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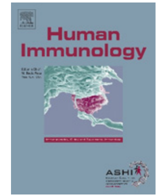

Major histocompatibility complex class I–related chain A allele mismatching, antibodies, and rejection in renal transplantation

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Even when kidney allografts are well matched for human leukocyte antigen (HLA) and anti-HLA antibodies are not detected, graft rejection can still occur. There is evidence that some patients who lose their graft have antibodies specific for major histocompatibility complex (MHC) class I–related chain A (MICA) antigens. We investigated whether mismatching MICA alleles associates with MICA antibody production and graft rejection or dysfunction. MICA and HLA antibody screening in 442 recipients was performed, and specificities were confirmed in a subgroup of 227 recipients using single-antigen multiplex technology. For assignment of MICA antibody specificity, we used three independent assays. In addition, MICA alleles of 227 recipients and donors were determined by DNA sequencing. In all, 17 patients (7.5%) had MICA antibodies, and 13 patients (6%) developed MICA donor-specific antibodies (DSA). Multivariate analysis revealed MICA mismatching, as an independent significant factor associated with the presence of MICA antibodies ($p = 0.009$), and 14 mismatched MICA residues significantly correlated with MICA antibody production. MICA and HLA antibodies significantly associated with acute rejection (AR) and MICA DSA and HLA DSA correlated with decreased graft function by univariate and multivariate analysis. We conclude that mismatching for MICA epitopes in renal transplantation is a mechanism leading to production of MICA antibodies that associate with AR and graft dysfunction.

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1. Introduction

Renal transplantation is the treatment of choice for patients with end-stage renal disease, facilitating a return to near normal health and extending life expectancy. Improvements in immunosuppressive therapy, aimed at limiting the effects of T-cell–mediated immune responses to the graft, have increased overall graft survival [1] and reduced acute rejection (AR). However, rejection caused by antibody-mediated graft damage arising from B-cell responses to mismatched human leukocyte antigen (HLA) antigens remains a problem. The production of post-transplantation, *de novo* donor-specific antibodies (DSA) to HLA antigens is associated with acute and chronic allograft rejection [2,3]. Despite renal transplant rejection being strongly associated with HLA antibodies, some 11–20% of patients without HLA antibodies develop chronic allograft dysfunction [3,4]. Furthermore, hyperacute rejection can occur in the absence of HLA antibodies, thus implicating other non-HLA alloantigens [5,6] including the major histocompatibility complex (MHC) class I–related chain A (MICA) and MHC class I–related chain B (MICB) antigens also encoded by genes within the human MHC [7]. Both MICA and MICB are highly polymorphic, and we have previously developed strategies for allele identification [8–11] and contributed to the elucidation and analysis of genetic variability at these loci [11–14].

MICA expression is limited to monocytes, keratinocytes, dendritic cells, epithelial and endothelial cells, and fibroblasts [15], as well as activated $CD4^+$ and $CD8^+$ T cells [16]. MICA can be up-regulated on tumors and infected cells as a marker of stress [17,18] and recognized by NK cells using the NKG2D lectin-like receptor [19]. MICA and MICB expression and the activation of complement by MIC-specific antibodies have been described in kidney and heart allografts [20–24]. Meta-analyses of MICA antibodies in renal transplant recipients have established correlations with poor graft outcome [25], confirming previous study findings [26–28]; and kidney recipients with pretransplantation MICA antibodies were found to have decreased graft survival, even when well matched for HLA antigens and in the absence of HLA antibodies [23]. These

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observations render MICA proteins as potential antigens for allogeneic immune recognition in transplantation.

The mechanisms by which individuals develop antibodies to MICA are largely unknown. It has been suggested that pregnancy can induce MICA antibodies [20]. By contrast, the role of blood transfusions in the induction of MICA antibodies is unclear [23,29]. However, the effect of MICA mismatching in organ transplantation has not been fully addressed, largely because of a paucity of information on donor and recipient MICA types. In this study, we have addressed these issues by typing 227 renal transplant donor and recipient pairs by direct DNA sequencing. We have also characterized MICA antibody profiles in transplant recipients using multiple sets of recombinant MICA proteins coupled to polystyrene microbeads in three independent assays to confirm the presence of MICA antibodies. Finally, we analyzed estimated glomerular filtration rate (eGFR) in recipients to evaluate whether the presence of MICA and HLA antibodies is associated with chronic graft dysfunction.

2. Subjects and methods

2.1. Subjects

Our single-center cohort study included 442 renal transplant recipients, comprising 386 primary and 56 retransplantations performed at The Royal Free Hospital (London, UK). Recipients included in this study were screened for HLA and MICA antibodies using LABScreen Mixed assay (LSM12, One Lambda, Inc., Canoga Park, CA), during 2007 and 2008, and all patients with DNA available for the recipient and donor were recruited to the study. MICA allele typing was performed on 227 of the above recipients and their donors transplanted between 2004 and 2008. Biopsies were performed in 391 of the larger cohort of 442 recipients and mean follow-up was 5.9 years. Maintenance immunosuppression for graft recipients included cyclosporine A or tacrolimus, mycophenolic acid or azathioprine and prednisolone. In addition, MICA antibody profiles were measured in 116 normal, healthy, unrelated control subjects.

2.2. Ethical considerations

All renal patients included in this study provided written consent for diagnostic testing for the presence of alloantibodies that may be a contraindication to transplantation. The analysis of data in this study did not require any additional patient samples or consent. Sera and DNA from normal healthy volunteers to the Anthony Nolan hematopoietic stem cell donor register were anonymized and used for quality control validation of the methods as approved by the Anthony Nolan UK Medical and Scientific Advisory Committee in 2008.

2.3. MICA and HLA antibody detection

Pre- and post-transplantation screening of patients was performed for HLA and MICA antibodies using LABScreen Mixed assay (LSM12; One Lambda, Inc., Canoga Park, CA). Mean time of testing for antibodies was 7 months post-transplantation. MICA antibodies were confirmed using three different methods: MICA LABScreen MICA single antigen kit (One Lambda, Inc., CA), Lifecodes LSA MICA kit (Gen-Probe Incorporated, San Diego, CA), and an "in-house" bead-based MICA antibody detection assay performed at the Southwestern Medical Center (University of Texas, Dallas, TX), as previously described [24,30]. All three methods identify MICA IgG antibodies directed against MICA*001, *002, *004, *007, *008, *009, *012, *017, *018, *019, and *027. Recommended mean fluorescence intensity (MFI) values for each method were used as the threshold for antibody positivity. MICA antibody specificities were considered positive if confirmed by at least two methods. We chose to use a consensus of three Luminex assays because of unusual reactions observed with one of the commercial assays, that were not confirmed by the other two assays as previously reported [31]. DSA against MICA were identified if any of the antibodies were directed against the known MICA mismatches of the donor.

HLA antibody specificity was confirmed after positive screening among the 227-patient cohort, with the same serum sample used to detect MICA antibodies. Single antigen bead testing for HLA antibodies was performed using LABScreen HLA Class I—Combi and HLA Class II — Group 1(LS1A04 and LS2A01; One Lambda, Inc., Canoga Park, CA). These beads detect antibody specificities against HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1. DSA was defined as a positive reaction, above the empirically determined MFI cut-off of 2000, with antigens expressed by the donor.

2.4. MICA and HLA allele typing

MICA allele profiles were determined by sequence-based typing (SBT) of exons 2–4, using Assign analysis software (Connexio Genomics, Fremantle, Australia), as previously described [11]. The GCT triplet number was determined by separate analysis of exon 5 sequences [32]. Unambiguous MICA allele profiles were obtained with the exception of MICA*009:01 and *049, which differ by one nucleotide in exon 6. Assessment of MICA amino acid residue mismatches was performed by alignment of predicted protein sequences of alleles detected in organ recipient and donor pairs, including mismatches where amino acid residues encoded by donor alleles were not encoded by either of the recipient's alleles. All patients and donors were typed for HLA-A, B, C, DRB1, -3, -4, -5, and DQB1 by PCR-SSP (One Lambda, CA) or PCR-SSO (Luminex, One Lambda, Canoga Park, CA).

2.5. Diagnosis of rejection

AR was diagnosed histologically, using guidelines of the Banff Conference Report, as updated in 2007 [33], and designated type I and type II T cell mediated rejection (for simplicity, referred to here as acute cellular rejection [ACR]) or acute antibody-mediated rejection (AAMR). AAMR with complement deposition was investigated by immunohistological investigation of C4d following Banff guidelines [34] using a rabbit anti-C4d antibody (Biomedica, Vienna, Austria) in an indirect immunoperoxidase method on paraffin sections, following antigen retrieval by heating to 100°C in ethylenediaminetetraacetic acid (EDTA) buffer, pH 8. As in the Banff guidelines [34], significant C4d deposition was defined by immunostaining in the endothelium of at least half of intertubular capillaries. Biopsy samples that showed C4d deposition were not identified separately but were included in either the ACR or AAMR group, depending on associated features.

2.6. Assessment of renal function and chronic renal damage

To investigate associations between MICA antibodies, renal function and clinical course, eGFR was calculated from serum creatinine values (expressed as ml/min/1.73 m^2) according to the four-point MDRD [35]. Measurements of eGFR were taken 1, 2 and 3 years post-transplantation for patients alive and available for follow-up, including 205 of 227 of the MICA-typed pairs. Variables included in the analysis of graft function were the presence of MICA DSA or HLA class I/II DSA, recipient and donor age and gender, deceased or living donor, retransplantation, ACR, and AAMR.

2.7. Statistical analysis

The prevalence of various risk factors, clinical characteristics and immunologic features and the presence or absence of MICA antibodies was compared using Pearson χ^2 test or Fisher's exact test. For odds ratios (OR), 95% confidence intervals (CI) were used. A two-sided p value of less than 0.05 was considered significant. Bonferroni's correction (p_c) for multiple comparisons was applied for analysis of MICA amino acid mismatches between patients and

donors. Corrected p values (p_c) of less than 0.05 were considered highly significant. Data examining age differences, eGFR and follow-up in study cohorts were expressed as mean ± standard deviation, and differences between means were assessed by Student's t test for independent variables. Binary logistic and linear regression was used for multivariate analysis. SPSS 17.0 was used for analysis (SPSS, Inc., Chicago, IL).

3. Results

3.1. Patient demographics

The demographics of the large cohort of 442 renal transplant recipients, stratified for MICA antibody status, are given in Table 1. Univariate analysis revealed associations between MICA antibody production and retransplantation, ACR, MICA allele and residue mismatching, and the presence of HLA antibodies ($p < 0.05$). Analysis of 116 healthy control subjects revealed only one subject with MICA antibodies (0.9%) and five subjects (4%) with HLA class II antibodies (data not shown). Binary logistic regression analysis confirmed that MICA residue mismatching ($p = 0.009$) and retransplantation ($p = 0.011$) were independent significant variables associated with the presence of MICA antibodies.

3.2. MICA and HLA antibodies and acute rejection

A subgroup of 227 transplant recipients and their donors were MICA typed by SBT. In these patients, the co-production of antibodies to HLA and MICA significantly associated with ACR ($p < 0.05$). A trend of association between MICA antibodies alone and ACR was also observed, and further associations between antibodies to HLA class I and II and ACR were identified ($p < 0.05$, Table 2). Analysis of patients with AAMR established strong associations with the presence of antibodies with HLA class I and II ($p < 0.001$), but not MICA. The three recipients with MICA DSA (but not HLA antibodies) detected pretransplantation, as shown in Table 3, have had no ACR or AAMR episodes against their current grafts. Only one recipient with MICA DSA antibodies developed AAMR, with no C4d deposition detected, whereas three of the six recipients (50%) with HLA class I+II DSA and AAMR had significant C4d deposition.

3.3. MICA amino acid residue mismatching

By aligning MICA allele profiles present in the subgroup of 227 renal graft recipients and their respective donors, it was possible to establish the precise position of amino acid mismatches that correlate strongly with MICA antibody production. Figure 1 shows 15 MICA residues mismatched between patient and donor across the $\alpha1$–$\alpha3$ regions (exons 2–4), 14 of which significantly correlated with MICA antibody production. Mismatching at residues 36, 129, 173, 175, 213, and 251 showed the strongest association with MICA antibody production in transplant recipients ($p_c < 0.05$). Residues 91, 125, 156, and 221 were also mismatched between recipients and donors, but were not significantly associated with MICA antibody production (data not shown).

The most common MICA alleles mismatched between recipient and donor had differences at residues 206, 210, and 215, defining the MICA-G1 or MICA-G2 immunodominant motifs in the $\alpha3$ domain, as previously described [30]. The strongest association with residue mismatching was at positions 213 and 251 (Fig. 1), reflect-

Table 1
Patient characteristics and MICA antibody profiles in 442 renal graft recipients

Graft recipient	Total (n = 442)	MICA Ab negative (n = 409)	MICA Ab positive (n = 33)	MICA Ab (%)	Univariate p value	Multivariate OR (95% CI)	Multivariate p value[a]
Gender, no (%)					0.332		
Male	259 (59)	237 (58)	22 (67)	8.5			
Female	183 (41)	172 (42)	11 (33)	6			
Recipient age (y), mean ± SD		42.2 ± 16.0	43.0 ± 16.2		0.179		
Donor age (y), mean ± SD		45.5 ± 16.4	49.0 ± 14.2		0.510		
Time of follow-up (y), mean ± SD		6.7 ± 5.8	7.1 ± 5.8		0.174		
Transplantation, n (%)					<0.001	7.1 (1.6–30.5)	0.011
Retransplantation	56 (13)	45 (11)	11 (33)	20			
Primary transplantation	386 (87)	364 (89)	22 (67)	6			
ACR, n (%)					0.021	1.2 (0.3–4.2)	0.797
Yes	70/391 (18)	61/365 (17)	9/26 (35)	13			
No	321/391 (82)	304/365 (83)	17/26 (65)	5			
AAMR, n (%)					0.421		
Yes	31/391 (8)	30/365 (8)	1/26 (4)	3			
No	360/391 (92)	335/365 (92)	25/26 (96)	7			
Donor status					0.433		
Living	113/417 (27)	107/387 (28)	6/30 (20)	5			
Deceased	304/417 (73)	280/387 (72)	24/30 (80)	7.9			
HLA class I sensitization, n (%)					0.004	0.8 (0.2–4.2)	0.801
Class I antibodies	109 (25)	92 (22)	17 (52)	16			
No class I antibodies	333 (75)	317 (78)	16 (48)	5			
HLA class II sensitization, n (%)					0.007	0.6 (0.1–3.8)	0.622
Class 2 antibodies	85 (19)	71 (17)	14 (42)	16			
No class II antibodies	357 (81)	338 (83)	19 (58)	5			
MICA allele, MM, n/total N (%)					0.006	–	
0	75/227 (33)	75/210 (36)	0/17 (0)	0			
1	113/227 (50)	102/210 (49)	11/17 (65)	10			
2	39/227 (17)	33/210 (15)	6/17 (35)	15			
MICA residue, MM, n/total N (%)					<0.001	16.1 (2.0–131)	0.009
Yes	124/227 (55)	108/210 (51)	16/17 (94)	13			
No	103/227 (45)	102/210 (49)	1/17 (6)	1			
HLA-A+B+DR MM, n/total N (%)					0.376		
0–3	97/227 (43)	88/210 (42)	9/17 (53)	9			
4–6	130/227 (57)	122/210 (58)	8/17 (47)	6			

MICA, major histocompatibility complex class I-related chain A; Ab, antibody; OR, odds ratio; CI, confidence interval; MM, mismatch; ACR, acute cellular rejection; AAMR, acute antibody-mediated rejection.
[a]Binary logistic regression (including covariates in the model where $p \leq 0.100$).

Table 2
Univariate analysis of categories of HLA and MICA antibodies associating with acute cellular and acute antibody-mediated rejection in MICA-typed renal transplant recipients

Antibodies	ACR[a] n (%)		p Value	χ^2	Odds ratio (95% CI)	AAMR[b] n (%)		p Value	χ^2	Odds ratio (95% CI)
	Negative N = 184	Positive N = 41				Negative N = 207	Positive N = 17			
HLA	51 (28)	16 (39)	0.152	—		54 (26)	12 (70)	<0.001	15.0	6.8 (2.3–20.2)
MICA[c]	11 (6)	5 (12)	0.161	—		15 (7)	1 (6)	0.834	—	
MICA DSA	8 (4)	5 (12)	0.051	3.8	3.1 (0.9–9.9)	12 (6)	1 (6)	0.924	—	
HLA and MICA	4 (2)	4 (10)	0.018	5.6	4.9 (1.2–20.3)	7 (3)	1 (6)	0.684	—	
HLA class I	40 (22)	15 (37)	0.045	4.0	2.1 (1.0–4.3)	43 (21)	11 (65)	<0.001	16.6	7.0 (2.5–20.0)
HLA class II	35 (19)	12 (29)	0.144	—		36 (17)	11 (65)	<0.001	21.2	8.7 (3.0–25.0)
HLA class I + II	24 (13)	11 (27)	0.028	4.9	2.4 (1.1–5.5)	25 (12)	10 (59)	<0.001	26.0	10.4 (3.6–29.8)
HLA class I DSA	11 (6)	4 (10)	0.381	—		8 (4)	7 (41)	<0.001	35.0	17.4 (5.3–57.6)
HLA class II DSA	10 (5)	5 (12)	0.117	—		7 (4)	8 (47)	<0.001	48.0	25.4 (7.5–85.5)
Class I + II DSA	6 (3)	2 (5)	0.613	—		2 (1)	6 (35)	<0.001	53.8	56 (10.1–310)

MICA, major histocompatibility complex class I–related chain A; DSA, donor-specific antibody; ACR, acute cellular rejection; AAMR, acute antibody-mediated rejection; CI, confidence interval.
[a] $n = 225$ (two patients had no biopsy details).
[b] $n = 224$ (three patients had no biopsy details).
[c] One of the MICA antibody–positive recipients did not have a biopsy available to diagnose ACR or AAMR.

ing a polymorphism present in MICA*008 and *019 alleles. Analysis of the three-dimensional structure of the MICA molecule revealed that the above mismatched residues would appear accessible to antibodies in the extracellular environment, situated on highly exposed loop regions in the α1 and α2 domains and β-strands and loop regions of the α3-domain (Fig. 2).

3.4. MICA allele mismatching and post-transplantation antibody production

The precise MICA allele types of recipients and donors, together with the MICA antibody profiles of the 17 MICA antibody-positive recipients, are given in Table 3. The majority of these recipients (10 of 17 individuals, 59%) developed *de novo* donor-specific MICA antibodies post-transplantation and another three patients had detectable MICA DSA, but no HLA antibodies, before transplantation. Post-transplantation MICA non DSA (NDSA) were also often detected against related allelic products with common amino acids at specific positions. For example, MICA*002 and *017 were often detected together and have glycine at residue 14. Similarly, MICA*001, *012 and *018 were commonly detected together with all three bead assays, reflecting expression of threonine at residue 24. Broad antibody reactivity, identifying MICA antigens sharing polymorphisms related to the MICA-G1 and MICA-G2 lineages, was also observed (Table 3, patients 3 and 13).

Overall, six of the 17 patients with MICA antibodies had either HLA class I or II NDSA, whereas three patients had HLA class I or II DSA. However, eight MICA-positive recipients (47%) and seven with MICA DSA (41%) had no HLA antibodies.

3.5. Correlating MICA and HLA antibodies with measurable graft function as defined by eGFR

Long-term follow-up of graft function in our 227 MICA-typed recipients revealed that patients with MICA DSA had significantly decreased eGFR 2 and 3 years post-transplantation (Student's *t* test, $p < 0.001$ and $p = 0.038$, respectively), with an equivalent trend apparent in year 1 (Table 4). Significantly reduced eGFR was also

Table 3
Recipient and donor MICA types and MICA antibody profiles detected in renal transplant recipients pre- and post-transplantation

Patient no.	Gender	Donor	Year	Patient MICA type	Donor MICA type	MICA Ab pre-Tx[a]	MICA Ab post-Tx[a]	Re-Tx	HLA class I[b]	HLA class II[b]
1	F	DD	2006	002, 004	008, 00901/049	Negative	008, 019	Yes	No	DSA
2	M	DD	2006	004	001, 00901/049	Negative	001, 007, 012, 018	No	No	No
3	M	1. DD 2. LD	1. 2004 2. 2006	008, 00902	1.002 2.004, 00902	Negative	001, 002, 007, 012, 017, 018	Yes	NDSA	No
4	M	DD	2006	002	002, 008	001, 004, 007, 008, 009, 012, 018, 019	001, 004, 007, 008, 009, 012, 018, 019	Yes	NDSA	NDSA
5	M	DD	2007	002, 01801	011, 016	004, 008, 009, 016[c], 019	004, 008, 009, 016[c], 019	Yes	NDSA	NDSA
6	M	DD	2004	008	004, 008	Negative	004	No	No	No
7	M	DD	2005	011, 016	002, 008	Negative	002, 017	No	No	No
8	F	LD	2005	004, 008	008, 00901/049	001, 002, 007, 017	001, 002, 007, 017	Yes	DSA	No
9	M	LD	2006	010, 011	008, 010	Negative	001, 012, 018	Yes	No	NDSA
10	M	DD	2007	002, 008	008, 00901/049	Negative	004, 009	Yes	DSA	DSA
11	M	DD	2007	008	002, 041	Negative	002, 017	No	No	No
12	M	LD	2007	008	011, 01801	Negative	001, 018	No	NDSA	No
13	M	LD	2008	002	002, 00901/049	Negative	004, 008, 009, 019	No	No	No
14	M	DD	2008	004, 010	008	Negative	001, 012, 018	No	No	No
15	F	DD	2008	008	004, 008	Negative	001, 004, 008[d], 009, 019	No	No	No
16	M	DD	2008	00902	002, 008	Negative	002, 017	No	No	NDSA
17	F	DD	2008	00901/049, 017	008, 01201	001, 012, 018	001, 012, 018	No	No	No

Tx, transplantation; MICA, major histocompatibility complex class I–related chain A; M, male; F, female; DD, deceased donor; LD, living donor; DSA, donor-specific antibody; NDSA, non–donor-specific antibody; Ab, antibody; Tx, transplantation; Re-Tx, retransplantation.
[a] Antibodies to MICA* 027 are not shown (same specificity as MICA* 008).
[b] Confirmed post-transplantation with single antigen Luminex testing for HLA class I/II.
[c] MICA* 016 antibody was detected using the Tepnel Lifecodes Luminex assay but not confirmed.
[d] The MICA* 008 antibody detected in recipient 15 is an autoantibody and this patient has severe psoriasis.

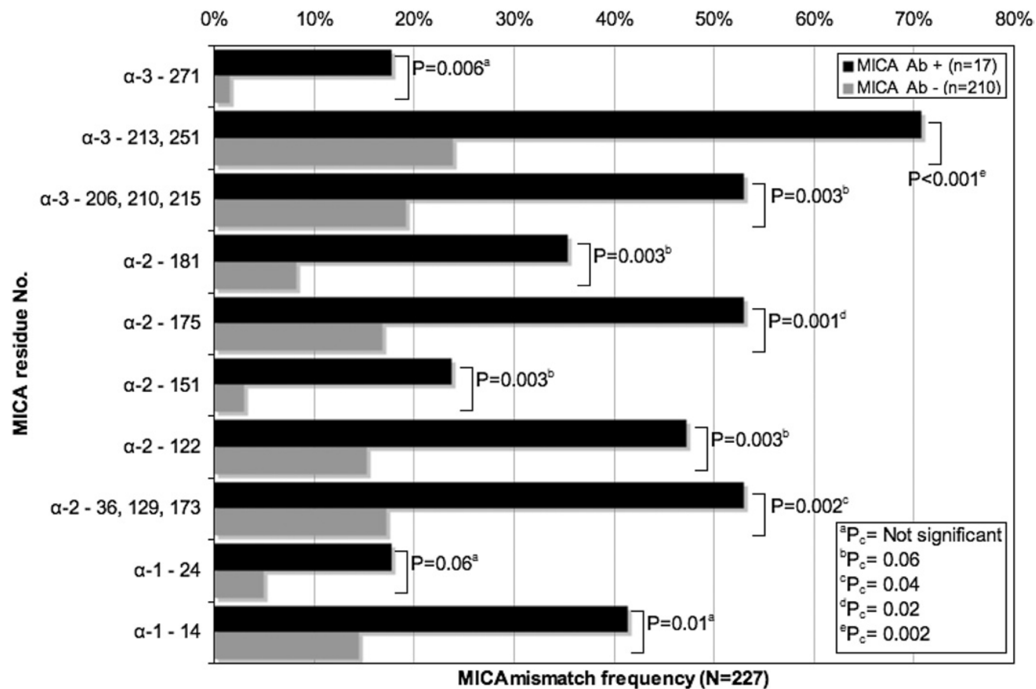

Fig. 1. Histogram comparing the frequency of mismatched MICA amino acid residues in MICA antibody positive ($n = 17$) and MICA antibody negative ($n = 210$) renal graft recipients and donors. Fourteen MICA residues mismatched between patients and donors across the $\alpha1$–$\alpha3$ regions (exons 2–4) significantly correlated with MICA antibody production. A trend for MICA antibody production was associated with mismatching at residue 24 and four additional mismatched residues showed no significant correlation with the presence of MICA antibodies (not shown). The corrected p values (p_c) for 19 comparisons are also indicated.

associated with HLA class I DSA in all 3 years post-transplantation, and the presence of class II DSA correlated with reduced function after 1 year and 3 years (Table 4). Moreover, there was a significant association of graft dysfunction with the presence of MICA DSA alone after 2 years ($p = 0.001$). By contrast, the presence of HLA DSA alone associated with reduced eGFR after 3 years post-transplantation ($p = 0.001$).

3.6. Additional factors significantly associated with graft dysfunction

In addition to DSA, significant univariate associations with reduced graft function were observed with patient and donor gender, age, status of donor (living or deceased), number of transplants, ACR and AAMR (Table 5). For example, female donors were associated with inferior graft function after 1 year ($p = 0.006$) and 2 years ($p = 0.007$) post-transplantation, as did patients more than 50 years of age ($p = 0.02$) after 1 year. However, donors in the higher age group associated with significantly worse graft function at all three time points ($p < 0.001$). Lower eGFR was also detected in transplants from deceased donors at 1 year post-transplantation ($p = 0.021$), whereas patients who had undergone retransplantation had significantly lower eGFR after 3 years ($p = 0.013$). ACR associated with reduced eGFR after 1 year post-transplantation ($p = 0.001$), and this remained significant after years 2 and 3 ($p < 0.001$). By contrast, AAMR was associated with graft dysfunction only after year 1 ($p = 0.001$).

3.7. Multivariate analysis of factors associated with graft dysfunction

Linear regression analysis of variables associated with graft dysfunction for each of the three analysis time points is also shown in Table 5. Variables independently associated with graft dysfunction after 1 year were ACR ($p < 0.001$), donor age greater than 50 years ($p < 0.001$), deceased donor ($p = 0.022$), and female donor ($p = 0.007$), but not MICA DSA or HLA DSA. After 2 years post-transplantation, independent variables associating with graft dysfunction were ACR ($p = 0.001$), donor age greater than 50 years ($p < 0.001$), female donor ($p = 0.026$), HLA class I DSA ($p = 0.05$), and MICA DSA ($p = 0.01$). Finally, our analysis after 3 years revealed ACR ($p < 0.001$), donor age greater than50 years ($p < 0.001$), and HLA class I DSA ($p = 0.046$) as independent variables associated with graft dysfunction.

4. Discussion

Previous meta-analyses of renal transplant cohorts have indicated that MICA antibodies detected both pre- and post-transplantation are associated with poor graft outcome and dysfunction [21,23]. However, information relating to MICA mismatching was unavailable for these and similar studies [23,25–28]. The primary aims of our study were to establish whether mismatching of MICA alleles associates with development of donor-specific MICA antibodies, and whether such antibodies correlate with measurable parameters of graft dysfunction in renal transplant recipients.

By sequencing MICA alleles in a relatively large number of donor and recipient pairs, we have detected significant associations with MICA allele residue mismatching and post-transplantation MICA antibody production, determined using three independent assays (Fig. 1, Table 3). It is likely that MICA mismatching, confirmed by multivariate analysis as an independent significant factor for the presence of MICA antibodies, is a mechanism leading to MICA sensitization. Furthermore, analysis of our larger cohort of 442 recipients indicated that retransplantation significantly associated with the production of MICA antibodies, confirming previous studies [20,22,23,25,27,28]. Thus, as with HLA, MICA antibody production may be a consequence of an alloresponse to a previous graft.

Our analysis of donor–recipient MICA allele combinations at the amino acid level revealed several mismatches correlated with production of MICA antibodies, most corresponding to amino acids involved in recognition by MICA antibodies, as previously defined [30], or predicted by computer analysis [36]. Identification of mul-

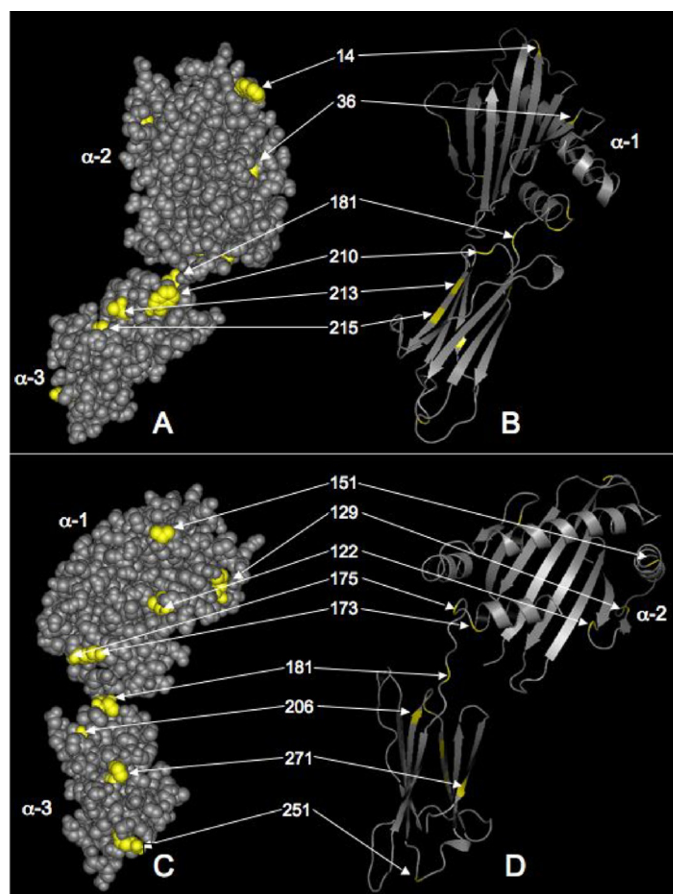

Fig. 2. The $\alpha 1$, $\alpha 2$, and $\alpha 3$ extracellular domains of the MICA molecule. Space-filling models are used in A and C and ribbon models in B and D. The models are shown in two orientations (AB and CD) to reveal the relative location of all mismatched amino acid residues in recipient and donor pairs (as indicated). Most mismatched residues across the $\alpha 1$–$\alpha 3$ domains appear to be exposed to the extracellular environment. Images were generated from the Molecular Modeling Database (MMDB) and the National Centre for Biotechnology Information (NCBI), using Pymol (DeLano WL, The PyMOL Molecular Graphics System (2002) on the World Wide Web at http://www.pymol.org, and Cn3D at http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.sht.

tiple significant mismatches across the $\alpha 1$–$\alpha 3$ domains, highlights the potential immunogenicity of the MICA molecule. Two broad reciprocal allele groups, termed MICA-G1 and MICA-G2, as defined by polymorphisms in the MICA $\alpha 1$ and $\alpha 3$ domains [30], were often mismatched and commonly associated with the production of MICA antibodies, as was mismatching of individual high frequency alleles with unique polymorphisms, such as MICA*004, *008, and *009.

The MICA typing of recipient and donor pairs shown in Table 3 revealed that *de novo* MICA DSA were present in MICA mismatched recipients, often in addition to NDSA. The presence of NDSA may relate to antibody recognition of MICA group-specific epitopes shared with donor MICA antigens [30]. A correlation between MICA antibodies and ACR suggests that cellular mechanisms culminate in MICA sensitization. Further analysis revealed that only patients with MICA antibodies showing high-MFI values developed ACR ($p < 0.001$; data not shown), suggesting that high-titer MICA antibodies are more significant, as reported for HLA antibodies [37]. It is likely that T-cell indirect allorecognition of mismatched MICA epitopes can give rise not only to T-cell help for the production of IgG antibodies against MICA, but also cell-mediated immunity resulting in cellular rejection. By comparison, a study investigating MICA matching and graft-versus-host disease in hematopoietic stem cell transplantation found a significantly higher rate of grade II–IV acute graft-versus-host disease in MICA-mismatched patients, indicating a T-cell response to mismatched MICA antigens [38].

There is evidence that HLA antibodies not only correlate strongly with AAMR, but also associate with ACR [39,40]. Our analysis of MICA antibodies revealed a similar trend with ACR (Table 2). However, the risk of ACR increased if graft recipients developed both MICA and HLA antibodies (Table 2). By contrast, MICA antibodies did not correlate with AAMR in our transplant cohort, whereas the presence of HLA class I DSA and class II DSA were significantly associated.

Our analysis of eGFR in kidney graft recipients, in particular those with MICA DSA, provides a novel insight into the possible pathogenic role of MICA antibodies on graft function and premature graft loss resulting from chronic damage (Table 4). Chronic damage to renal grafts, as indicated by significantly decreased eGFR, independently associated with MICA DSA as well as HLA class I DSA 2 years post-transplantation. These observations concur with previous reports of HLA class I antibodies associating with more rapid rejection of renal transplants, compared with HLA class II antibodies [39–41], which is probably related to the more ubiquitous expression of HLA class I antigens. It is possible that immune responses to mismatched HLA antigens result in increased expression of MICA on renal endothelial surfaces, which in turn invokes cellular and humoral responses directed against mismatched MICA antigens. This process may not have fully developed at the time of our sampling and analysis of MICA and HLA antibodies, which could explain the absence of association between MICA antibodies and AAMR. Furthermore, recipients with MICA DSA but not HLA antibodies were significantly associated with reduced eGFR 2 years post-transplantation ($p = 0.001$), whereas patients with HLA DSA and no MICA antibodies associated with significantly decreased eGFR after 3 years ($p = 0.001$) as shown in Table 4. Thus, the kinetics of antibody responses to HLA and MICA differ, indicating they may have distinct pathogenic roles, but once initiated the development of MICA antibodies may lead to accelerated graft dysfunction.

Recently, an integrative genomics approach to the analysis of serologic responses against MICA antigens after renal transplantation has identified the glomerulus as a specific target [42,43]. Immunohistochemistry studies have further identified localized expression of MICA to podocytes within the glomeruli of renal transplant recipients with AR, together with infiltrating mononuclear cells, B cells, $CD8^+$ T cells, and NK cells [43]. Furthermore, post-transplantation events associated with significant damage in the renal cortex include AR, infection, and allorecognition of compartment-specific antigens. Thus, it is feasible that MICA antibodies react with overexpressed MICA antigens in these compartments. This in turn may activate complement-dependent cytotoxicity and also facilitate direct lysis by degranulation of NK cells via NKG2D engaging MICA, or via activation of FcγRIIIA (CD16) through antibody interaction. Alternatively, binding of antibodies with MICA may initiate complement-independent mechanisms of graft damage by inducing a prothrombotic phenotype resulting in vascular thrombosis and loss of graft function [22].

Our study has some limitations. For example, we have not considered the effect of immunosuppression protocol differences, although the use of calcineurin inhibitors (CNI) is known to associate with nephrotoxicity and chronic renal damage [44]. Nevertheless, a previous study of 161 kidney recipients found no association with drug therapy and MICA antibodies [45], whereas others have shown a moderate increase in mycophenolic acid but not CNI use in MICA antibody positive recipients [23]. However, CNI use and association with eGFR was not tested in these studies. Likewise, we

Table 4
Comparison of mean eGFR in renal transplant recipients with different categories of HLA and MICA donor-specific antibodies at 1, 2, and 3 years post-transplantation

Antibody	Years post-Tx	eGFR mean ± SD (1 year, n = 205; 2 years, n = 180; 3 years, n = 112)					
		Negative	n	Positive	n	p Value	Mean difference
HLA DSA	1	53.3 ± 18.8	186	43.8 ± 23.0	19	NS	9.5
	2	53.3 ± 20.1	164	41.4 ± 22.2	16	0.027	11.9
	3	51.6 ± 19.2	104	24.8 ± 15.4	8	<0.001	26.8
MICA DSA	1	53.1 ± 19.6	192	43.2 ± 12.1	13	0.073	9.9
	2	53.4 ± 20.5	168	35.7 ± 11.2	12	<0.001	17.7
	3	50.7 ± 20.1	106	33.2 ± 13.2	6	0.038	17.5
HLA DSA alone	1	53.0 ± 18.8	189	45.6 ± 24.1	16	NS	7.4
	2	52.9 ± 20.2	167	43.4 ± 23.0	13	0.100	9.5
	3	51.2 ± 19.5	106	24.2 ± 14.9	6	0.001	27.0
MICA DSA alone	1	52.7 ± 19.5	198	45.5 ± 11.8	7	NS	7.2
	2	52.8 ± 20.5	174	34.8 ± 7.6	6	0.001	18.0
	3	50.1 ± 20.2	109	34.3 ± 8.6	3	NS	15.8
Class I DSA	1	53.2 ± 19.0	193	40.6 ± 21.5	12	0.028	12.6
	2	53.0 ± 20.4	170	39.6 ± 18.9	10	0.045	13.4
	3	50.8 ± 19.8	107	26.2 ± 13.5	5	0.007	24.6
Class II DSA	1	53.2 ± 18.7	191	42.6 ± 24.8	14	0.047	10.6
	2	52.8 ± 20.0	169	42.4 ± 20.6	11	NS	10.4
	3	51.1 ± 19.5	106	25.5 ± 16.4	6	0.002	25.6
Class I + II DSA	1	53.0 ± 19.0	198	35.7 ± 23.6	7	0.019	17.3
	2	52.6 ± 20.3	175	40.0 ± 24.5	5	NS	12.6
	3	50.3 ± 20.0	109	28.7 ± 13.6	3	0.066	21.6

MICA, major histocompatibility complex class I–related chain A; eGFR, estimated glomerular filtration rate (ml/min/1.73m^2); SD = standard deviation; Tx, transplantation; DSA, donor-specific antibody.

have also not considered primary disease in our recipients, although in another study this was not significantly associated with the presence of MICA antibodies [45]. Given our stringent definition that the presence of MICA antibody depended on concordant results from two of three independent bead assays, we may have underestimated the prevalence of MICA antibody because of variations between methodologies. Moreover, as the immobilized MICA proteins used in our assays cover the most common MICA alleles defined, it is possible that antibodies reactive with rare MICA proteins were undetected.

In conclusion, we have shown that mismatching MICA alleles leads to the development of MICA antibodies in some renal graft recipients. We have also demonstrated that the presence of MICA DSA independently associated with decreased eGFR and poorer graft outcome, which warrants further investigation in larger collaborative studies. Furthermore, MICA typing of patients and donors together with MICA antibody screening, especially for patients who have undergone retransplantion, may identify those at risk for graft dysfunction and may influence the management of transplant survival.

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Table 5
Linear regression analysis of factors significantly associated with decreased eGFR in MICA-typed renal transplant recipients

Year (N)	Variable	n	Univariate p value	B[a]	CI (95%)	Multivariate p value
1 (205)	ACR	36	0.001	12.3	5.5–19.1	<0.001
	AAMR	13	0.001	17.2	3.6–30.7	0.022
	Donor age ≥50 y	95	<0.001	11.1	5.9–16.4	<0.001
	Patient age ≥50 y	77	0.020	3.7	–	0.172
	Deceased donor	148	0.021	6.9	1.0–12.7	0.022
	Female donor	82	0.006	7.1	1.9–12.2	0.007
	HLA class I DSA	12	0.028	3.6	–	0.575
	HLA class II DSA	14	0.047	8.8	–	0.166
	MICA DSA	13	0.073	6.5	–	0.251
2 (180)	ACR	34	<0.001	12.8	5.4–20.1	0.001
	Donor age ≥50 y	84	<0.001	17.3	11.7–22.8	<0.001
	Female donor	75	0.007	6.3	0.8–11.9	0.026
	HLA class I DSA	10	0.045	11.2	0.4–22.3	0.050
	MICA DSA	12	<0.001	16.1	3.9–28.2	0.010
3 (112)	ACR	26	<0.001	15.9	8.2–23.6	<0.001
	Donor age ≥50 y	51	<0.001	14.5	8.1–20.9	<0.001
	Retransplantation	17	0.013	1.0	–	0.985
	HLA class I DSA	5	0.007	18.8	0.3–37.4	0.046
	HLA class II DSA	6	0.002	1.7	–	0.860
	MICA DSA	6	0.059	10.8	–	0.172

MICA, major histocompatibility complex class I–related chain A; CI, confidence interval; DSA, donor-specific antibody; ACR, acute cellular rejection; AAMR, acute antibody-mediated rejection.
Patient gender was not significantly associated with decreased eGFR at any time point.
[a]B (coefficient of variation) indicates difference in eGFR between positive and negative cases (ml/min/1.73 m^2). Covariates included in the model were those originally identified in univariate analysis giving significant and trend p values of ≤0.1.

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