



Original Article

Alloreactive adaptive natural killer cells in renal transplantation: Potential contribution to allograft microvascular inflammation



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ABSTRACT

Inhibitory killer cell immunoglobulin-like receptors (iKIRs) are randomly expressed by natural killer (NK) cell subsets and recognize motifs shared by HLA class-I (HLA-I) allotypes. Such interactions prevent NK cell autoreactivity while enhancing their response against cells lacking those HLA-I molecules (*missing self*), a situation defined in transplantation as iKIR-HLA-I mismatch (iKIR-MM), whose genotypic prediction has been associated with microvascular inflammation (MVI). Herein, we compared iKIR-MM in kidney transplant recipients with MVI ≥ 2 ($n = 19$) and controls with MVI ≤ 1 ($n = 36$). In parallel to genetic analysis of iKIR-MM, which was more frequent in MVI ≥ 2 patients, putative alloreactive

Abbreviations: Ab, antibody; AMR, antibody-mediated rejection; CI, confidence interval; CMV, cytomegalovirus; HLA, human leukocyte antigen; HLA-I, HLA class-I; iKIR, inhibitory killer cell immunoglobulin-like receptor; iKIR-MM, iKIR-HLA-I mismatch; IQR, interquartile range; KT, kidney transplantation; KTR, kidney transplant recipients; MVI, microvascular inflammation; NK, natural killer; PBMC, peripheral blood mononuclear cell.

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iKIR-MM NK cells were defined by flow cytometry as NKG2A(–) cells bearing self-specific but lacking donor-specific iKIR. Although iKIR-MM NK cells were detected in both groups, their pretransplant numbers were higher in MVI ≥ 2 patients (median = 11.02, interquartile range = 0–58.31 vs median = 0, interquartile range = 0–9.46), especially in the presence of donor-specific antibodies or C4d, and correlated with MVI grade. Pretransplant, a subset of MVI ≥ 2 patients showed high proportions and numbers of oligoclonal iKIR-MM NK cells, which displayed an NKG2C(+) adaptive phenotype associated with cytomegalovirus infection. This pilot study provides a novel perspective on the contribution of iKIR-MM NK cells to MVI, with potential practical implications.

1. Introduction

The alloreactive immune response of the recipient against donor polymorphic molecules, mainly human leukocyte antigen (HLA), limits graft survival after kidney transplantation (KT). This leads to T cell-mediated or antibody (Ab)-mediated rejection (AMR), mainly defined according to biopsy detection of interstitial inflammation and tubulitis for T cell-mediated rejection, or glomerulitis and peritubular capillaritis, which are signs of significant microvascular inflammation (MVI), for AMR. The presence of circulating antibodies against donor HLA (donor-specific antibodies [DSAs]) or other polymorphic molecules, and/or the presence of C4d staining in peritubular capillaries, support AMR diagnosis. However, a number of patients with MVI are identified in the absence of detectable DSA or C4d.^{1–3} Transcriptomic and microscopic analyses of graft biopsies revealed a relationship between AMR signs and NK cell infiltration and activation signatures. These observations were interpreted as a contribution of Ab-induced CD16A⁺ NK cell activation to AMR/MVI^{4–6} but were also reported in the absence of detectable DSA.^{3,7,8}

NK cell functions are triggered when activating signals overcome the control by inhibitory receptors, whose interaction with self HLA class-I (HLA-I) ligands prevents autoreactivity while favoring acquisition of NK cell functional competence.^{9–11} Among a variety of inhibitory NK cell receptors, killer cell immunoglobulin-like receptors (iKIRs) and CD94/NKG2A, which respectively recognize structural motifs shared by different classical HLA-I allotypes and HLA-E, are important players in the control of NK cell functions. The diversity of KIR haplotypes containing different sets of genes and their random clonal expression determines the existence in every individual of multiple subsets of NK cells displaying different combinations of these receptors.^{12–14} It is conventionally accepted that iKIR and NKG2A interaction with self-HLA-I molecules enhances NK cell responsiveness against cells that lack/downregulate HLA-I ligands (*missing self*) through a process termed *licensing*, which is incompletely defined at the molecular level.^{15,16} These “educated” NK cells contribute to the response against infections and tumors but may also react against normal allogeneic cells. In transplantation, detection in the recipient of an iKIR gene specific for a self HLA-I ligand absent in the donor is defined as an iKIR-HLA-I mismatch (iKIR-MM). Of note, due to its limited polymorphism, HLA-E interaction with CD94/NKG2A has been

reported to also promote NK cell education¹⁵ but not alloreactivity.¹⁷ Reminiscent of F1 hybrid resistance¹⁸ against hematopoietic but not solid tissues in animal models of NK cell alloreactivity, donor-to-recipient iKIR-MM in hematopoietic stem cell transplantation does not cause graft-vs-host disease but may mediate a graft-vs-leukemia effect, leading to the safe use of alloreactive NK cells for immunotherapy of hematopoietic malignancies.^{19–21}

Nevertheless, there is evidence that alloreactive NK cells might contribute to chronic solid organ transplant rejection, presumably resulting from allograft expression of NK receptor-activating ligands induced by proinflammatory stimuli such as other alloreactive insults (eg, AMR, infections, or cold ischemia).^{22–24} Some reports have suggested an association between genetic prediction of iKIR-MM and kidney allograft rejection,²⁵ which has not confirmed by others.^{26,27} Recently, genetic iKIR-MM was associated with MVI ≥ 2 and decreased MVI ≥ 2 -free graft survival in kidney transplant recipients (KTRs) without detectable DSA or C4d,^{28,29} as well as in cases with DSA without evidence of complement activation in the microvasculature.^{2,29,30} These latter observations suggested that iKIR-MM might enhance Ab-dependent cell-mediated cytotoxicity triggered by the NK cell Fc γ RIIIa (CD16A) receptor.

On the other hand, a relationship between kidney allograft rejection and NKG2C copy number, rather than with iKIR-MM, was recently reported.²⁷ Expression of the activating CD94/NKG2C receptor specific for HLA-E, together with other phenotypic features, characterize NK cells, which undergo adaptive differentiation and expansion in response to cytomegalovirus (CMV) infection.^{31,32} This process is promoted by CD94/NKG2C recognition of HLA-E bound to a peptide derived from the UL40 CMV protein,³³ influenced by NKG2C copy number^{34,35} and favored by the NKG2C*02 allele.³⁶ Adaptive NKG2C⁺ NK cells display an oligoclonal expression of iKIR specific for self HLA-I molecules together with other phenotypic features^{37,38} and appear particularly efficient in mediating Ab-dependent effector functions.^{38–40} In KTR, pretransplant adaptive NK cells were reported to reduce the risk of CMV infection^{41,42} and to expand in response to post-transplant infection.^{43,44}

The stochastic expression of KIR genes during NK cell differentiation determines the existence in every individual of a huge variety of NK cell subsets displaying different KIR combinations, whose proportions may change as a result of oligoclonal

expansion in response to other events, particularly CMV infection.^{31,32,37,45} Thus, genetic prediction of iKIR-MM is not informative on the proportions/numbers of circulating alloreactive NK cells, which might be relevant to allograft damage; the relationship of this variable with the potential development of NK cell alloreactivity is unknown. To approach this issue, a pilot study was conducted in a cohort of KTRs categorized according to MVI detection, combining conventional genetic analysis with a flow cytometry-based phenotypic assessment of the frequencies and numbers of putative alloreactive circulating iKIR-MM NK cells.

2. Materials and methods

2.1. Study population

The study population was selected from KTRs recruited at Hospital del Mar having (1) follow-up or indication biopsies between 1 and 3 years after the transplantation; (2) monitoring of serum anti-HLA Ab data pretransplant and at the time of biopsy using single antigen bead kits (Lifecodes LifeScreen Deluxe; Immucor Diagnostics Inc); (3) donor and recipient HLA-A, -B, and -C genotype data; and (4) available recipient DNA samples. Patients diagnosed at some point with Banff⁴⁶ categories 3, 4 (including mixed rejection), or 6 and patients treated with lymphodepleting therapies were excluded. Biopsies with pyelonephritis or that were nondiagnosable were not considered. MVI grade was calculated as the sum of the scores of glomerulitis and peritubular capillaritis. A group of cases with MVI ≥ 2 at any of the biopsies ($n = 19$) was selected. Control patients with MVI ≤ 1 at all biopsies analyzed ($n = 36$) were selected by frequency matching to have similar ($<10\%$ difference) mean age and proportions of sex and living donors. In patients with >1 biopsy, the most pathologic biopsy was considered for patient categorization and analyses involving MVI grade. Patients were considered posttransplant DSA⁺ according to the positive results for donor HLA antigens in any of their anti-HLA Ab determinations. Demographic, clinicopathological, and immunologic data were collected until death, graft loss (defined as retransplant or return to dialysis) or loss to follow-up on January 1, 2024. CMV DNAemia was systematically monitored as previously described.^{42,44} CMV infection was diagnosed when CMV DNAemia reached a quantifiable level and was treated with antiviral therapy.

The Parc de Salut Mar Ethical Research Board (2010/3904/I) approved the study, and all patients gave written informed consent. Clinical and research activities reported herein are consistent with the Principles of Istanbul and Helsinki Declarations.

2.2. HLA-I genotyping

HLA-A, -B and -C genotyping was routinely performed at the Histocompatibility Laboratories of Hospital Clínic of Barcelona or Hospital Puerta de Hierro using standard next-generation sequencing, probe hybridization, or sequence-specific primer PCR methods (details available upon request). For HLA-I

genotypes with low resolution, high-resolution alleles were deduced by frequency and linkage using HaploStats (<https://www.haplostats.org/>), a web-based application of the US National Marrow Donor Program Bioinformatics Group, considering origin or descent of recipients and donors. In addition, linkage disequilibria between HLA-C*16:01 and HLA-B*44 and between HLA-C*16:02 and HLA-B*51 were considered.^{47,48} KIR-binding motifs of the different HLA-B and -C alleles were assigned according to standard HLA nomenclature (https://www.ufrgs.br/immunovet/molecular_immunology/hla.html) or, for alleles absent in that classification, according to the presence of the KIR-binding motifs in the HLA-I allele sequences in the Immuno Polymorphism Database⁴⁹ (<https://www.ebi.ac.uk/ipd/>).

2.3. KIR genotyping and analysis of iKIR-MM

DNA was purified from whole blood with Puregene Blood Core kit (Qiagen). Recipient KIR genotypes (*KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR3DL1*, and *KIR3DL2*) were assessed with a commercial reverse oligonucleotide probe-hybridization method based on the Luminex xMap Technology (LabType SSO Test, One Lambda Inc); ambiguous results were verified by sequence-specific primer PCR.⁵⁰ iKIR-MM was defined when the recipient expressed an iKIR that recognized an HLA-I ligand in the recipient that was absent from the donor. The interactions considered were: KIR2DL1-HLA-C2, KIR2DL2/3-HLA-C1, KIR3DL1-HLA-Bw4, and KIR3DL2-HLA-A*03 and -A*11.^{14,19}

2.4. Flow cytometry analysis

Pretransplant, 3 months and 12-36months posttransplant cryopreserved peripheral blood mononuclear cell (PBMC) samples were analyzed by flow cytometry. PBMCs (6×10^5) were first stained with unconjugated Abs specific for KIR3DL2 (clone DX31, IgG2a, or clone Q66, IgM, provided by Drs Lewis Lanier and Daniela Pende, respectively), followed by secondary immunostaining with polyclonal anti-mouse IgG-APC (Biolegend) or anti-mouse IgM-DyLight650 (Invitrogen). Subsequently, a mixture of the following Abs specific for surface antigens was added: anti-CD3-PerCP (clone SpVT3b, provided by Dr J. de Vries, conjugated by Immunostep) or anti-CD3-PerCP (clone SK7, Biolegend), anti-CD56-BV510 (clone NCAM16.2, BD Bioscience), anti-NKG2C-BV786 (clone 134591, BD Bioscience), anti-NKG2A-FITC (clone Z199, provided by Dr A. Moretta, in-house conjugated), or anti-NKG2A-CFBlue (clone Z199, conjugated by Immunostep), anti-KIR2DL1-PE (clone HP-DM1, Biolegend), anti-KIR2DL2/L3-biotin (Rea147; Miltenyi Biotec), and anti-KIR3DL1-BV421 (DX9, Biolegend). A fourth staining step with streptavidin-BV605 (BD Horizon) was performed. For intracellular staining of Fc ϵ R γ chain, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Bioscience) and stained with anti-Fc ϵ R γ -FITC polyclonal antibody (Merck Millipore). Samples were acquired with a Cytex Aurora spectral flow cytometer (Cytex Bioscience Inc) using Spectroflo software

Table 1
Demographic characteristics of the study population.

Characteristic	MVI ≤ 1 (N = 36)	MVI ≥ 2 (N = 19)	95% CI	P
At transplantation				
Recipient				
Age (y), mean $\pm$ SD	56.78 $\pm$ 12.38	56.21 $\pm$ 17.60	−7.60, 8.74 ^a	.784
Sex (female), n (%)	11 (30.56)	7 (36.84)	0.24, 2.39 ^b	.637
Preemptive KT, n (%)	2 (5.56)	3 (15.79)	0.05, 1.70 ^b	.209
Time on dialysis before KT (mo), median (IQR)	18.45 (9.46–34.83)	27.14 (19.45–36.73)	−2.46, 15.81 ^c	.105
Race (White), n (%)	31 (86.11)	16 (84.21)	0.28, 5.46 ^b	.559
Detection of pre-KT anti-HLA DSA, n (%)	0	4 (21.05)	0.00, 0.51 ^b	.004
Previous KT, n (%)	0	0	NA ^b	1.000
Cause of renal failure				.794
Glomerulonephritis, n (%)	5 (13.89)	2 (10.53)	0.24, 7.43 ^b	
Autoimmune disease, n (%)	1 (2.78)	0	NA ^b	
Diabetes mellitus, n (%)	5 (13.89)	4 (21.05)	0.14, 2.22 ^b	
Other, n (%)	25 (69.44)	13 (68.42)	0.30, 3.41 ^b	
Donor				
Donor type (living donor), n (%)	5 (13.89)	4 (21.05)	0.14, 2.22 ^b	.781
Age (y), mean $\pm$ SD	58.89 $\pm$ 12.44	58.16 $\pm$ 14.75	−6.81, 8.28 ^a	.958
Sex (female), n (%)	15 (41.67)	12 (63.16)	0.14, 1.30 ^b	.130
Race (White), n (%)	36 (100)	19 (100)	NA ^b	
Transplantation				
Cold ischemia time (h), mean $\pm$ SD	11.51 $\pm$ 5.76	8.97 $\pm$ 4.80	−0.56, 5.64 ^a	.106
Delayed graft function, n (%)	10 (27.78)	2 (10.53)	0.68, 16.09 ^b	.141
No. of HLA-A/-B/-C/-DRB1 mismatches, median (IQR)	6.00 (5.00–7.00)	6.00 (4.00–7.00)	−1.00, 1.00 ^c	.971
CMV serostatus pre-KT, n (%)				.187
R−D−	2 (5.56)	0	NA ^b	
R−D+	1 (2.78)	3 (15.79)	0.01, 1.13 ^b	
R+D−	5 (13.89)	1 (5.26)	0.33, 35.89 ^b	
R+D+	28 (77.78)	15 (78.95)	0.28, 3.55 ^b	
Detection of CMV viremia post-KT, n (%)	10 (27.78)	8 (42.11)	0.18, 1.58 ^b	.282
Immunosuppression				
Induction therapy				
Anti-CD25, n (%)	36 (100)	18 (94.74)	NA ^b	.165
Initial maintenance therapy				
Tacrolimus & mTOR inh. & corticosteroids, n (%)	7 (19.44)	11 (57.89)	0.05, 0.64 ^b	.004
Tacrolimus & MPA & corticosteroids, n (%)	29 (80.56)	8 (42.11)	1.57, 18.31 ^b	.004
Biopsy and DSA data				
Detection of post-KT anti-HLA DSA, n (%)	2 (5.56)	11 (57.89)	0.01, 0.23 ^b	<.001
No. of biopsies, n (%)				.031
1	7 (19.44)	10 (52.63)	0.06, 0.80 ^b	

(continued on next page)

Table 1 (continued)

Characteristic	MVI ≤ 1 (N = 36)	MVI ≥ 2 (N = 19)	95% CI	P
2	27 (75.00)	9 (47.37)	0.97, 10.39 ^b	
3	2 (5.56)	0	0.00, 4.10 ^b	
At the end of follow-up				
Exitus, n (%)	8 (22.22)	4 (21.05)	0.28, 3.62 ^b	.920
Death-censored graft loss, n (%)	6 (16.67)	6 (31.58)	0.13, 1.50 ^b	.203
Loss to follow-up, n (%)	0	1 (5.26)	0.00, 4.75 ^b	.165
Follow-up time after KT (y), mean $\pm$ SD	7.67 $\pm$ 1.86	6.63 $\pm$ 2.60	−0.18, 2.25 ^a	.094

Statistically significant *P* values shown in bold.

CI, confidence interval; CMV, cytomegalovirus; CNI, calcineurin inhibitor; D, donor; DSA, donor-specific antibody; HLA, human leukocyte antigen; inh., inhibitor; IQR, interquartile range; KT, kidney transplantation; MPA, mycophenolic acid; mTOR, mammalian target of rapamycin; MVI, microvascular inflammation; NA, not applicable; R, recipient; SD, standard deviation.

^a 95% CI of the difference of the means.

^b 95% CI of odds ratio.

^c 95% CI of the difference of the medians.

(version 3.1.0, Cytex Bioscience Inc) and analyzed with Flowjo (version 10.8, BD Bioscience). Only samples with a minimum of 500 total NK cells (CD3⁺ CD56⁺) were used. NK cell subpopulations defined by the combined expression of iKIR, NKG2A, and NK2G2C were determined by Boolean gating. Expression of iKIR-MM relative to the donor and recipient HLA-I was determined for every subpopulation. Putative alloreactive iKIR-MM NK cells were operationally defined by their expression of at least an iKIR specific for a recipient HLA-I ligand and lacking NKG2A and iKIR specific for any donor HLA-I allotype. To calculate the frequency of total iKIR-MM NK cells for each patient, the proportions of iKIR-MM subsets were summed. Calculation of the absolute numbers of iKIR-MM NK cells per microliter was based on lymphocyte counts obtained with a hemacytometer (DxH 500, Beckman Coulter). NK cell subsets were considered as expansions when they represented a percentage of total NK cells higher than the third quartile + 1.5 $\times$ interquartile range (IQR) of the pool of subsets from the analyzed patients, following the Turkey method.

2.5. Data presentation and statistical analysis

Quantitative variables following or not following a normal distribution were expressed as mean and standard deviation or median and IQR, respectively, except where otherwise stated. Categorical variables were described as counts and percentages and compared between groups with the chi-square test. The Baptista-Pike method was used to calculate 95% confidence interval (CIs) of odds ratios of tabular data. Comparison of quantitative variables between 2 groups was expressed with 95% CI of the difference of the means or medians and was performed by *t* test or Mann-Whitney U test, for variables following or not following a normal distribution, respectively, except where otherwise indicated. Correlations between quantitative variables were assessed with the Spearman test and 95% CI Spearman rank correlation coefficients (*r*). Differences were considered

significant at a 2-sided *P* < .05. Where indicated, *P* values were corrected for false discovery rate using the Benjamini-Hochberg procedure. Only significant *P* values are shown in figures. Statistical analysis was performed using GraphPad Prism 8.0 (version 8.0.1, GraphPad Software), SPSS (version 23, SPSS Inc), or R (version 4.3.2).

3. Results

3.1. Relation of genetic iKIR-MM with MVI

To analyze the effect of genetic and phenotypic iKIR-MM on MVI, we designed a case-control study in which genetic and phenotypic iKIR-MM parameters were compared between cases with MVI ≥ 2 and controls with MVI ≤ 1 selected from a cohort of KTRs without T cell-mediated rejection who had not received lymphodepleting therapy. Cases displaying MVI ≥ 2 in any of the biopsies (*n* = 19) were compared with controls without overt MVI lesions (MVI ≤ 1 , *n* = 36). The MVI ≥ 2 group included 12 patients diagnosed as AMR according to Banff22⁴⁶ (9 DSA⁺, 1 C4d⁺, and 2 DSA⁺ C4d⁺), observed on ≥ 1 occasion; the remaining 7 patients were simply categorized as MVI. The MVI ≤ 1 group included 2 DSA⁺, currently considered probable AMR, and 3 C4d⁺ patients (Tables 1 and 2). Presence of pretransplant DSA, posttransplant DSA and/or C4d and initial treatment with mTOR inhibitors were significantly more frequent in the MVI ≥ 2 group whereas initial treatment with mycophenolate acid was less frequent (Table 1, Fig. 1).

Genetic iKIR-MM was conventionally defined based on donor/recipient HLA-I and recipient KIR genotypes, considering their specific interactions. In the context of this study, only iKIR with well-defined polymorphic HLA-I ligands were considered. A greater frequency of patients with ≥ 1 iKIR-MM was observed in the MVI ≥ 2 group (73.68%) than in the MVI ≤ 1 group (52.78%), although the difference did not reach statistical significance, and patients without genetic iKIR-MM were found in

Table 2

Comparison of histological features between groups.

Characteristic	MVI $\leq$ 1	MVI $\geq$ 2	95% CI	<i>P</i>	MVI $\leq$ 1	MVI $\geq$ 2	95% CI	<i>P</i>
Biopsies studied	36	18			29	10		
Months after KT, median (IQR)	13.80 (12.53-16.00)	12.76 (11.07-14.98)	−2.66, 0.79 ^b	.291	38.57 (36.99-41.20)	48.59 (37.49-58.38)	−2.04, 18.99 ^b	.143
Reason for biopsy (follow-up), n (%)	34 (94.44)	13 (72.22)	1.09, 34.64 ^c	.022	27 (93.10)	7 (70.00)	0.97, 35.36 ^c	.06
Tubulitis ^a , mean $\pm$ SD	0.06 $\pm$ 0.33	0.17 $\pm$ 0.38	−0.09, 0.31 ^d	.079	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	NA ^d	1.000
Interstitial inflammation ^a , mean $\pm$ SD	0.03 $\pm$ 0.17	0.17 $\pm$ 0.38	−0.01, 0.29 ^d	.069	0.07 $\pm$ 0.37	0.00 $\pm$ 0.00	−0.31, 0.17 ^d	.557
Glomerulitis ^a , mean $\pm$ SD	0.11 $\pm$ 0.32	1.56 $\pm$ 0.92	1.10, 1.79 ^d	<.001	0.17 $\pm$ 0.38	1.90 $\pm$ 0.99	1.29, 2.17 ^d	<.001
Peritubular capillaritis ^a , mean $\pm$ SD	0.14 $\pm$ 0.35	1.06 $\pm$ 0.80	0.60, 1.23 ^d	<.001	0.21 $\pm$ 0.49	0.70 $\pm$ 0.82	0.06, 0.93 ^d	.035
Intimal arteritis ^a , mean $\pm$ SD	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	NA ^d	1.000	0.00 $\pm$ 0.00	0.10 $\pm$ 0.32	−0.02, 0.22 ^d	.089
C4d deposition ^a , mean $\pm$ SD	0.19 $\pm$ 0.58	0.61 $\pm$ 0.92	0.01, 0.83 ^d	.023	0.10 $\pm$ 0.41	0.30 $\pm$ 0.67	−0.17, 0.56 ^d	.246
Interstitial fibrosis ^a , mean $\pm$ SD	1.03 $\pm$ 0.76	1.50 $\pm$ 0.79	0.02, 0.92 ^d	.03	1.03 $\pm$ 0.57	1.70 $\pm$ 0.67	0.22, 1.11 ^d	.007
Tubular atrophy ^a , mean $\pm$ SD	1.00 $\pm$ 0.70	1.39 $\pm$ 0.78	−0.04, 0.81 ^d	.081	1.10 $\pm$ 0.56	1.60 $\pm$ 0.70	0.05, 0.94 ^d	.045
Transplant glomerulopathy ^a , mean $\pm$ SD	0.06 $\pm$ 0.33	0.28 $\pm$ 0.67	−0.05, 0.49 ^d	.072	0.03 $\pm$ 0.19	1.30 $\pm$ 1.16	0.82, 1.71 ^d	<.001
Vascular fibrous intimal thickening ^a , mean $\pm$ SD	1.08 $\pm$ 0.87	0.82 $\pm$ 0.88	−0.78, 0.26 ^d	.314	1.39 $\pm$ 0.79	1.40 $\pm$ 0.70	−0.56, 0.58 ^d	.986
Arteriolar hyalinosis ^a , mean $\pm$ SD	0.36 $\pm$ 0.64	0.44 $\pm$ 0.78	−0.32, 0.48 ^d	.861	1.21 $\pm$ 0.90	1.20 $\pm$ 1.03	−0.70, 0.69 ^d	.932
Mesangial matrix expansion ^a , mean $\pm$ SD	0.06 $\pm$ 0.33	0.06 $\pm$ 0.24	−0.18, 0.18 ^d	.634	0.21 $\pm$ 0.49	0.80 $\pm$ 0.63	0.20, 0.99 ^d	.003

Statistically significant *P* values shown in bold.

CI, confidence interval; IQR, interquartile range; KT, kidney transplantation; MVI, microvascular inflammation; NA, not applicable; SD, standard deviation.

^a Variables not following a normal distribution shown as mean $\pm$ SD for more intuitive representation of data distribution.^b 95% CI of the difference of the medians.^c 95% CI of odds ratio.^d 95% CI of the difference of the means.

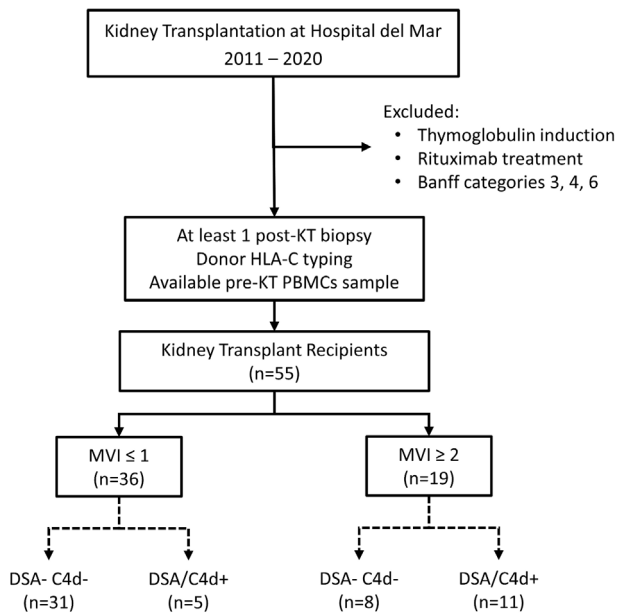


Figure 1. Flowchart of patient selection. KT, HLA, DSA, PBMC, MVI.

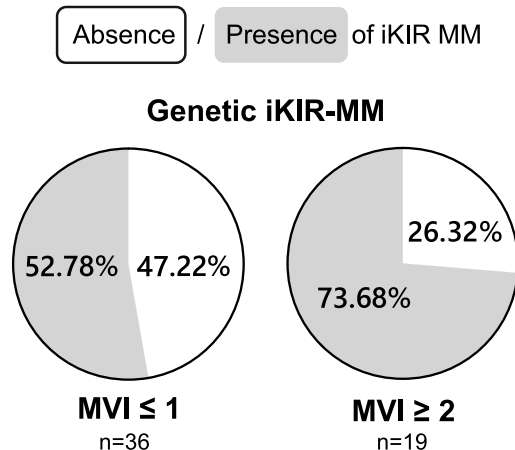


Figure 2. Distribution of genetic iKIR-MM. Genetic iKIR-MM was predicted based on HLA-I and iKIR genotyping in a cohort of KTR categorized as MVI≤1 (n=36) or MVI≥2 (n=19) according to posttransplant biopsies; frequencies of cases and controls with or without iKIR-MM are displayed. iKIR-MM, KTR, MVI.

both the MVI ≤1 and MVI ≥2 groups (47.22% and 26.32%, respectively) (Fig. 2).

3.2. Phenotypic analysis of iKIR-MM NK cells and relation with MVI

Identification of putative alloreactive circulating NK cells with an iKIR-MM phenotype was assessed by flow cytometry (Supplementary Fig. 1) pre- and posttransplant (3 and/or 12–36 months). Different percentages of pretransplant NK cells with this profile were noticed between MVI ≤1 (median 0%, IQR 0–6.73) and MVI ≥2 (median 3.86%, IQR 0–25) groups ($P = .058$,

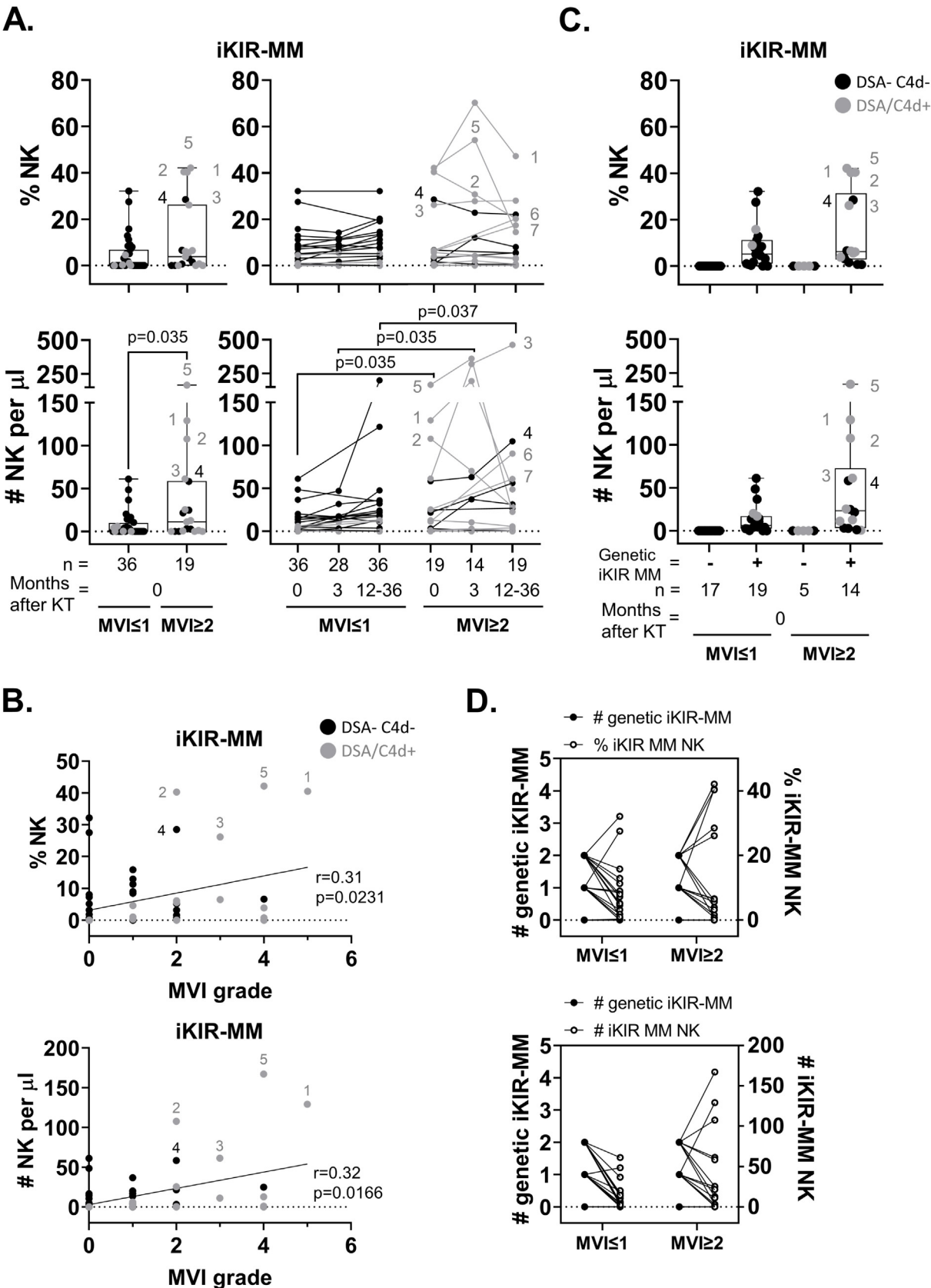
95% CI of difference of medians 0.00–6.09), reaching >20% in 2 MVI ≤1 and 5 MVI ≥2 KTRs (4 AMR, 1 MVI) including 3 with >40% (Fig. 3A). Moreover, pretransplant numbers of iKIR-MM NK cells were significantly higher in MVI ≥2 (median 11.02 cells/μL, IQR 0–58.31) than in MVI ≤1 cases (median 0 cells/μL, IQR 0–9.46) ($P = .035$, 95% CI of difference of medians 0.00–21.54) (Fig. 3A). This difference was noticeable in the subgroup of DSA⁺ and/or C4d⁺ patients but not in DSA[−] C4d[−] patients (Supplementary Fig. 2A). Both proportions and numbers of iKIR-MM NK cells significantly correlated with MVI grade (percentages: $r = 0.31$, $P = .023$, 95% CI 0.04–0.53; numbers: $r = 0.32$, $P = .0166$, 95% CI 0.05–0.55) (Fig. 3B).

In cases with genetic iKIR-MM, different levels of iKIR-MM NK cells were identified pretransplant in both the MVI ≤1 (median 6.15 cells/μL, IQR 2.28–16.98) and MVI ≥2 (median 23.17 cells/μL, IQR 3.09–72.83) groups ($P = .089$, 95% CI of difference of medians −1.24 to 47.51), being, by definition, undetectable in the absence of genetic iKIR-MM (Fig. 3C). No relationship was found between the number of genetic iKIR-MM and the proportions/numbers of iKIR-MM NK cells (Fig. 3D).

Of note, distinct posttransplant evolution patterns of iKIR-MM NK cells were noticed (Fig. 3A). In the majority of MVI ≤1 and MVI ≥2 cases displaying low proportions/numbers of iKIR-MM NK cells, their levels tended to remain stable throughout the follow up, with 2 exceptions showing a late increase (patients 6 and 7). In contrast, among patients with higher pretransplant iKIR-MM NK cells, the levels also remained stable in 2 MVI ≤1 and MVI ≥2 cases (patients 3 and 4). By contrast, 3 MVI ≥2 patients (patients 1, 2, and 5) showed a marked reduction at 11, 13, and 16 months, respectively preceded by an increase at 3 months in 2 of them (patients 1 and 5).

3.3. Adaptive NK cell phenotype of iKIR-MM NK cell expansions detected in MVI ≥2 cases

The flow cytometry panel designed for NK cell phenotyping allowed detection of NKG2C, a marker of adaptive NK cells, a subset that differentiates and expands in response to CMV infection. Variable proportions of NKG2C⁺ NK cells were detected in KTRs from both groups, indicating that they were independent of MVI (Fig. 4A). However, greater pretransplant percentages/numbers of NKG2C⁺ iKIR-MM NK cells were detected in MVI ≥2 patients (MVI ≥2: median 0.23%, IQR 0–3.24; median 0.39 cells/μL, IQR 0–7.58; MVI ≤1: median 0%, IQR 0–0.69; median 0 cells/μL, IQR 0–0.55; $P(\%) = .032$, 95% CI of difference of medians (%) 0.00–0.47; $P(\text{cells}/\mu\text{L}) = .018$, 95% CI of difference of medians (cells/μL) 0.00–2.76) (Fig. 4B). Again, this difference was observed in the subgroup of DSA⁺ and/or C4d⁺ patients but not in DSA[−] C4d[−] (Supplementary Fig. 2B). Moreover, pretransplant percentages/numbers of NKG2C⁺ iKIR-MM NK cells correlated with higher MVI grade (percentages: $r = 0.36$, $P = .007$, 95% CI 0.10–0.58; numbers: $r = 0.39$, $P = .003$, 95% CI 0.13–0.60) (Fig. 4C). In 4 MVI ≥2 cases (patients 1, 2, 4, and 5), NKG2C was found to be coexpressed by 27% to 95% of



(caption on next page)

iKIR-MM NK cell subsets pretransplant. Consistent with their adaptive phenotype, these alloreactive NKG2C⁺ NK cells showed downregulation of FcεRγ chain expression (Supplementary Figs. 3 and 5A) and displayed an oligoclonal iKIR expression pattern, bearing ≥1 iKIR-MM (ie, KIR2DL2 in patient 1, KIR2DL3 in patient 4, KIR3DL2 in patient 5; and KIR2DL3/KIR3DL2 in patient 2) (Fig. 5B). Patient 3 displayed an FcεRγ(-) iKIR-MM oligoclonal NK cell expansion without NKG2C expression bearing a double iKIR-MM (KIR3DL1/KIR3DL2). At 12 months, some patients with low pretransplant proportions/numbers of iKIR-MM NK cells (patients 6 and 7) showed an increase of these cells, which coexpressed NKG2C (Fig. 3A); of note, these patients had experienced CMV viremia. Among the 2 MVI ≤1 patients who had ≥20% iKIR-MM NK cells, 1 displayed an oligoclonal expansion showing FcεRγ chain downregulation together with KIR2DL1 and KIR2DL2/3 MM (Fig. 5B).

In summary, our observations indicated that expansions of adaptive NK cells displaying an iKIR-MM relative to the donor were more frequent and enlarged in MVI ≥2 patients.

4. Discussion

In this pilot study, we assessed the relationship between the frequencies of putative alloreactive and CMV-adaptive NK cells and MVI detection/grade, in parallel to a standard genetic analysis of iKIR-MM.

In line with previous reports,^{28–30} we observed a higher proportion of patients with genetic iKIR-MM in the group with MVI ≥2. Pretransplant circulating iKIR-MM NK cells were identified in KTR with genetic iKIR-MM from both MVI ≤1 and MVI ≥2 groups. Their presence in MVI ≤1 cases unequivocally indicated that circulating alloreactive NK cells are insufficient to cause MVI. On the other hand, MVI ≥2 cases without iKIR-MM confirmed that iKIR-MM NK cells are not necessary for MVI development. However, the numbers of iKIR-MM NK cells were significantly different between MVI ≤1 and MVI ≥2 cases, especially in DSA⁺ or C4d⁺ cases, and correlated with MVI grade, suggesting that their presence might increase the probability of MVI development.

Moreover, although patients with relatively high pretransplant proportions of iKIR-MM NK cells were detected in both groups, differences in their phenotype and posttransplant evolution patterns were noticed. In 4 of 5 MVI ≥2 cases with elevated pretransplant oligoclonal iKIR-MM NK cells, an important fraction of them expressed NKG2C and displayed FcεRγ chain downregulation. These features characterize NK cells which undergo an adaptive differentiation and expansion in response to CMV infection and have been reported to efficiently mediate Ab-

dependent effector functions,^{38–40} possibly related to coupling of CD16 with ξ instead of γ chain adaptor.⁵¹ Of note, in the remaining case, most iKIR-MM NK cells showed FcεRγ chain downregulation in the absence of NKG2C expression, a phenotype which has been also associated with CMV infection.³⁹ Only one MVI ≤1 patient showed iKIR-MM NK cell expansions with adaptive features pre- or posttransplant, ie, FcεRγ chain downregulation. Although the proportions/numbers of iKIR-MM NK cells remained generally stable throughout the follow-up, differences in their posttransplant evolution were also noticed. Increases of iKIR-MM NK cells with adaptive features likely reflected posttransplant CMV infection events, supported by clinical records, whereas the sharp reduction observed at 12 to 36 months in 3 MVI ≥2 cases might be related with their putative persistent involvement in renal lesions, possibly leading to a progressive depletion from circulation. Importantly, from a practical standpoint, these observations indicate that phenotypic analysis of iKIR-MM should also be performed pretransplant and eventually monitored in cases with posttransplant CMV viremia, which is known to promote adaptive NK cell expansions.^{43,44} We did not observe a relation of mTOR immunosuppression with posttransplant variations of iKIR-MM NK cell levels (data not shown).

Some studies have associated genetic recipient-to-donor iKIR-MM with solid organ transplant rejection suggesting a contribution of NK cell alloreactivity,²⁵ presumably in combination with stimuli inducing graft expression of activating NK receptor ligands.²² Genetic iKIR-MM has been associated with MVI in the absence of DSA, suggesting that it may have an Ab-independent role.^{28,29} However, it is of note that genetic iKIR-MM has been associated with MVI in DSA⁺ patients as well,^{29,30} suggesting that it may contribute to MVI development by enhancing the effects of alloantibody-mediated NK cell activation by FcγR-IIIa (CD16) and eventually other DSA-independent insults. In our study, the difference in iKIR-MM NK cell levels between MVI ≥2 and MVI ≤1 patients was observed in the DSA⁺ and/or C4d⁺ subgroup, suggesting that iKIR-MM NK cells may contribute with DSA to develop MVI. No differences were perceived in DSA⁻ C4d⁻ patients; however, given the limited number of MVI ≥2 DSA⁻ C4d⁻ patients, our results do not rule out an effect of iKIR-MM NK cells independent of DSA, as previously reported.^{28,29}

Altogether, the data suggest that MVI risk/severity may be enhanced by increased circulating pretransplant adaptive NK cells with iKIR-MM relative to the donor HLA-I; the possibility that they may also contribute to MVI when present in smaller proportions is not excluded. In this regard, there is evidence that posttransplant CMV infection may promote adaptive NKG2C⁺ expansions,^{43,44} which, in case of iKIR-MM, might enhance their

Figure 3. Phenotypic analysis of iKIR-MM NK cells in MVI ≤1 and MVI ≥2 patients and relation with MVI grade. (A) Proportions (top) and numbers (bottom) of iKIR-MM NK cells detected pretransplant (bar graphs) and their evolution at 3 and 12 to 36 months posttransplant (connected dots graphs) are represented comparing all MVI ≤1 and MVI ≥2 patients. Numbers of samples available for analysis is indicated. Statistically significant differences (Mann-Whitney test) between groups at equivalent time frames adjusted for false discovery rate are shown. (B) Relation of pretransplant proportions (top) and numbers (bottom) of iKIR-MM NK cells with MVI grade. Significant results of Spearman tests are shown. (C) Pretransplant proportions (top) and numbers (bottom) of iKIR-MM NK cells of MVI ≤1 and MVI ≥2 patients segregated according to absence or presence of genetic iKIR-MM. Differences were only tested between groups with genetic iKIR-MM. (A–C) Patients with DSA and/or C4d (DSA/C4d⁺) are highlighted in grey and patients without DSA nor C4d (DSA⁻ C4d⁻) are represented in black as indicated. Individual MVI ≥2 patients with high iKIR-MM NK cells levels are identified with numbers. (D) Relation between pretransplant proportions (top) or numbers (bottom) of iKIR-MM NK cells and the number of genetic iKIR-MM. DSA, iKIR-MM, KT, MVI, NK.

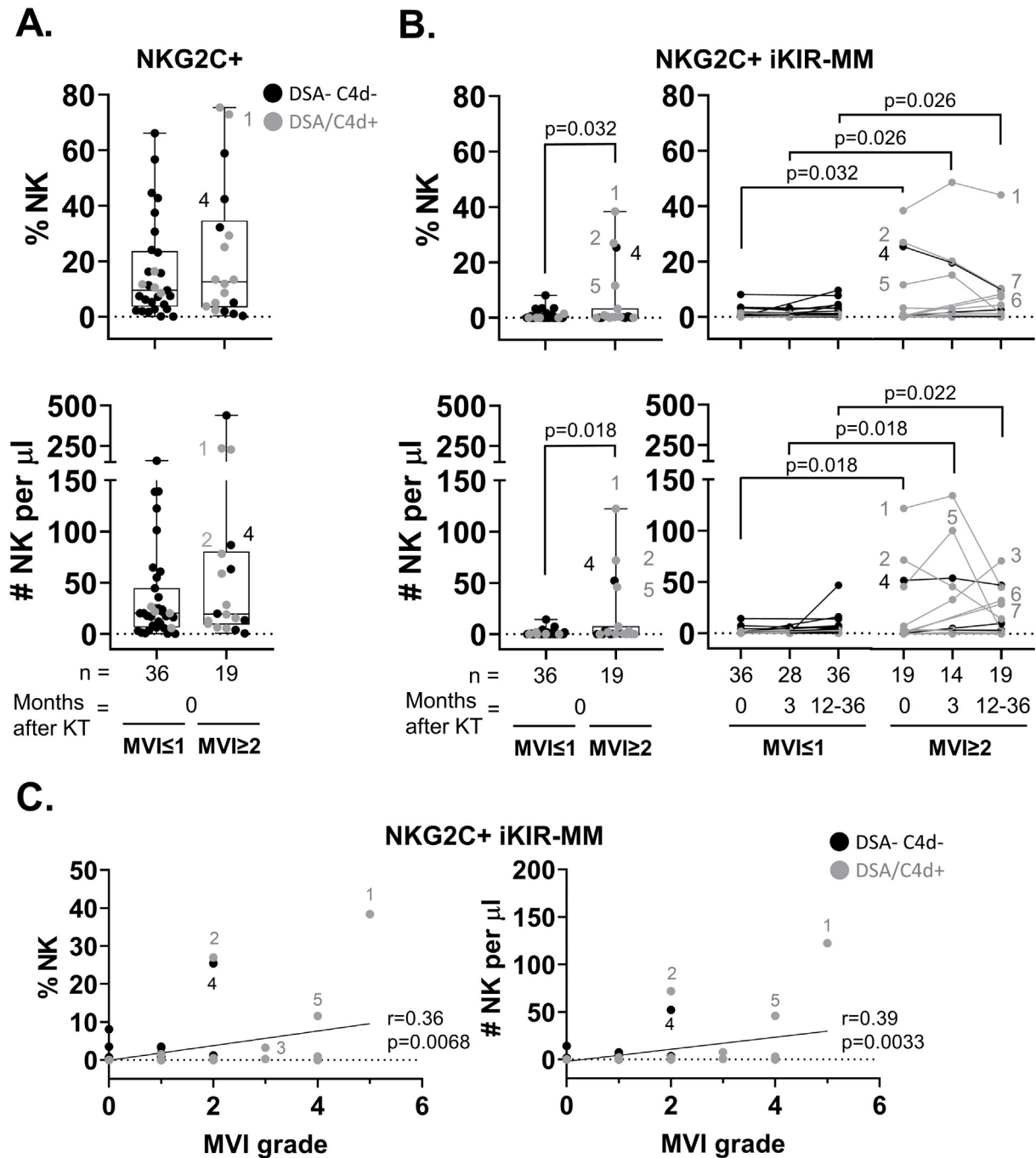


Figure 4. Detection of adaptive NKG2C+ iKIR-MM NK cells in kidney transplant recipients categorized according to MVI. (A, B) Proportions (top) and numbers (bottom) of NKG2C+ NK cells (A) or of NKG2C+ iKIR-MM NK cells identified pretransplant (A, B, bar graphs) and their evolution at 3 and 12-36 months posttransplant (B, connected dots graphs) are represented for all MVI ≤ 1 and MVI ≥ 2 patients. Statistically significant differences (Mann-Whitney test) between groups at equivalent time frames adjusted for false discovery rate are shown; the numbers of patient samples analyzed at each time frame are indicated. (C) Relation between pretransplant proportions (left) or numbers (right) of NKG2C+ iKIR-MM NK cells with MVI grade; significant results of Spearman tests are shown. (A-C) Cases with DSA and/or C4d (DSA/C4d+) are highlighted in grey and patients without DSA nor C4d (DSA- C4d-) are represented in black as indicated. Individual MVI ≥ 2 cases showing high levels of iKIR-MM NK cells are identified with a number code. DSA, iKIR-MM, KT, MVI, NKG2C, NK.

potential contribution to MVI. In fact, the increased iKIR-MM numbers observed in some MVI ≥ 2 cases at 3 months coincided with CMV viremia detection in that period. The NKG2C (*KLRC2*) gene copy number,⁵² which is related to adaptive NK cell expansions,^{32,35} has been associated with AMR by

others.^{27,53} Moreover, expression by renal graft infiltrating NK cells of CD57, a marker shared by adaptive NK cells,⁴³ has also been reported.⁵⁴ Additional, precise studies on the adaptive phenotype of infiltrating NK cells in MVI renal lesions are warranted.

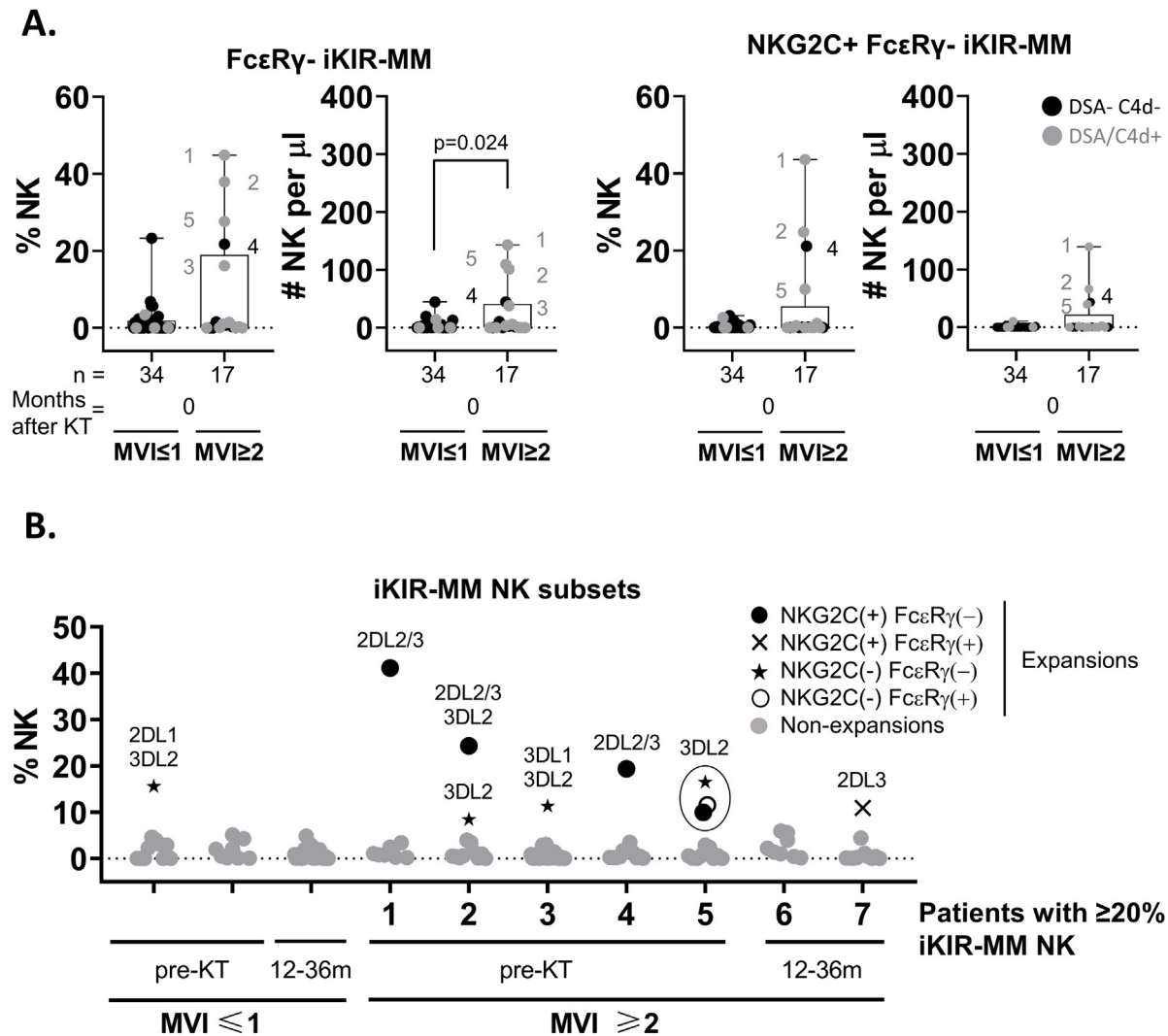


Fig. 5. Adaptive phenotypic profile of oligoclonal iKIR-MM NK cells expanded in MVI $\geq$ 2 cases according to NKG2C expression and FcεRγ-down-regulation. (A) Proportions and numbers of FcεRγ- (left) and NKG2C+ FcεRγ- iKIR-MM NK cells (right) detected in MVI $\leq$ 1 and MVI $\geq$ 2 patients; the time and the number of samples analyzed are indicated, and statistically significant differences (Mann-Whitney test) between groups are shown. MVI $\geq$ 2 patients with high percentages and numbers of iKIR-MM NK cells are identified with number codes. (B) Proportions of iKIR-MM NK cell subsets defined by the combined expression of KIR2DL1, KIR2DL2/3, KIR3DL1, KIR3DL2, NKG2A, NKG2C and FcεRγ expression are shown in cases reaching $\geq$ 20% of iKIR-MM NK cells, pre or posttransplant. KIR MM and adaptive features are specified for the expanded subsets. iKIR-MM, KT, MVI, NKG2C, NK.

Our retrospective study is exploratory and has several limitations. First, the patient sample size is small, due to the limited availability of donor HLA-C typing and of cryopreserved PBMC samples, as well as the need to exclude patients who had received lymphodepleting therapies that may reduce posttransplant NK cells or had suffered cellular rejection, to better delineate the role of iKIR-NK MM in the pathogenesis of MVI. Furthermore, considering the wide potential diversity of NK cell subsets⁴⁵ and the limited number of available cryopreserved cells, the sensitivity of our analysis does not allow a reliable detection of minor subsets, thus leaving uncertain their putative contribution to MVI by enhancing the effects of other concomitant pathogenic stimuli (ie, DSAs).

In summary, in our experience, phenotypic evaluation of peripheral alloreactive iKIR-HLA NK MM improves the understanding of the participation of NK cells in AMR/MVI, compared to just genotypic analysis. Further studies in larger KTR cohorts

are required to confirm whether identification and quantification of circulating alloreactive iKIR-MM NK cell subsets with an adaptive phenotype may contribute to predicting MVI risk.

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Declaration of competing interest

The authors of this manuscript have no conflicts of interest to disclose as described by *American Journal of Transplantation*.

Data availability

The data that support the findings of this study are available on request from the corresponding authors. The data are not publicly available due to privacy or ethical restrictions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ajt.2025.04.024>.

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