-cfDNA results (**Figure 4B**). The therapy with daratumumab will be extended to 9 months and time intervals will be increased under close monitoring.

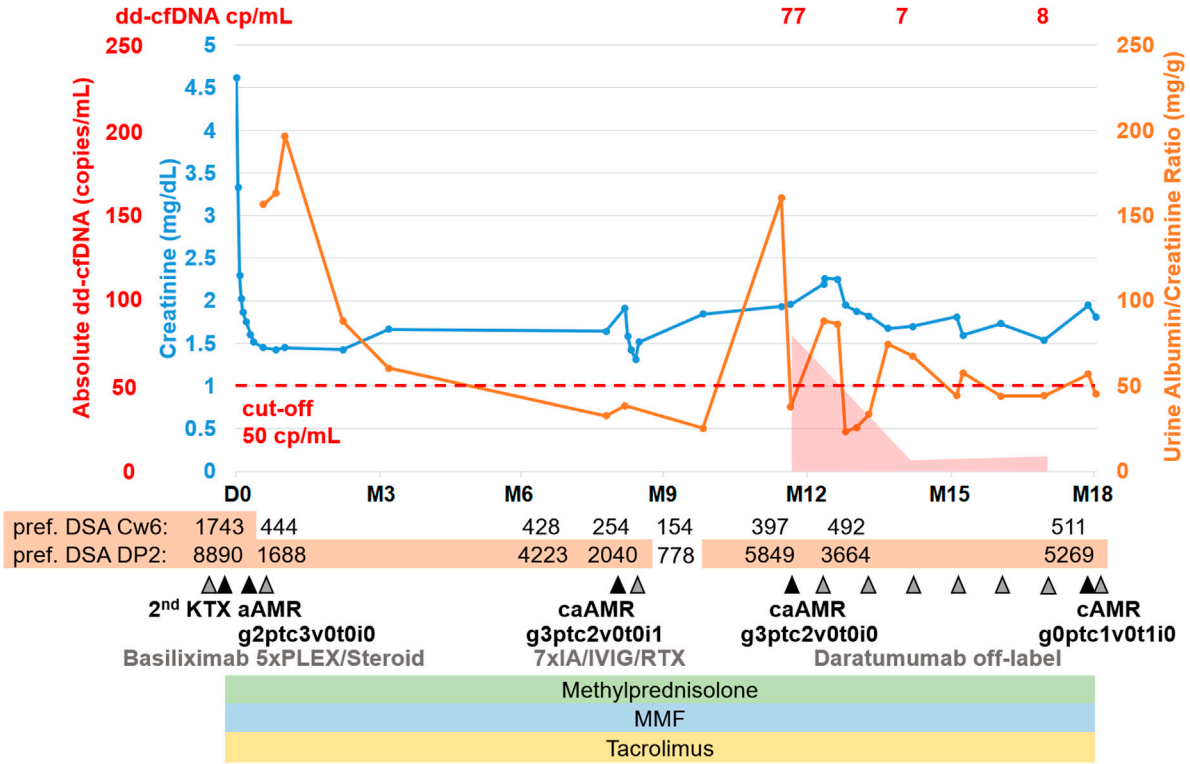


FIGURE 3 | Case summary including key clinical events, allograft biopsy results, therapeutic interventions, routine biomarkers (creatinine, urine albumin/creatinine ratio) as well as donor-derived cell-free DNA (dd-cfDNA). The red shaded area depicts the absolute dd-cfDNA levels and numeric values are provided at the top of the figure. The prespecified cut-off of 50 copies/mL derived from previous validation studies is shown. Grey arrowheads indicate therapeutic interventions, black arrowheads indicate clinical events including kidney allograft biopsies. Orange shape indicate the time periods when DSA MFI were above 1,500. dd-cfDNA, donor-derived cell-free DNA; cp, copies; D0, day of transplantation; M, months after transplantation; DSA, donor-specific anti-HLA antibodies; KTX, kidney transplantation; PLEX, plasma exchange; IVIG, intravenous immunoglobulins; RTX, rituximab; aAMR, active antibody-mediated rejection; cAMR, chronic inactive antibody-mediated rejection; MMF, mycophenolate mofetil.

DISCUSSION

This is the first case series to demonstrate the use of dd-cfDNA to monitor treatment response to anti-CD38 therapy with daratumumab in two sensitized patients with AMR after kidney transplantation.

Histologically, we observed partial response of AMR activity in the first patient, and virtually complete resolution of AMR activity in the second one. After treatment initiation with daratumumab, the injury biomarker dd-cfDNA showed a strong decline below the previously established threshold for rejection in both patients. However, in the patient with remaining AMR activity after 6 months of treatment, dd-cfDNA remained higher than the median of 25 copies/mL that was established in stable phase patients in the original validation study, while the second patient showed dd-cfDNA at the lower limit of detection [16].

In both patients, serum creatinine levels eventually stabilized and uACR declined, which - together with histopathological parameters and dd-cfDNA course - we interpreted as clinically meaningful treatment response.

Clinical routine parameters showed a slower and a less pronounced response to treatment initiation than dd-cfDNA. This is due to chronic damage in both patients and the general disadvantages of creatinine and uACR as markers of injury. While uACR responded faster after treatment initiation than creatinine, chronic transplant glomerulopathy may represent chronic structural damage and can lead to persistent albuminuria, which hence does not necessarily reflect AMR activity.

Given the natural course of caAMR with progressive worsening of kidney function, this anecdotal evidence still suggests a meaningful therapeutic effect of daratumumab. Unfortunately, both patients had already developed mild to moderate chronic lesions (cg2-3, ci1, ct1, and cv0-1) at initiation of treatment, which are known to indicate limited long-term success [18].

Recently, a phase 2 trial of felzartamab in AMR demonstrated acceptable safety and tolerability, and promising results with 81.8% of patients showing complete histological resolution of AMR after 24 weeks of treatment as compared to 20% in the placebo group as well as improvement in microvascular

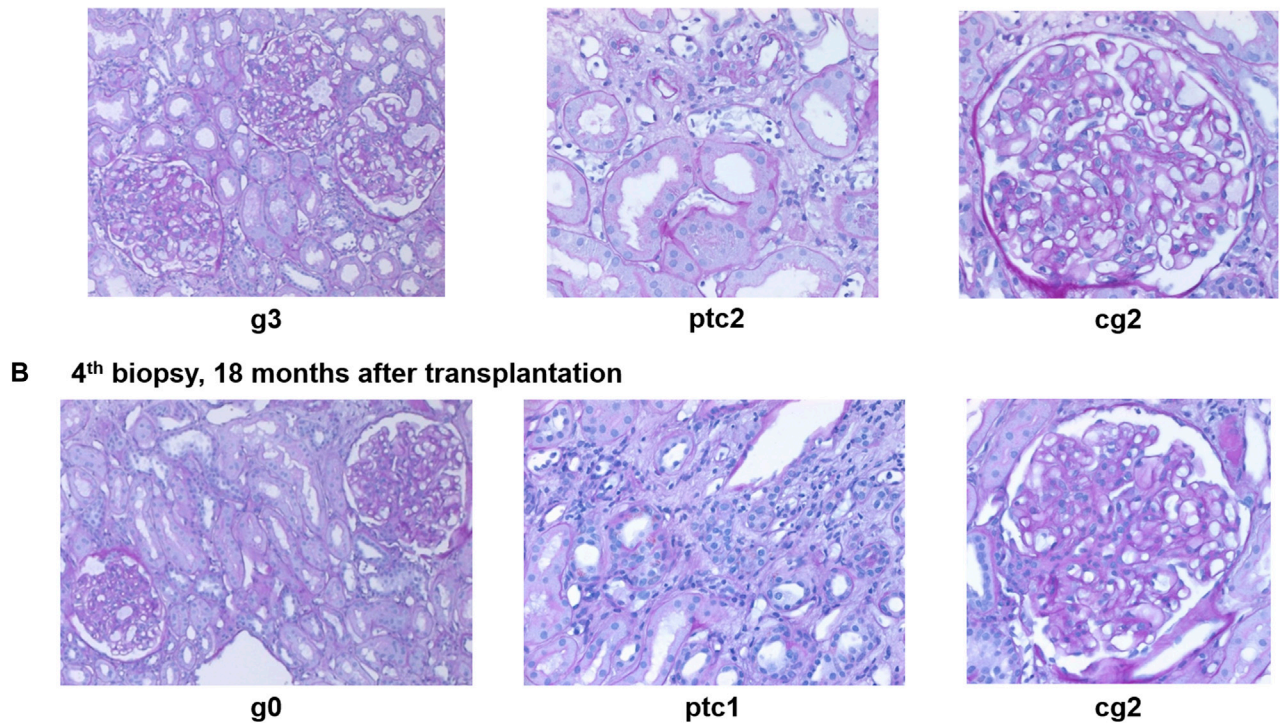


FIGURE 4 | Representative histopathological findings for patient 2 showing glomerulitis (g), peritubular capillaritis (ptc), and chronic transplant glomerulopathy (cg) at **(A)** 12 months after transplantation (before daratumumab) and **(B)** 18 months after transplantation (6 months after treatment with daratumumab).

inflammation in normal histology, AMR-related transcripts, and dd-cfDNA as marker of injury [12].

While daratumumab is the first fully human anti-CD38 monoclonal antibody, and induces complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), as well as antibody-dependent cellular phagocytosis (ADCP), felzartamab has a λ instead of κ light chain, and only induces ADCC and ADCP [19]. The third anti-CD38 monoclonal antibody available, isatuximab, additionally induces direct apoptosis of plasma cells. Different effects on the bone marrow microenvironment have been discussed in multiple myeloma, but the clinical relevance is not yet understood in the context of AMR [19, 20].

Practically, felzartamab and daratumumab are usually given as an induction therapy (e.g., 4 weekly doses for felzartamab), followed by maintenance therapy (e.g., 5 monthly doses for felzartamab), which is derived from the treatment of multiple myeloma [12, 21]. For the feasibility of outpatient treatment, we initiated daratumumab as 4-weekly infusions without an induction period with weekly infusions, discordant to previously established regimens for multiple myeloma and to the protocol used in the felzartamab trial [12]. Both patients developed nausea during first dose administration, but the absence of a severe first dose reaction could be due to the extensive premedication and slow initial infusion rate (25 mL/h). In the felzartamab trial, the rate of infusion-related reactions in the felzartamab group was 72.7% [12].

Even without an induction period, we observed a pronounced and persistent reduction of NK cells in both patients, which was comparable to the one seen in the felzartamab trial [12]. While we did not assess plasma cell depletion in bone marrow biopsies, the vanishing AMR activity in the presence of detectable DSA in the second patient suggests that depletion of NK cells instead of antibody-producing plasma cells is the mechanism of action.

Accordingly, the felzartamab trial showed depletion of NK cells, but only moderate reduction in DSA MFI, which led the authors to similar conclusions [12]. In line with this, it has been shown that NK cell-mediated endothelial cytotoxicity plays an important role in DSA-negative recipients with histological evidence of microvascular inflammation [22]. If this hypothesis holds true, monitoring peripheral blood NK cell count and assessing treatment response with dd-cfDNA could enable personalized dosing intervals and treatment regimens, which has also been proposed by the authors of the felzartamab trial [12].

Future studies need to evaluate whether an induction period as established for myeloma is necessary for AMR treatment, especially if NK cell depletion can be confirmed to be the main mechanism of action. Furthermore, subcutaneous instead

of intravenous application of daratumumab is more feasible and was noninferior in patients with multiple myeloma but showed an improved safety profile and should be considered depending on the reimbursement and local availability [21].

In the felzartamab trial, there was a high recurrence rate of 1/3 in the initial felzartamab responders at week 52, after treatment has been stopped for 6 months. In light of these results, we decided to continue treatment with increasing dosing intervals under close monitoring of dd-cfDNA and NK cell count, as well as routine biomarkers [12].

Nevertheless, both cases also demonstrate that sensitized patients undergoing transplantation against preformed DSA are at high-risk for AMR and potentially inferior graft survival and that currently used desensitization regimens are insufficient to prevent AMR. Therefore, rigorous patient information and selection is necessary to avoid complications and the need for intensified immunosuppressive regimens. If desensitization remains the only option, adding daratumumab or another anti-CD38 agent to novel regimens may reduce the risk of AMR and improve clinical outcomes [23].

In summary, we suggest that dd-cfDNA may be a useful companion biomarker for longitudinal monitoring of anti-CD38 treatment in patients with AMR.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving humans were approved by the ethics commitee of Charité - Universitätsmedizin Berlin. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

BO and JK wrote the article. All authors reviewed and commented the article. JB, KB-K, MO, ES, AA, and BO were responsable for dd-cfDNA testing. JK, BK, and AB treated the patients and contributed clinical data. CL performed HLA testing. JV performed histopathology. KB, MO, and ES supervided the project. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors of this manuscript have conflicts of interest to disclose. BO received travel reimbursement from Oncocyte. MO acts as a consultant to Oncocyte. JB, KB-K, and ES are employees of Chronix Biomedical GmbH, a subsidiary of Chronix Biomedical Inc. (an Oncocyte company), which holds intellectual property rights (EP 3004388B1, EP3201361B1, and US10570443B2).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
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The Potential Role of the Leucocyte Immunoglobulin-Like Receptors in Kidney Transplant Rejection: A Mini Review

Jovanne Palvair[1], *Imane Farhat*[1,2], *Mélanie Chaintreuil*[2], *Ludivine Dal Zuffo*[1], *Lennie Messager*[1], *Claire Tinel*[1,2] *and Baptiste Lamarthée*[1]*

[1]Université de Franche-Comté, EFS, INSERM, UMR RIGHT, Besançon, France, [2]Centre Hospitalier Universitaire de Dijon, Service de Néphrologie et Transplantation Rénale, Université de Bourgogne, Dijon, France

Antibody-mediated rejection (ABMR) remains one of the main causes of long-term graft failure after kidney transplantation, despite the development of powerful immunosuppressive therapy. A detailed understanding of the complex interaction between recipient-derived immune cells and the allograft is therefore essential. Until recently, ABMR mechanisms were thought to be solely caused by adaptive immunity, namely, by anti-human leucocyte antigen (HLA) donor-specific antibody. However recent reports support other and/or additive mechanisms, designating monocytes/macrophages as innate immune contributors of ABMR histological lesions. In particular, in mouse models of experimental allograft rejection, monocytes/macrophages are readily able to discriminate non-self via paired immunoglobulin receptors (PIRs) and thus accelerate rejection. The human orthologs of PIRs are leukocyte immunoglobulin-like receptors (LILRs). Among those, LILRB3 has recently been reported as a potential binder of HLA class I molecules, shedding new light on LILRB3 potential as a myeloid mediator of allograft rejection. In this issue, we review the current data on the role of LILRB3 and discuss the potential mechanisms of its biological functions.

Keywords: kidney transplant, allograft rejection, monocyte, LILRs, innate immunity

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***Correspondence**
Baptiste Lamarthée,
baptiste.lamarthee@inserm.fr

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INTRODUCTION

Two major types of rejection are classically described after kidney transplantation: T cell-mediated rejection (TCMR) and antibody-mediated rejection (ABMR), named after their respective presumed mechanism of injury: T lymphocytes and anti-human leucocyte antigen (HLA) donor-specific antibody (DSA). However, with regard to ABMR, current antibody-targeting treatments are proving to be disappointing, highlighting a lack of a comprehensive understanding of all the immune mechanisms involved [1]. Furthermore, some rejections show both typical and concomitant lesions of ABMR and TCMR and are sometimes referred to as "Mixed rejection," suggesting that potentially common cellular mechanism are at play in these cases. New advances indicate that allograft lesions can be induced by myeloid cells, independently of DSA. A high heterogeneity in the graft infiltrating cells has been demonstrated, with myeloid cells accounting for up to 80% of those upon rejection [2]. Importantly, murine models of solid organ transplantation suggest that monocyte/macrophage may recognize non-self determinant [3] independently of adaptive immunity [4, 5]. The specific depletion of monocyte/macrophages may additionally preserve the allograft from rejection lesions [6–8].

Recently in mice, an elegant report has identified receptors on the surface of recipient-derived monocytes/macrophages capable of interacting specifically with donor cells via their major histocompatibility complexes and promoting rejection [9]. The authors of this study showed previously that initiation of the primary alloresponse of recipient's monocytes requires the interaction of their CD47 ligand with SIRP-α [5]. This initiation leads to paired immunoglobulin-like receptors (PIRs) modulation at monocytes membrane and notably PIR-A was identified as responsible of donor's MHC-I recognition. Deletion of these receptors and/or inhibition of their interaction with the complexes improved long-term allograft survival. Inversely, deletion of PIR-B lead to rapid rejection, underlying the opposite mechanism of both receptors and the balance existing between them. Deletion of one precipitates/accelerates/determine the fate of the grafted organ. They validated their findings in two solid organ transplantation murine models, in both heart and kidney allograft. Importantly, as long as PIRs were expressed on the surface of monocytes, an innate memory response against non-self MHC persisted [9]. These so-called PIRs are the murine orthologs of the human leukocyte immunoglobulin-like receptors (LILRs). In humans, half of these LILRs were described as ligand for class I HLA and could thus play a role in myeloid responses in an allogeneic context [10–13]. The present review aims to understand the involvement of these receptors, with a special focus on LILRB3, in recognizing donor tissue as non-self and activating the recipient's myeloid cells against the transplanted organ.

LILRs as an Immunomodulatory Family

LILRs are a family of immunomodulatory receptors expressed on myeloid and lymphoid cell lines [14]. These receptors are structurally similar to killer-cell immunolobulin-like receptors (KIRs), whose role in allograft rejection has been highlighted over the past decade [15]. As for KIRs, their encoding genes are located on chromosome 19q [16], and clustered in centromeric and telomeric regions with opposite transcription directions. Depending on the haplotype, *LILRA6* and *LILRB3* genes have different copy numbers [17, 18]. Within the LILRs' family, LILRAs and LILRBs subtypes were further defined according to their activators (LILRAs) or inhibitors (LILRBs) functions, the distinction being made according to the activating or inhibitory motifs of their intracellular domain [10]. Both LILRAs and LILRBs are characterized by an extracellular structure comprising several immunoglobulin (Ig) domains. The LILRA-dependent signaling pathways are activated by the recruitment of Src and Syk kinases following the phosphorylation of immunoregulatory tyrosin-based activator motifs (ITAM) sequences present in the intracellular compartment. The Syk and Src kinases phosphorylate PI3K and PLC γ2, leading to activation of the PI3K/Akt, NFAT, Ras/ERK, NF-κB, JNK and MAPK pathways. On the opposite, LILRBs signal transduction include a cytoplasmic domain rich in immunoregulatory tyrosin-based inhibitory motifs (ITIM). After binding of the LILRBs ligands, the phosphorylation of the intracellular ITIM sequences induces the recruitment of the Src homology domain phosphatases SHP-1 or SHP-2. Activation of these phosphatases leads to dephosphorylation of ITAM sequences, particularly those carried by neighboring LILRAs, as well as kinases involved in their downstream signaling. Thereby, LILRB ITIM sequences can interfere with effector functions, cytokine secretion as well as immune cell maturation [19]. For instance, activation of LILRB3 was recently reported to induce NF-κB pathway in cancer cells [20].

LILRB3, a Potential Myeloid Immune Checkpoint

LILRB3, also know n as ILT5 (immunoglobulin-like transcript 5), is emerging as a key player in the modulation of the immune response and has attracted growing interest in biomedical research. In the tumor microenvironment, the surface expression of LILRB3 on myeloid cells was recently correlated to a poor patient survival [21, 22]. In addition, a remarkable work reported LILRB3 binding capacity to class I HLA [23], suggesting a new role in regulating immune cells interaction, and not only in regulating immune cell functions. Recently using single-cell RNA sequencing, we showed that *LILRB3* expression was increased in kidney allograft infiltrating monocytes during ABMR, suggesting LILRB3 involvement in monocyte activation during rejection [24, 25]. Interestingly the high polymorphism of this gene [26] leads to the translation of slightly different molecules. In 2009, Pfistershammer and colleagues reported two major variants of the LILRB3 protein, ILT5v1 and ILT5v2 based on their respective sequences [27]. Later, Bashirova and colleagues described six different "allotypes" of LILRB3 in the European population. Allotypes 2, 3, 4 and 6 share the same phylogenetic root and correspond to ILT5v1 while allotypes 1 and 5 corresponds to ILT5v2 [26]. These differences may carry the affinity of each variant for a specific ligand. Hence in 2016, Hofer and colleagues investigated LILRB3 variants binding capacity and determined that ILT5v2 bound complement factor C4d and class I HLA unlike ILT5v1 [28] C4d molecules binding to LILRB3 in activated monocytes inhibited TNF-α and IL-6 secretion in a dose dependant manner. Given the major role of complement activation in ABMR [29], the potential interaction of recipient-derived monocyte LILRB3 and C4d in transplant rejection context has yet to be determined.

The advent of *in silico* tools for modelling protein interactions such as HOMology modeling of COmplex Structure (HOMCOS) [30], allows to speculate that LILRB3 may interact with the heavy chain of antibodies (Fab heavy chain). The ability of LILRB3 to link a DSA Fab, while Fc fragment is bound to another immune cell, is an interesting process to investigate. This interaction could be sufficient to create an immunological synapse between two cells and to inhibit LILR specific pathways (including MAPK, PI3K/Akt or Jak/STAT) leading the monocytes to an anti-inflammatory phenotype. Imbalance of this interaction could switch the phenotype and favor a pro-inflammatory state therefore inducing donor cells destruction.

A large number of proteins have since been reported as potential ligands for LILRB3 including apolipoprotein-E (APOE) [31] and angiopoietin-like (ANGPTL). ANGPTL is involved in diabetes mellitus, in which it promotes the development of adipose tissue, chronic inflammation and systemic insulin resistance in response to

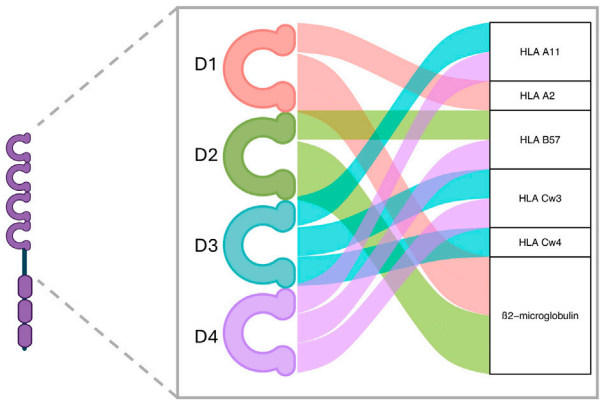

FIGURE 1 | LILRB3's predicted interaction domains with HLA molecules. Based on sequence alignment, several interaction sites with class I HLA are found on LILRB3 proteins. Domains 1 and 2 are predicted to mainly link the β2-microglobulin HLA class I invariant chain, whereas domains 3 and 4 are predicted to link several polymorphic HLA class I α-chains. Created with ggalluvial R package and BioRender.com.

T-cell and macrophages activation [32]. In addition, ANGPTL increases expression of TGF-ß and macrophage recruitment, leading to the development and progression of fibrosis in chronic kidney disease [33]. More recently, Galectin 4 and 7 were shown to bind and activate LILRB3 on the surface of myeloid cells and induce an immunosuppressive phenotype [21]. It's a safe bet that the future will bring the discovery of other LILRB3 ligands, enriching LILRB3 with new functions, not only in the allograft context.

LILRB3 as a Potential Non-Self Class I HLA Discriminator

To date, no LILRs have been described as potentially interacting with HLA class II molecules. However, with regard to LILRB3's ability to bind HLA class I, the literature is contradictory. Indeed, the earliest reports claimed that LILRB3 was unable to bind HLA [34–37] whereas more recent reports [23, 28] contradict this assertion. These discrepancies could be due to the earlier mentioned LILRB3 various allotypes. *In silico* predictions suggest several potential contact sites for LILRB3 and HLA class I molecules interaction. Of the 4 immunoglobulin domains present in the extracellular part of LILRB3, domains 1 and 2 present binding sites to the invariant HLA class I ß2-microglobulin chain, as well as class I A2 molecule. In addition, domains 3 and 4 appear to have the most interaction sites with HLA I molecules A11, B57, Cw3, Cw4 (**Figure 1**). LILRB3 polymorphism, especially in domains 3 and 4, could be of utmost importance if balancing the affinity of a particular LILRB3 allotype to an allogeneic ligand, and thus determine the activation status of the recipient myeloid cells toward the donor cells.

Sequence Homology With LILRA6

Intriguingly at the protein level, LILRB3 shows a strong homology of its extracellular domain with LILRA6, another polymorphic receptor with opposite functions [18, 26, 38]. As expected, this similarity is also found in the gene sequence encoding the two proteins, their homology making them difficult to distinguish either by transcriptomic approaches or using specific antibodies [26]. Unlike LILRB3, several copies of *LILRA6* gene can be present in the genome [38]. The homology between these two receptors implies that they bind the same proteins but induce opposite downstream signalization (**Figure 2**). In basal conditions, a balance exists between the LILRA6 positive signals and the LILRB3 negative signals. Upon strong binding to some LILRB3 allotypes, a disruption of this balance may happen, driving the cell into a specific phenotype. Overexpression of LILRB3 was mostly found on immunosuppressive cells in inflammatory contexts, supporting the hypothesis that LILRB3 induce anti-inflammatory signals in monocytes and leads to pro-resolutive phenotype [21]. With a greater copy number in addition to its high polymorphism, LILRA6 could outperform LILRB3 signals and lead to a pro-inflammatory phenotype. In the future, this sequence homology will have to be taken into account with the utmost vigilance, so that biological functions can be distinctly attributed to LILRB3 or LILRA6.

Immunologic Synapse Hypothesis Between Recipient-Derived Macrophages and Donor Renal Cells During Graft Rejection

All known or putative interactions between LILRB3/A6 and their potential ligands of interest, leading to monocyte modulation, are summarized in **Figure 2**. In the context of kidney transplantation, the recipient-derived monocyte LILRB3 may interact with soluble C4d, directly with donor class I HLA but also with the Fc fragment of HLA-DSA bound to the surface of donor's renal cells. Other ligands could also be secreted in the synapse for instance ANGPTL, APOE or Galectins, inducing downstream signalization. The ITIM sequences expressed in the LILRB intracellular domain recruits SHP1/2 phosphatases and induce inhibition of central signaling pathways such as PI3K/Akt, Jak/STAT and Pkc dependent pathways. Oppositely LILRA6 intracellular domain interact with ITAM leading to the recruitment of Syk and Src kinases. The recruitment of these proteins activates the same pathways and induce activation, differentiation and proliferation of the monocytes.

DISCUSSION

For many years, monocytes were regarded as mere second-knives, responding to non-specific danger signals and unable to trigger an allogeneic rejection on their own. New data derived from mouse models, have shown the potential of LILRs as key players in alloimmunity, suggesting their involvement in human allograft rejection. LILRs have been investigated in several context as modulators of the innate immune system activation. Yet their specific mechanism of action remains

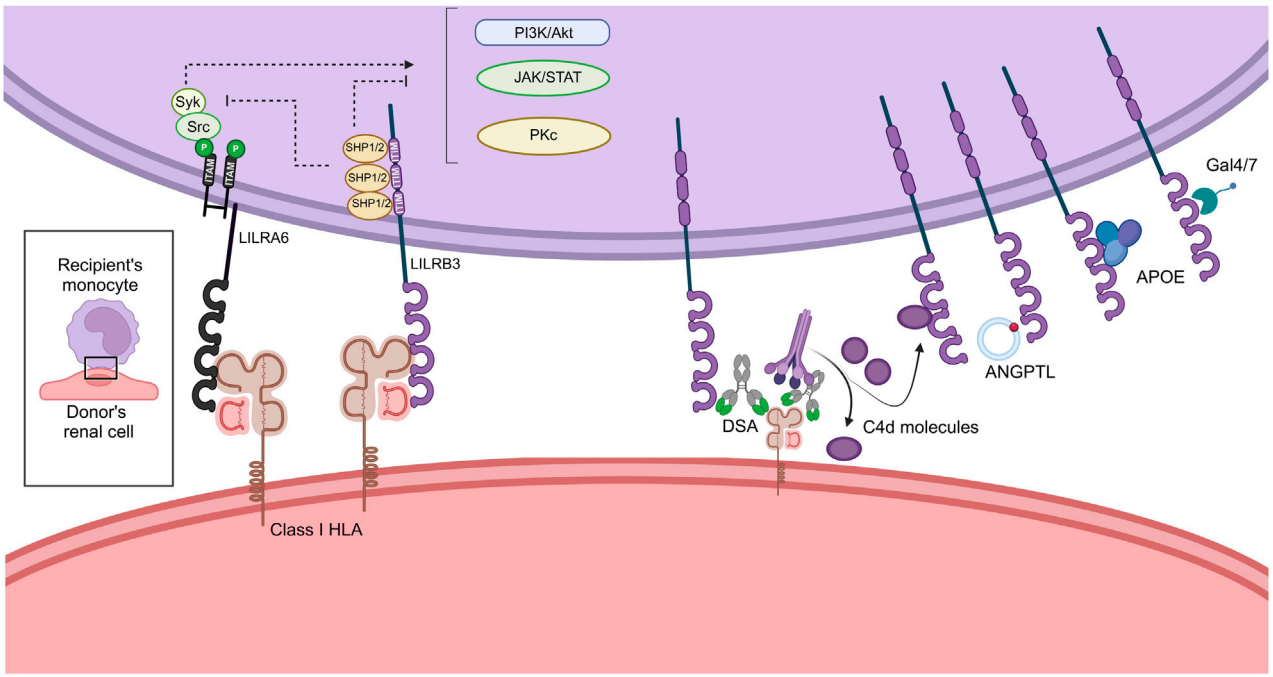

FIGURE 2 | Immunologic synapse hypothesis between the donor's renal cell and the recipient's monocytic cell. ANGPTL, Angiopoietin-Like; APOE, Apolipoprotein-E; PI3K, Phosphoinositide 3 Kinase; DSA, Donor Specific Antibody; HLA, Human Leukocyte Antigen; ITAM, Immunoregulatory Tyrosin-based Activator Motifs; ITIM, Immunoregulatory Tyrosin-based Inhibitory Motifs; LILR, Leukocyte Immunoglobulin Like Receptor. Created with BioRender.com.

unclear. Recent founding proved the importance of LILRB3 in the cancer context, leading to the rapid development of anti-LILRB3 antagonist antibodies and chimeric-antigen receptor T-cells (CAR T-cells) to boost the anti-tumoral response [22]. In the context of transplant rejection, an opposite mechanism is expected, favoring a pro inflammatory phenotype and educating the recipient immune system against the donor's cells. As LILRs high polymorphism drive their ligation capabilities, i.e., their ability to interact with donor cells, genotyping the recipient LILRB3 allotypes could improve our understanding of the monocytes-driven mechanisms of allorecognition. Interestingly, in African American transplant recipients, Sun and colleagues reported a potential association of the polymorphism in the *LILRB3* gene with long-term allograft outcomes, suggesting that this receptor is crucial in the allogeneic context. In fact, this polymorphism could be a genetic risk factor for graft outcome in this population [39]. Furthermore, a disruption in the balance between LILRB and LILRA expression on recipients' monocytes in response to interaction with donor cells may be decisive in modulating the immunological synapse. This monocyte-driven allogeneic response is still at its infancy and many issues remain to be addressed. At first, it would be relevant to investigate whether LILRs expression is modulated on the surface of the recipient monocytes after allograft transplantation, and if the LILRA6/LILRB3 balance is tipped in the event of rejection. *In vitro*, what are the functional consequences for monocytes, if LILR expression is artificially increased or decreased? Can LILRs various allotypes sense the donor-recipient incompatibility? If so, it will be necessary to specify whether the trigger for monocyte activation is the presence of non-self HLA or rather the absence of self HLA ("missing-self") as proposed for NK cells? Research teams will also need to focus on *in vitro* activation of human primary monocytes (a laboratory challenge given the "messiness" of these fragile cells) to better understand the cellular implications of LILR ligation to their various ligands. *In silico* binding predictions will need to be confirmed by experimental data to specify the range of molecules bound by each LILR.

Overall, these discoveries in innate immunity challenges the idea that ABMR rejection phenotype is solely caused by HLA and non-HLA DSA. It is likely that technological advances enabling cell phenotyping on an individual scale, will facilitate these investigations and provide more granularity in the involvement of innate immunity by causing or amplifying allogeneic response. Future developments include the opportunity of new therapeutic targets, whose need is clear to improve ABMR prognosis and long-term graft survival. Given that LILRs activate the PI3K/Akt and NFAT pathways, we can therefore speculate that calcineurin inhibitors or mTOR inhibitors might control monocyte activation in the context of allotransplantation. This field of LILRs activation pathways specific inhibitors is already a reality, with the Syk pathway inhibitor Fostamatinib FDA approved for the treatment of chronic immune thrombocytopenia.

AUTHOR CONTRIBUTIONS

JP, MC, LD, LM, IF, CT, and BL reviewed the literature and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Natural Killer Cell Presence in Antibody-Mediated Rejection

Matthias Diebold[1,2†], Evan A. Farkash[3†], Jenna Barnes[3], Heinz Regele[4], Nicolas Kozakowski[4], Martina Schatzl[1], Katharina A. Mayer[1], Susanne Haindl[1], Hannes Vietzen[5], Luis G. Hidalgo[6], Philip F. Halloran[7], Farsad Eskandary[1‡] and Georg A. Böhmig[1‡]*

[1]Division of Nephrology and Dialysis, Department of Medicine III, Medical University of Vienna, Vienna, Austria, [2]Clinic for Transplantation Immunology and Nephrology, University Hospital Basel, Basel, Switzerland, [3]Department of Pathology, University of Michigan, Ann Arbor, MI, United States, [4]Department of Pathology, Medical University of Vienna, Vienna, Austria, [5]Center for Virology, Medical University of Vienna, Vienna, Austria, [6]Department of Surgery, University of Wisconsin, Madison, WI, United States, [7]Alberta Transplant Applied Genomics Centre, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada

Transcript analyses highlight an important contribution of natural killer (NK) cells to microvascular inflammation (MVI) in antibody-mediated rejection (ABMR), but only few immunohistologic studies have quantified their spatial distribution within graft tissue. This study included 86 kidney transplant recipients who underwent allograft biopsies for a positive donor-specific antibody (DSA) result. NK cells were visualized and quantified within glomeruli and peritubular capillaries (PTC), using immunohistochemistry for CD34 alongside CD16/T-bet double-staining. Staining results were analyzed in relation to histomorphology, microarray analysis utilizing the Molecular Microscope Diagnostic System, functional NK cell genetics, and clinical outcomes. The number of NK cells in glomeruli per mm^2 glomerular area (NK_{glom}) and PTC per mm^2 cortical area (NK_{PTC}) was substantially higher in biopsies with ABMR compared to those without rejection, and correlated with MVI scores (NK_{glom} Spearman's correlation coefficient [SCC] = 0.55, $p < 0.001$, NK_{PTC} 0.69, $p < 0.001$). In parallel, NK cell counts correlated with molecular classifiers reflecting ABMR activity ($ABMR_{prob}$: NK_{glom} 0.59, NK_{PTC} 0.75) and showed a trend towards higher levels in association with high functional *FCGR3A* and *KLRC2* gene variants. Only NK_{PTC} showed a marginally significant association with allograft function and survival. Our immunohistochemical results support the abundance of NK cells in DSA-positive ABMR.

Keywords: natural killer cell, antibody-mediated rejection, microvascular inflammation, immunohistochemistry, genetics

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***Correspondence**
Georg A. Böhmig,
georg.boehmig@meduniwien.ac.at

†These authors share first authorship

‡These authors share last authorship

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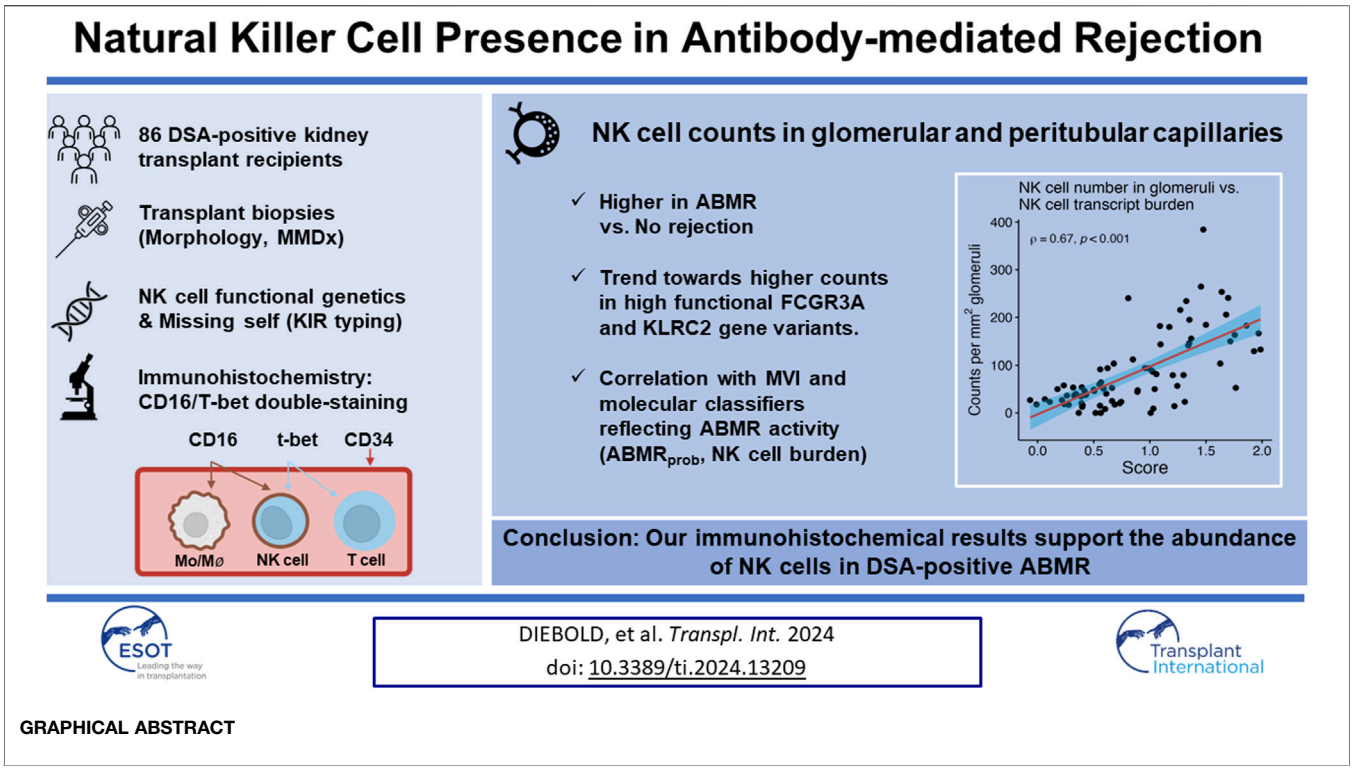
INTRODUCTION

Antibody-mediated rejection (ABMR) is a leading cause of graft failure after kidney transplantation [1]. The current paradigm of the pathogenetic sequence underlying ABMR may involve alloantigen-driven B cell activation and subsequent differentiation into plasma cells producing donor-specific antibodies (DSA) [2, 3]. DSA directed against donor HLA molecules bind to the respective alloantigen expressed on the endothelial surface and may initiate inflammation/injury in the microvasculature, through direct cellular effects, complement activation and/or Fc receptor-mediated activation of innate immune cells, in particular, natural killer (NK) cells and



GRAPHICAL ABSTRACT

macrophages [4–7]. However, this traditional view may not fully account for the diversity of rejection phenotypes [8]. For instance, a significant subset of patients presenting with microvascular inflammation (MVI), the hallmark lesion of ABMR, may not exhibit detectable antibodies [8, 9]. MVI, DSA negative and C4d negative, may be due to high affinity DSA absorbed to the allograft, or, alternatively, could encompass rejection triggered by "missing self" or other genetically determined mechanisms of NK cell activation [8, 10, 11]. Notwithstanding, in MVI, the role of NK cells as effector cells remains paramount, regardless of the initial trigger of immune activation [4, 8, 12–14]. NK cells are commonly defined as $CD3^{-}CD56^{+}NKp46^{+}$, and are further categorized into a variety of subsets, including $CD56^{bright}$ and $CD56^{dim}$ cells [15]. Among these, $CD56^{dim}$ cells, particularly those expressing high levels of CD16, may be the predominant subset involved in ABMR [16, 17].

The current assumption of a critical involvement of NK cells in ABMR is primarily based on the results of molecular studies, including bulk and spatial transcriptomics or single cell sequencing [4, 12–14, 18]. However, immunohistologic studies to visualize the actual extent and compartmental distribution of NK cell infiltrates in rejecting allografts are scarce. Available studies mostly relied on single antigen staining (CD56, CD16, or NKp46), which may impede the unambiguous identification of NK cells [6, 19, 20].

The goal of the present study was to provide a detailed morphologic analysis of the presence of capillary NK cells in a well-characterized cohort of 86 DSA-positive renal allograft recipients using immunohistochemical double staining. To gain a more comprehensive understanding of NK cell-associated injury, immunohistologic results were evaluated in relation to genetic determinants of NK cell functionality, the results of detailed morphologic and molecular biopsy analysis, as well as clinical outcome parameters, including graft function and survival.

MATERIALS AND METHODS

Study Design and Patients

The present study comprised 86 DSA-positive kidney transplant recipients who had been recruited during the cross-sectional screening phase (between October 2013 and February 2015) of a single-center randomized controlled trial assessing the effect of bortezomib in late ABMR (BORTEJECT; ClinicalTrials.gov: NCT01873157) [21]. ABMR screening involved 741 adult recipients with stable allograft function at least 180 days post-transplantation (estimated glomerular filtration rate [eGFR] >20 mL/min per 1.73 m^2). Among 111 DSA-positive recipients 86 underwent renal allograft biopsies [21]. The study was approved by the institutional review board of the Medical University of Vienna and adhered to the principles of the Declaration of Helsinki 2008 and the Declaration of Istanbul. We followed the STROBE guidelines for reporting the study results [22].

Transplant Biopsies

Morphologic Evaluation

As outlined previously [21], we used formalin-fixed and paraffin-embedded tissue sections for histomorphologic and

immunohistochemical evaluation. Eighty-four of the 86 study biopsies underwent additional ultrastructural evaluation. The biopsies were analyzed by two experienced renal transplant pathologists (N.K., H.R.). Following the rules of the 2019 Banff schema [23], rejection phenotypes were categorized according to a combination of histologic, immunohistochemical (C4d), ultrastructural and molecular (molecular ABMR classifier) criteria. MVI was quantified using a sum score of glomerulitis (g) and peritubular capillaritis (ptc). Notably, g or ptc scores could not be ascertained for 5 biopsies, limiting the calculation of MVI scores to 81 biopsies.

The number and distribution of glomerular and peritubular capillary NK cells were visualized applying T-bet (T cells; NK cells) and CD16 (NK cells; monocytes/macrophages) double-staining as well as CD34 staining to highlight layers of endothelial cells (**Figure 1**) [24]. For immunohistochemistry, we used polyclonal rabbit CD34-specifc IgG (1:100/Thermo Fisher Scientific, Waltham, Massachusetts, United States) and an alkaline phosphatase (AP)-labeled goat anti-rabbit IgG polyclonal antibody (1:100/Southern Biotech, Birmingham, Alabama, United States), a mouse anti-T-bet IgG1 monoclonal antibody (1:50/4B10/BioLegend, San Diego, California, United States) and an AP-labeled goat anti-mouse IgG1 polyclonal antibody (1:50/Southern Biotech, Birmingham, Alabama, United States), as well as a mouse CD16-specific IgG2a monoclonal antibody (1:20/2H7/GeneTex, Irvine, California, United States) and a horse radish peroxidase-labeled goat anti-mouse IgG2a polyclonal antibody (1:50/Southern Biotech, Birmingham, Alabama, United States). Stained slides were scanned utilizing an Aperio AT2 scanner and visualized using Aperio ImageScope software (Leica, Wetzlar, Hesse, Germany). NK cell staining results were analyzed independently by two experts blinded to the histologic results. Dual-stained cells in glomeruli were manually quantified, and the glomeruli were counted and manually outlined to quantify the glomerular area using ImageScope (Leica, Wetzlar, Hesse, Germany). The same approach was used to quantify dual-stained cells in the peritubular capillaries (PTC) and to calculate the cortical area. The median of the two calculations was divided by the area of the glomeruli for NK cells in the glomeruli or by the area of the cortex (excluding the area of the glomeruli) for NK cells in the PTC. For two patients (2.3%), biopsy specimens were not adequate for evaluation of NK cells in glomeruli. **Figure 1** depicts representative examples of immunohistological NK cell staining. There was a strong agreement between the measurements of the two evaluators (**Supplementary Figure S1**).

Molecular Analysis

Eighty-three of the 86 study biopsies were subjected to gene expression profiling using the Molecular Microscope Diagnostic System (MMDx) [25]. Employing a reference dataset of 1,679 indication biopsies, specific classifiers indicative of rejection (ABMR, "all rejection") and pathogenesis-associated transcript sets were generated as previously detailed [25].

HLA Antibody Detection

DSA were detected and defined as described previously [21]. Briefly, for HLA antibody detection, LABscreen single-antigen flow-bead assays (One Lambda, Canoga Park, CA) were used. Serum specimens were pretreated with heat inactivation for 30 min at 56°C to mitigate complement-mediated interference. Donor specificity of antibody patterns was analyzed in relation to low- or high-resolution HLA typing for HLA-A, HLA-B, HLA-Cw, HLA-DR, HLA-DQ, and/or HLA-DP. Assay results were reported as mean fluorescence intensity (MFI) of the immunodominant DSA, with an MFI value above 1,000 defined as a positive result.

KIR Typing for Missing Self Calculation and Functional Single Gene Variants

The inhibitory killer cell Ig-like receptors (KIRs) receptors 2DL1, 2DL2, 2DL3, 3DL1, and 3DL2 were genotyped using the Olerup SSP KIR Genotyping Kit (CareDx Inc., Brisbane, CA, United States), with DNA amplified via PCR-SSP and analyzed through electrophoresis on 2% E-Gel™ Agarose Gels as detailed previously [11]. Missing self was defined based on the absence of specific HLA class I molecules corresponding to the educated NK cells' KIR receptors [10, 11]. Due to the absence of donor HLA C typing in records before 2009 and unavailable biobanked donor DNA, missing self could only be assessed for 80 donor/recipient pairs.

All patients were genotyped for polymorphisms in four different single genes known to determine the functionality and phenotypic distribution of NK cells as previously detailed [11]. *FCGR3A*$^{V/F158}$ functional variants (rs396991) determining the affinity of Fc gamma receptor IIIA (FcγRIIIA) were genotyped utilizing the QuantStudio5 real-time polymerase chain reaction (RT-PCR) system (Applied Biosystems, Darmstadt, Germany) alongside the TaqMan SNP Genotyping Assay and TaqMan Genotyping Master Mix [5]. Additionally, *KLRC2*$^{wt/del}$ gene variants, which encode the NKG2C receptor, were genotyped through touchdown techniques [26]. *KLRK1*$^{LNK/HNK}$ variants (rs1049174) associated with NKG2D activity were identified using the TaqMan SNP Genotyping Assay and TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, United States). Lastly, the functionally relevant rs9916629-C/T polymorphism was examined via an in-house TaqMan assay [27].

Statistics

Categorical data are reported as frequencies (percentages) and continuous data as median and interquartile range (IQR), respectively. Group comparisons were conducted using the Mann-Whitney-U-Test for continuous variables and Pearson's chi-square test for categorical variables. Hypothesis testing was two-tailed, with a p-value of less than 0.05 indicating statistical significance. Associations between continuous variables were analyzed using Spearman's rank correlation. For analysis of time-to-event outcomes and calculation of the eGFR slopes, the total number of NK cells was stratified into values above and below the median. Death-censored graft survival was evaluated using Kaplan-Meier method, with group differences assessed using the log-rank test. The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) eGFR slope was

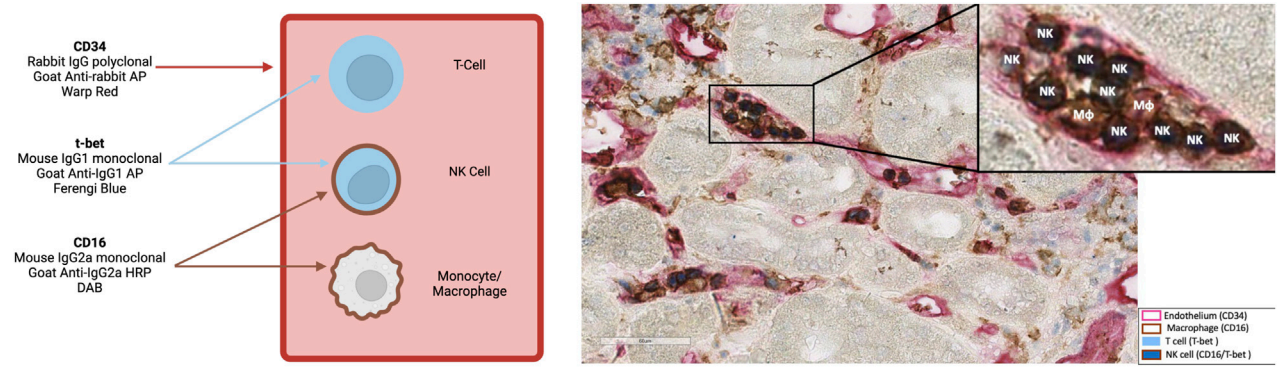


FIGURE 1 | A representative biopsy at 400x showing natural killer (NK) cells within glomerular and peritubular capillaries ("NK"). NK cells were identified through a dual CD16 (membrane, brown) and T-bet (nuclear, blue) staining. Endothelial cells are highlighted via CD34 staining (red). MΦ represent macrophages. Representation was created with BioRender.com.

determined using a linear mixed-effects model. This model incorporated time, the number of NK cells as well as the interaction between the number of NK cells and time, as fixed effects, and random intercepts and slopes through an unstructured covariance matrix. Statistical analyses were performed using R software, version R 4.0.2 (R Core Team 2020. R: a language and environment for statistical computing. R Foundation for Statistical Computing,[1]). A list of the packages used for this analysis is provided as **Supplementary Material**.

RESULTS

Patient Characteristics

The study cohort consisted of 86 renal allograft recipients, all of whom underwent allograft biopsies for a positive DSA screening result. Fifty patients were diagnosed with ABMR according to the Banff schema (active ABMR: n = 15; chronic active ABMR: n = 33; chronic [inactive] ABMR: n = 2), and the median MVI score was 2 (IQR 0–3). Baseline characteristics are provided in **Table 1**. The median recipient age was 47 years (IQR 36–54) and 39 (45.3%) patients were female. Fourteen (16.3%) patients were recipients of a living donor transplant, 25 (29.1%) recipients of a re-transplant, and 26 (30.2%) patients were pre-sensitized and were subjected to desensitization with immunoadsorption and depleting antibody induction at the time of transplantation. The median eGFR at the time of ABMR screening was 54 mL/min/1.73 m^2 (IQR 32–78), with most recipients being on tacrolimus-based maintenance therapy.

NK Cell Presence and ABMR Histomorphology

The number of glomeruli, the glomerular and the cortical area calculated for each sample were comparable between biopsies with and without ABMR (**Table 2**). The median sum of NK cells in glomerular capillaries was significantly higher in patients with ABMR compared to those without ABMR (NK cell number per mm^2 glomerular area: 103, IQR 47 to 180 versus 36, IQR 18 to 49; $p < 0.001$). NK cell counts in glomeruli were numerically highest in active ABMR, and lowest in chronic ABMR (**Supplementary Table S1**). The median number of NK cells in PTC per mm^2 cortical area was also higher in patients with ABMR (24, IQR 11 to 33 versus 3 IQR 2 to 5; $p < 0.001$), particularly in active and chronic active ABMR (**Supplementary Table S1**).

As shown in **Figure 2**, **Supplementary Table S2** and **Supplementary Figure S2**, glomerular NK cell counts were tightly correlated with MVI scores (Spearman's correlation coefficient [SCC] = 0.55, $p < 0.001$) as well as g (SCC = 0.51, $p < 0.001$) and ptc (SCC = 0.48, $p < 0.001$) Banff single lesion scores. Stronger correlations were found between peritubular capillary NK cell counts and MVI scores (SCC = 0.69, $p < 0.001$) as well as g (SCC = 0.54, $p < 0.001$) and ptc (SCC = 0.69, $p < 0.001$) Banff single lesion scores. No such correlations were observed for other single lesions, including tubulitis (t), interstitial infiltrates (i) or lesions reflecting chronic injury in the tubulo-interstitium (ci, ct), arteries (cv) or glomeruli (cg). No significant correlation was found between immunodominant DSA MFI and the number of NK cells in glomeruli (SCC = 0.21, $p = 0.058$) but we observed a weak correlation with NK cell counts in PTC (SCC = 0.32, $p = 0.003$). We did not find any associations with other clinical (eGFR, protein/creatinine ratio) or immunological characteristics (HLA mismatch) (data not shown).

NK Cell Presence and Gene Expression Patterns

NK Cells in Glomeruli

Next, we investigated associations between NK cell counts in glomeruli per mm^2 glomerular area and distinct MMDx-derived molecular scores (**Figure 3**). Strong correlations were found with molecular classifiers that reflect the probability of histologic ABMR ($ABMR_{prob}$; SCC = 0.59, $p < 0.001$), or the probability of a g score >0 (g_{prob}; SCC = 0.64, $p < 0.001$) or a ptc score >0 (ptc_{prob}; SCC = 0.65, $p < 0.001$). Conversely, no significant

[1]https://www.R-project.org

TABLE 1 | Baseline characteristics.

Parameter	No ABMR (n = 36)	ABMR (n = 50)	Total (N = 86)	*p*-value
Variables recorded at transplantation				
Recipient age (yr), median (IQR)	47 (39–54)	48 (35–54)	47 (36–54)	0.575
Female recipient sex, no. (%)	14 (38.9)	25 (50.0)	39 (45.3)	0.423
Donor age[a] (yr), median (IQR)	44 (36–54)	46 (33–58)	46 (36–58)	0.757
Prior kidney transplant, no. (%)	10 (27.8)	15 (30.0)	25 (29.1)	1.000
Living donor, no. (%)	6 (16.7)	8 (16.0)	14 (16.3)	1.000
ABO-incompatible live donor transplant[b], n (%)	1 (2.8)	0 (0.0)	1 (1.2)	0.868
Cold ischemia time[a] (hr), median (IQR)	11 (5–15)	12 (9–18)	12 (9–17)	0.187
HLA mismatch[a] (A, B, DR), median (IQR)	3 (3–4)	3 (2–3)	3 (2–4)	0.053
CDC panel reactivity[a] ≥10%, no. (%)	6 (16.7)	9 (18.0)	15 (17.4)	1.000
Preformed anti-HLA DSA[c], no. (%)	5 (13.9)	20 (40.0)	25 (29.1)	0.009
Induction with antithymocyte globulin, n (%)	6 (16.7)	22 (44.0)	28 (32.6)	0.015
Peritransplant immunoadsorption[d], n (%)	6 (16.7)	20 (40.0)	26 (30.2)	0.037
Variables recorded at the time of ABMR screening				
Time to biopsy (yr), median (IQR)	5 (2–12)	5 (2–13)	5 (2–13)	0.793
Recipient age (yr), median (IQR)	55 (48–63)	55 (43–61)	55 (45–62)	0.581
MFI of peak DSA	1,491 (1,205 to 3,446)	3,878 (2,223 to 10,484)	2,952 (1,485 to 6,781)	<0.001
eGFR (ml/min/1.73 m^2), median (IQR)	58 (40–82)	44 (30–76)	54 (32–78)	0.183
Urinary protein/creatinine ratio (mg/g), median (IQR)	167 (73–270)	258 (86–954)	192 (80–421)	0.052
Triple immunosuppression, no. (%)	27 (75.0)	38 (76.0)	65 (75.6)	1.000
Tacrolimus-based immunosuppression, no. (%)	21 (58.3)	31 (62.0)	52 (60.5)	0.905
Index biopsy results				
Active ABMR, no. (%)	0 (0.0)	15 (30.0)	15 (17.4)	0.001
Chronic active ABMR, no. (%)	0 (0.0)	33 (66.0)	33 (38.4)	<0.001
Chronic inactive ABMR, no. (%)	0 (0.0)	2 (4.0)	2 (2.3)	0.625
Microvascular inflammation (MVI)[e], median (IQR)	0 (0–0)	2 (2–4)	2 (0–3)	<0.001
C4d positivity, no. (%)	2 (5.6)	24 (48.0)	26 (30.2)	<0.001
Banff single lesion scores, median (IQR)				
Glomerulitis (g)	0 (0–0)	1 (1–2)	0 (0–2)	<0.001
Peritubular capillaritis (ptc)	0 (0–0)	2 (1–2)	0 (0–2)	<0.001
Interstitial inflammation (i)	0 (0–0)	0 (0–0)	0 (0–0)	0.704
Tubulitis (t)	0 (0–0)	0 (0–0)	0 (0–0)	0.341
Glomerular basement membrane double contours (cg)	0 (0–0)	1 (0–1)	0 (0–1)	<0.001
Interstitial fibrosis (ci)	1 (0–1)	1 (1–1)	1 (1–1)	0.028
Tubular atrophy (ct)	1 (0–1)	1 (1–1)	1 (0–1)	0.049
Total inflammation (ti)	1 (0–1)	1 (0–1)	1 (0–1)	0.735
Vascular fibrous intimal thickening (cv)[f]	1 (0–1)	1 (0–1)	1 (0–1)	0.854
Interstitial fibrosis and tubular atrophy (IFTA)	2 (0–4)	3 (2–4)	3 (1–4)	0.093

ABMR, antibody-mediated rejection; CDC, complement-dependent cytotoxicity; DSA, donor-specific antibody; eGFR, estimated glomerular filtration Rate; HLA, human leukocyte antigen; IQR, interquartile range; MFI, mean fluorescence intensity.

[a]Donor age, cold ischemia time, HLA, mismatch and CDC, panel reactivity were not recorded for 3, 5, 1, and 5 recipients, respectively.

[b]This patient underwent desensitization for ABO (AB, donor to O recipient) plus HLA, antibody (DSA+) barriers.

[c]Pretransplant single-antigen testing was available for 42 patients (solid-phase HLA, antibody screening on the wait list according to our local standard implemented in July 2009).

[d]Pre-sensitized patients (until 2009: ≥40% CDC, panel reactivity; since 2009: preformed DSA) were subjected to a protocol of peritransplant immunoadsorption as earlier detailed [35].

[e]g and/or ptc single lesions scores could not be ascertained for 5 biopsies, limiting the calculation of MVI, scores to 81 biopsies.

[f]The vascular fibrous intimal thickening score was not documented for two recipients in the bortezomib group and five recipients in the placebo group due to inadequate biopsy material for complete lesion scoring.

TABLE 2 | Intracapillary NK cell counts in relation to ABMR diagnosis.

Variables	No ABMR (n = 36)	ABMR (n = 50)	Total (N = 86)	*p*-value
Cortical area, mm^2 (IQR)	5 (3–7)	4 (3–6)	4 (3–7)	0.353
Glomerular area, mm^2 (IQR)	0.16 (0.11–0.31)	0.15 (0.08–0.26)	0.15 (0.10–0.26)	0.255
Number of glomeruli, median (IQR)	7 (4–12)	6 (4–10)	7 (4–10)	0.231
NK cells in glomeruli[a] per mm^2 glomerular area, median (IQR)	36 (18–49)	103 (47–180)	52 (25–130)	<0.001
NK cells in PTC per mm^2 cortical area, median (IQR)	3 (2–5)	24 (11–33)	10 (3–27)	<0.001

ABMR, antibody-mediated rejection; NK cell, natural killer cell.

[a]Sufficient material for calculation of glomerular and total NK, cell counts was available for 84/86 patients.

correlations were observed with the molecular classifier for TCMR diagnosis probability ($TCMR_{prob}$; SCC = 0.05, p = 0.659) even after excluding an outlier (SCC = 0.14, p = 0.202).

Among analyzed pathogenesis-based transcripts (PBT) sets, the strongest correlation was found for a PBT set reflecting the NK cell transcript burden (NKB; SCC = 0.67, $p < 0.001$)

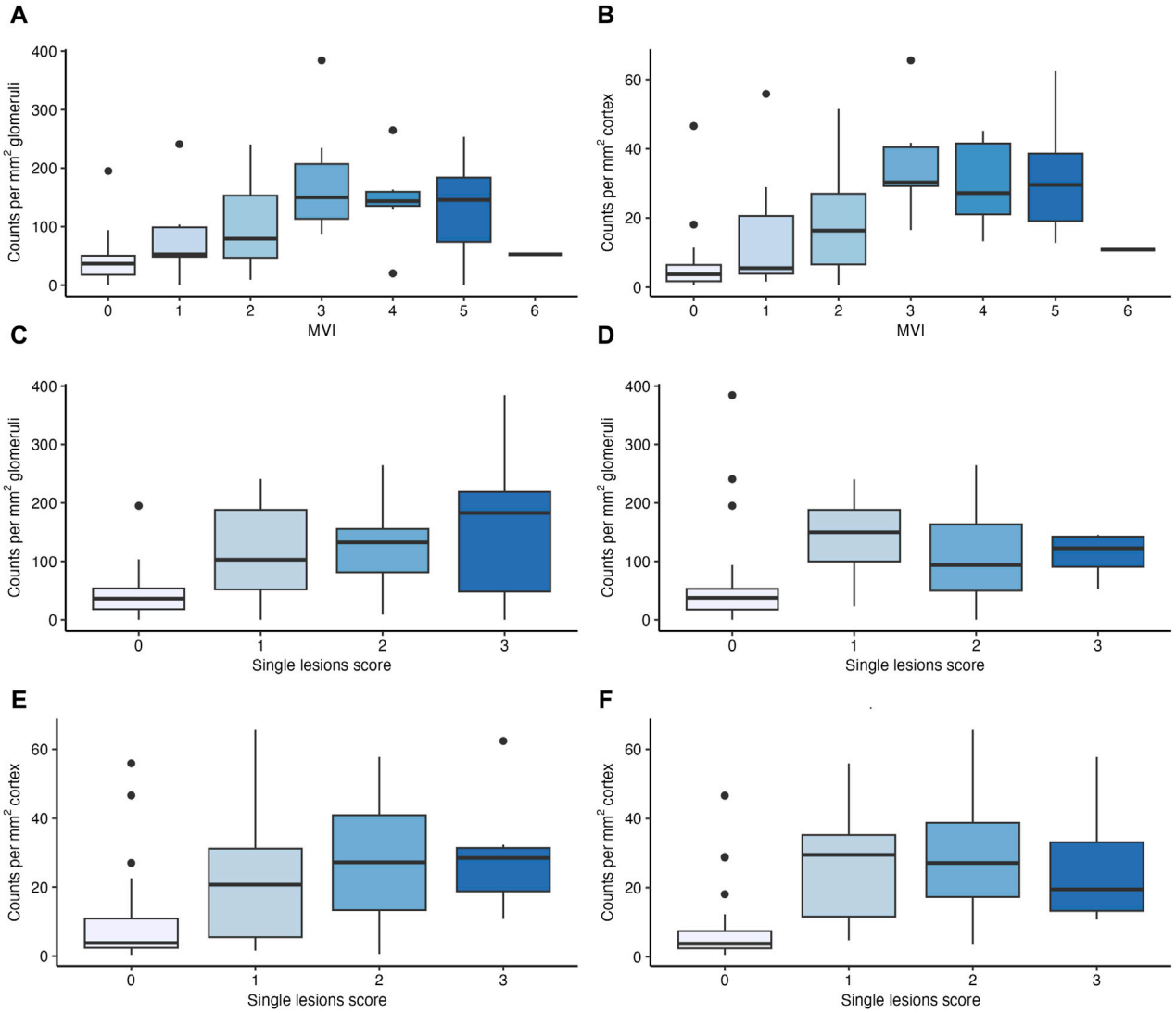


FIGURE 2 | Number of natural killer (NK) cells in relation to features of microvascular inflammation (MVI). Boxplots depicting the number of NK cells in glomeruli and peritubular capillaries (PTC) in relation to MVI scores **(A,B)**, to glomerulitis (g) scores **(C,E)** and peritubular capillaritis (ptc) **(D,F)** scores.

(**Figure 4**). Weaker correlations were observed for a DSA selective (DSAST; SCC = 0.59, $p < 0.001$) and interferon gamma-inducible PBT (GRIT, SCC = 0.49, $p < 0.001$). The correlation with the T cell burden (TCB) was only moderate (SCC = 0.25, $p = 0.022$). The correlations with all rejection' (Rej_{prob}; SCC = 0.63, $p < 0.001$), cg score >0 (cg_{prob}; SCC = 0.42, $p < 0.001$) and injury-repair response associated PBT (IRRAT; SCC = 0.15, $p = 0.145$) are shown in **Supplementary Figure S3**.

NK Cells in Peritubular Capillaries

In PTC, stronger correlations were found between NK cells per mm^2 cortical area and the three ABMR-related classifiers: $ABMR_{prob}$ (SCC = 0.75, $p < 0.001$), g_{prob} (SCC = 0.77, $p < 0.001$) and ptc_{prob} (SCC = 0.78, $p < 0.001$) (**Figure 3**). The PBT sets related to ABMR also showed strong correlations, with the strongest correlation for NKB (SCC = 0.77, $p < 0.001$). DSAST (SCC = 0.74, $p < 0.001$) and GRIT (SCC = 0.55, $p < 0.001$) showed strong correlations as well (**Figure 4**). TCB showed only a weak but significant correlation (SCC = 0.39, $p < 0.001$). A weak correlation was also noted with IRRAT (SCC = 0.34, $p < 0.001$). **Supplementary Figure S3** shows the correlations with "all rejection" probability (Rej_{prob}; SCC = 0.76, $p < 0.001$) and cg score >0 (cg_{prob}; SCC = 0.55, $p < 0.001$).

Molecular Archetypes Clusters

Investigating molecular rejection archetype clusters, the number of NK cells in glomeruli and in PTC was highest in early-stage ABMR (EABMR), followed by fully developed ABMR (FABMR),

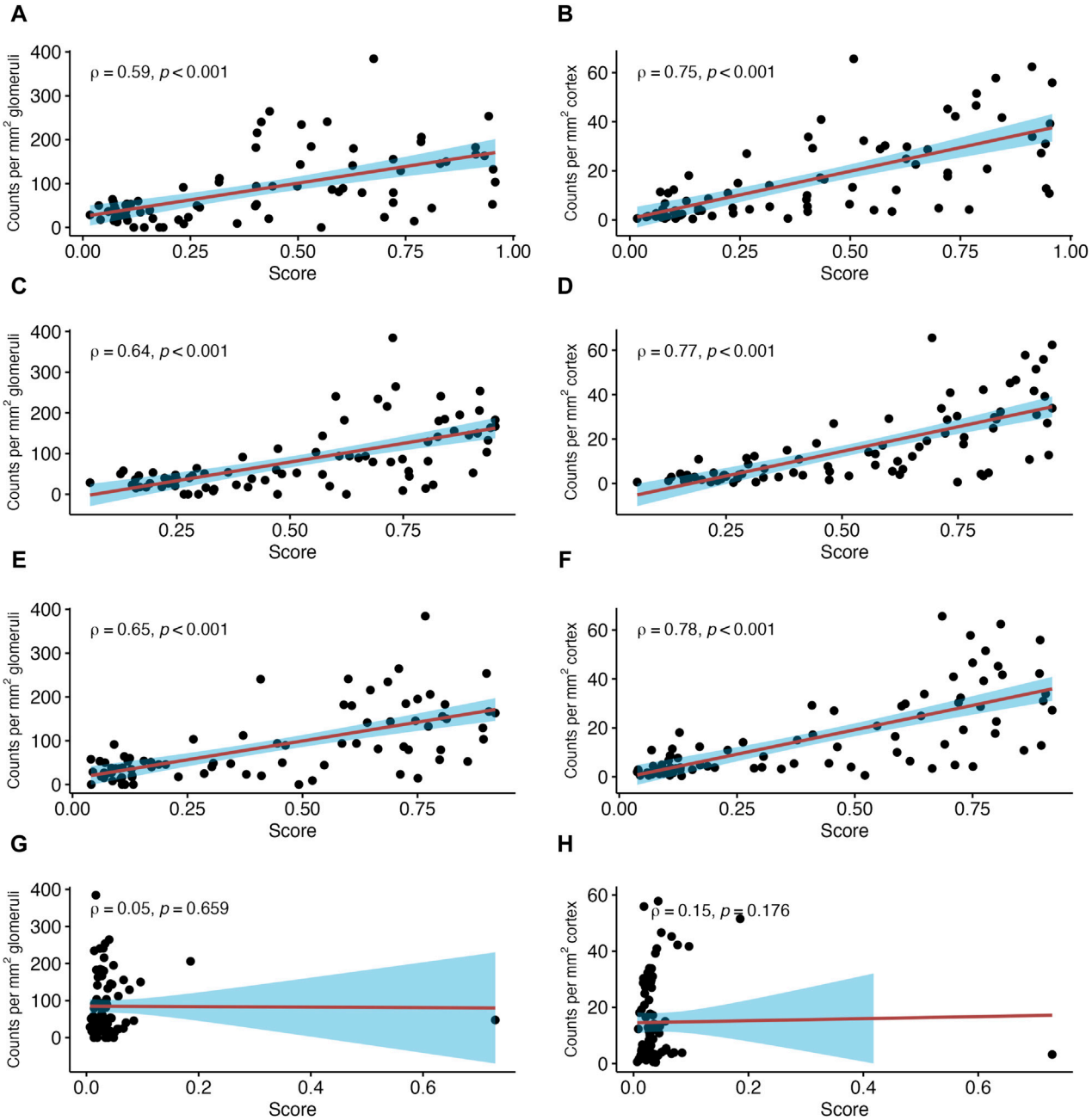


FIGURE 3 | Number of natural killer (NK) cells in relation to antibody-mediated rejection (ABMR)-related molecular classifier scores. Shown are correlations between NK cell counts in glomeruli and peritubular capillaries (PTC) per mm2 glomerular/cortical area and Molecular Microscope Diagnostic System (MMDx)-derived scores reflecting antibody-mediated rejection. ABMR activity ($ABMR_{prob}$) **(A,B)**, probability of lesion scores for glomerulitis >0 (g_{prob}) **(C,D)**, probability of peritubular capillaritis >0 (ptc_{prob}) **(E,F)** and TCMR classifier ($TCMR_{prob}$) **(G,H)**. Red lines indicate linear regression with the 95% confidence interval shown in blue. ρ denotes the Spearman's correlation coefficient.

late-stage ABMR (LABMR) and no rejection as well as TCMR (both $p < 0.001$) (**Supplementary Table S3**).

NK Cell Presence and Functional NK Cell Genetics

Next, we analyzed the association between immunohistochemical results and a set of functional NK cell gene polymorphisms, known to influence the number and functionality of NK cells, as well as missing self. **Supplementary Table S3** shows the median number of NK cells stratified by the degree of "missing self," calculated on the basis of KIR receptor and HLA polymorphisms, as well as individual functional NK cell gene variants, such as $FCGR3A^{V/F158}$, $KLRC2^{wt/del}$, $KLRK1^{LNK/HNK}$ and rs9916629-C/T.

The number of NK cell counts in glomeruli and PTC turned out to be numerically higher in patients with high functional variants *KLRC2*$^{wt/wt}$ and *FCGR3A*$^{V/F158}$, without reaching the conventional boundaries for statistical significance (**Supplementary Table S4**). Only NK cell counts in PTC among patients with *KLRC2*$^{wt/wt}$ were significantly higher. For the other variants, including missing self, no such associations were observed.

Number of NK Cells and Transplant Outcomes

The diagnosis of ABMR was significantly associated with inferior death-censored graft survival (**Figure 5**). Interestingly, the number of NK cells in PTC, but not glomeruli, did have a marginally significant impact on graft survival (p = 0.043). Nevertheless, neither the number of NK cells in glomeruli nor the number of NK cells in PTC did impact the yearly eGFR decline (**Supplementary Table S5**).

DISCUSSION

A major finding of the present study was that, among a subset of patients diagnosed with ABMR, there was a marked elevation of NK cell counts in glomerular and peritubular capillaries. This increase in NK cell counts was strongly correlated with distinct ABMR-related Banff single lesion scores and the extent of MVI, but not TCMR-related Banff single lesion scores. Additionally, we found a significant correlation between the presence of NK cells and molecular classifiers and transcript sets related to ABMR activity, NK cell burden or DSA effects, while no or only weak correlations were observed with classifiers related to TCMR. Notably, our results extended to functional polymorphisms in *KLRC2* and *FCGR3A* genes, which regulate the abundance and function of NKG2C$^+$ NK cells and determine the binding affinity of the FcγRIIIA receptor, respectively. However, despite associations with features of rejection, only a marginal association was found between the number of NK cells in PTC and inferior graft survival.

Several studies have underscored the pivotal role of NK cells in ABMR, primarily inferred from elevated NK cell-dependent transcripts in biopsies from patients with ABMR [12, 14, 18]. Nevertheless, there is a paucity of morphological studies visualizing NK cells in allograft biopsies. Existing data largely rely on single-marker staining (CD16, CD56, or NKp46), which does not allow for definitive identification of NK cells [6, 14, 16, 20, 28, 29]. Kildey et al. [16] successfully identified NK cells among lymphocytes from digested biopsy samples using flow cytometry; however, the methodology precluded analyses of spatial distribution. Our study supports and expands upon these findings by employing a double-staining technique for a more precise identification of NK cell infiltrates. The reliability of our staining technique was supported by the strong correlation observed between our immunohistochemical results and a pathogenesis-based transcript set reflecting the NK cell burden. In this regard, our study extends the original work by Hidalgo et al. [12], where only CD56 as a sole marker was used for the histological detection of NK cells.

NK cells may contribute to rejection through various mechanisms, including antibody-dependent cellular cytotoxicity (ADCC) or direct lysis, resulting from increased genetically determined activation or a lack of inhibition triggered by missing self [8, 17]. ADCC, for instance, may involve the binding of FcγRIIIA to the Fc portions of alloantigen-bound antibodies (e.g., HLA or non-HLA). A pivotal role of FcγRIIIA-positive effector cells in rejection was suggested by a recently published study employing innovative technologies to dissect the involvement of distinct cellular components, including single cell sequencing, spatial transcriptomics or multifluorescent staining [18]. Our previous work has shown that a polymorphism affecting the binding affinity of this receptor is associated with the occurrence and extent of MVI [5, 11]. In the present study, we found that the number of NK cells in transplant capillaries is increased in patients with genotypes including the high-affinity *FCGR3A*^{V158} allele, although not statistically significant. However, we found an association with a deletion polymorphism in the *KLRC2* gene, which not only influences the expression of the NKG2C receptor but also shifts the entire NK subset towards a higher proportion of NKG2C$^+$ NK cells. A potential relevance of this polymorphism, as suggested by its association with MVI, was discussed in previous studies [11, 26]. However, a notable difference was primarily observed in the quantity of NK cells within PTC and the absolute difference was relatively small. Investigating the proportion of NK cells in patients with a homozygous deletion of the *KLRC2* gene would have been of interest, but this aspect could not be explored in our study due to the absence of patients with such a genotype. Other functional polymorphisms had no impact on the number of NK cells. Likewise, no association with the presence of missing self was observed. This finding, however, was not unexpected, as missing self in the same cohort was not associated with the occurrence of MVI [11]. As previously discussed [11], it is possible that in a selected population of patients with circulating DSA, other mechanisms of NK cell activation, such as ADCC, may predominate.

Interestingly, we only found a marginal association between NK cells in PTC and inferior death-censored graft survival, but no association for NK cells in glomeruli. This finding aligns with Yazdani et al. [14], who showed a strong independent effect of NK cell infiltrates on graft survival. The difference of measure of effect could be explained by the different patient's population and the effect of the number of NK cells on graft survival would be higher in a population of kidney transplant recipients not selected by the presence of DSA. However, it is unclear why we did not find an association for NK cells in glomeruli. Due to the limited sample size, we refrained from conducting further subanalyses.

An interesting observation was that NK cells counts were highest in a molecular archetype reflecting early-onset AMR. This was paralleled by higher glomerular capillary NK cell counts in biopsies showing active, as compared to chronic active or chronic

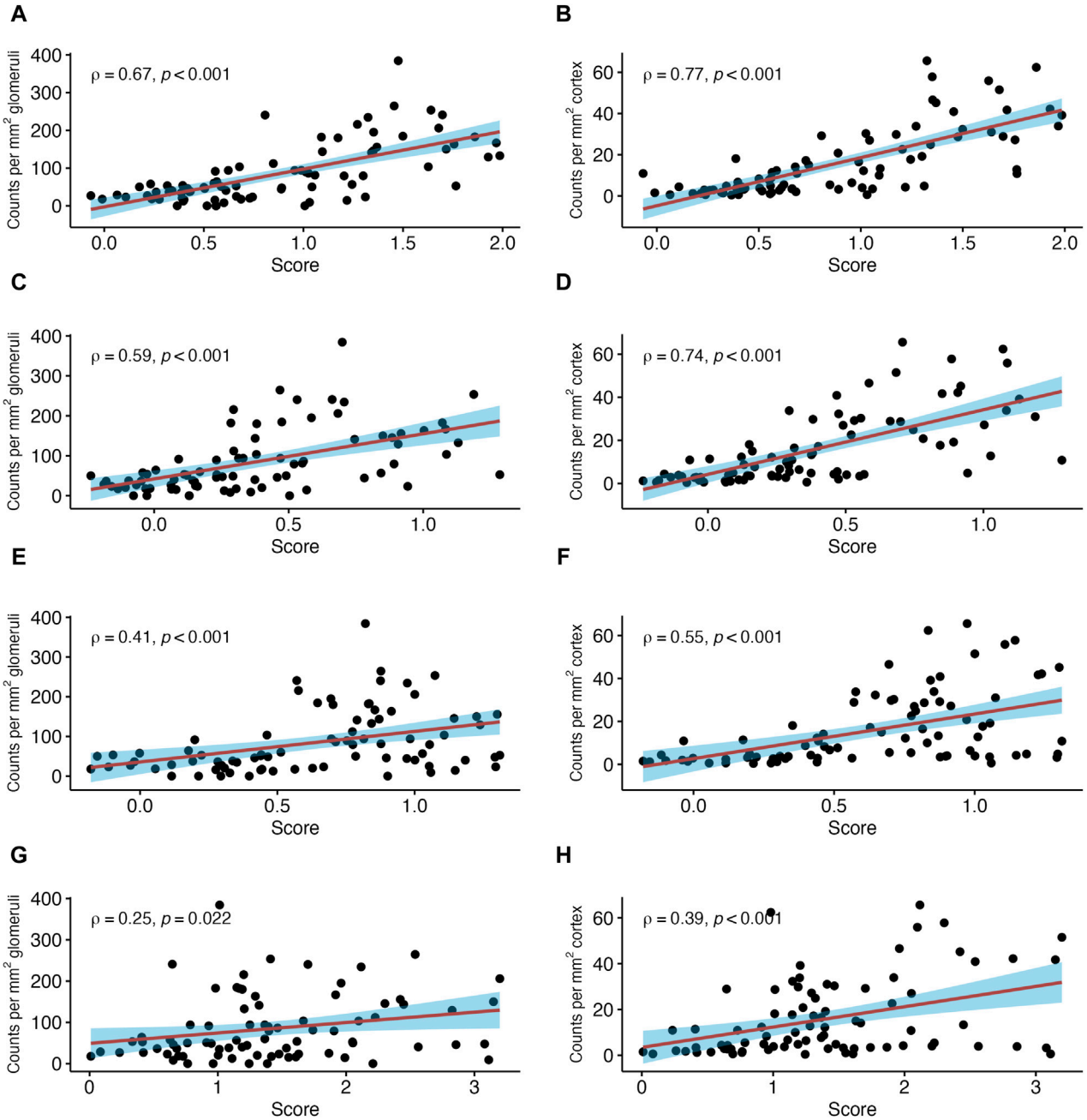


FIGURE 4 | Number of natural killer (NK) cells in relation to selected pathogenesis-based transcript sets (PBT). Shown are correlations between NK cell counts in glomeruli and peritubular capillaries (PTC) per mm2 glomerular/cortical area, respectively, and NK cell transcript burden **(A,B)**, donor-specific antibody (DSA) selective **(C,D)**, gamma inducible (GRIT) **(E,F)** and T cell burden (TCB) transcript sets **(G,H)**. The red line indicates a linear regression line with the 95% confidence interval shown in blue. ρ denotes the Spearman's correlation coefficient.

ABMR. In this respect, our data may be in some contrast to a study by Shah et al. [30], who used pathway enrichment analysis, single-cell RNA-Seq data, BayesPrism and immunohistochemistry (CD56 staining only) to identify NK cells. The authors found a higher NK cell–mediated cytotoxicity pathway and NK cell fractions in immunohistochemistry in chronic ABMR compared to active ABMR. However, in contrast to this study, where all but one ABMR cases were diagnosed within 8 weeks after transplantation, our present analysis included only cases of late ABMR (≥180 days post-transplantation; median 5 years post-transplantation). This may have pathophysiological implications leading to different results. For instance, it was suggested that the primary mechanism by which DSA mediate early ABMR is complement-dependent cytotoxicity, whereas NK cell-dependent ADCC is more prominent in late ABMR [30].

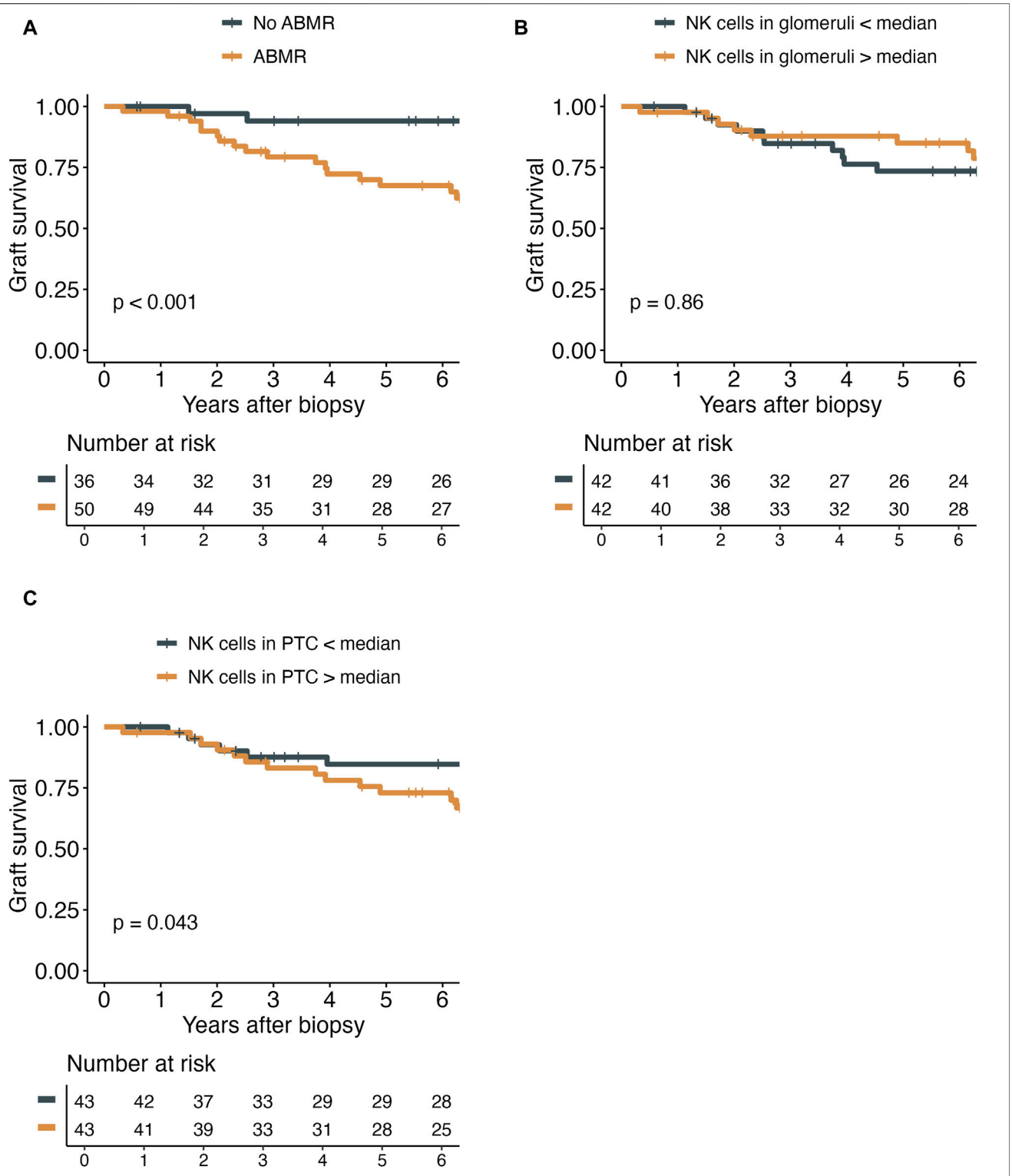


FIGURE 5 | Kaplan-Meier plots demonstrating the death-censored graft survival in relation to antibody-mediated rejection (ABMR) diagnosis **(A)**, the number of NK cells in glomeruli (g) per mm^2 of glomerular area **(B)**, and the number of NK cells in peritubular capillaries (PTC) per mm^2 cortical area **(C)**.

Our analysis offers several advantages, including the well-characterized patient population which allowed for a comprehensive analysis of immunohistochemical results with a set of different histological and molecular markers related to rejection, as well as genetic polymorphisms that may determine NK cell functionality and distribution. However, it is important to

acknowledge some remaining limitations. First, the patient population consists of a selected cohort of DSA-positive transplant recipients. While this facilitated accurate characterization, it may have reduced the generalizability and, due to the low number of patients, also diminished the power to recognize subtle differences. Our in-depth analysis of the BORTEJECT screening cohort allowed us to dissect the abundance of NK cells only in a very specific context, that is, DSA-positive ABMR. Other MVI variants (e.g., MVI, DSA negative and C4d negative) or TCMR and mixed rejection cases were not represented in our cohort, which precluded the evaluation of a potential role of NK cells in a broader sense. Secondly, it is crucial to recognize that correlations observed in the study do not imply causation. Although the role of NK cells in the pathogenesis of microvascular inflammation is increasingly understood and a relevant role seems very likely, this analysis only shows correlations. Further validations are necessary to strengthen the evidence and establish a causal relationship definitively. Specifically, in this analysis we were only able to morphologically describe the number of capillary NK cells. Whether they act as the primary effectors causing tissue injury or whether they are simply bystander cells cannot be deduced from this analysis. Further research with larger sample sizes and functional assays may be warranted to elucidate the specific role of NK cells in the context of allograft rejection. Notably, in a recently published phase 2 trial evaluating the CD38 antibody felzartamab in late active and chronic active ABMR, we demonstrated a marked reduction in MVI scores alongside a reduction in peripheral NK cell counts and donor-derived cell-free DNA release [31]. The results of this trial may provide further evidence that NK cells could play a role as effector cells promoting graft injury, and may be in line with previous experimental studies that have demonstrated that NK cell depletion can ameliorate rejection processes [4, 32]. One concern may be that our approach of immunohistochemical double-staining, which relies on the detection of CD16-and CD56-positive cells, may not reliably detect $CD56^{bright}CD16^{dim}$ cells. While this may have led to an underestimation of total NK cell counts, this distinct subset is most likely not contributing to NK cell-dependent ADCC [15]. However, it is important to note that NK cells are able to shed their CD16 receptor upon lysis of target cells to disassemble the NK cell immune synapse, which allows them to target several other cells in a row [33, 34]. Because of this phenomenon, it could be that some effector cells have escaped immunohistochemical detection in our study biopsies.

In conclusion, our study demonstrated an increased number of NK cells in the glomeruli and PTC, among patients with DSA-positive MVI. The quantity of NK cells showed correlations with histological markers of MVI, as well as with ABMR-related molecular classifiers and pathogenesis-based transcript sets. Polymorphisms in the *FCGR3A* and *KLRC2* genes, known to affect NK cell functionality, were found to correlate with the number of NK cells. However, despite these associations, only the number of NK cells within PTC was prognostic for transplant survival in our analysis. Further research, including analyses of unselected transplant cohorts, is needed to clarify the precise role of NK cells in allograft rejection and transplant outcomes.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The datasets presented in this article are not readily available because public sharing of individual participant data was not included in the informed consent of the BORTEJECT trial. Data can be made available to interested researchers upon reasonable request by mailing to georg.boehmig@meduniwien.ac.at. Requests to access these datasets should be directed to georg.boehmig@meduniwien.ac.at.

ETHICS STATEMENT

The studies involving humans were approved by the Medical University of Vienna; approval: August 2012; no. 1515/2012. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EF and JB conducted the experiments, MD, FE and GB conducted the analysis and wrote the first draft of the paper. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13209/full#supplementary-material

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Editorial Office

Avenue du Tribunal Fédéral 34
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