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# Impact of Immunosuppressive Drug Concentrations on Microvascular Inflammation, Negative Donor-Specific Antibodies, and C4d-Negative Status in Kidney Transplant Recipients

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## ABSTRACT

**Introduction:** This study investigated the impact of immunosuppressive drug concentrations on microvascular inflammation (MVI) in kidney transplant recipients with negative donor-specific antibodies (DSA) against human leukocyte antigen (HLA) and negative C4d deposition in peritubular capillaries.

**Methods:** We analyzed data from 268 living kidney transplant recipients at the Department of Urology, University of Osaka, Japan. Patients received immunosuppressive therapy comprising extended-release tacrolimus, mycophenolate mofetil (MMF), and/or everolimus, with or without steroids. Graft biopsies were routinely performed at 3, 12, 36 and 60 months post-surgery.

**Results:** No significant differences were observed between the MVI+DSA-C4d- and MVI-DSAC4d groups regarding graft survival rates (95.5% vs. 96.6%, $p = 0.772$) or patient survival rates (95.7% vs. 95.9%, $p = 0.735$). Lower tacrolimus and everolimus concentrations were significantly associated with an increased risk of MVI+DSA-C4d- (tacrolimus: OR, 0.169; 95% CI, 0.055–0.515; $p = 0.002$; everolimus: OR, 0.386; 95% CI, 0.171–0.874; $p = 0.022$). In contrast, MPA concentration was not significantly associated with MVI+DSA-C4d- (OR, 0.994; 95% CI, 0.554–1.780; $p = 0.984$). Steroid discontinuation did not significantly impact the risk of MVI+DSA-C4d- (OR, 1.980; 95% CI, 0.318–12.000; $p = 0.470$).

**Conclusion:** Lower trough levels of tacrolimus and everolimus correlated with a higher incidence of antibody-independent MVI, supporting the need for tailored immunosuppressive regimens in kidney transplantation.

**Abbreviations:** AMR, antibody-mediated rejection; CI, confidence interval; DSA, donor-specific anti-HLA antibodies; eGFR, estimated glomerular filtration rate; HLA, human leukocyte antigen; MVI, microvascular inflammation; MPA, mycophenolic acid; MMF, mycophenolate mofetil; NK cells, natural killer cells; OR, odds ratio.

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## 1 | Introduction

Kidney transplantation, the most established treatment for end-stage renal disease, significantly improves the quality of life and survival rates of patients compared with dialysis [1, 2]. However, the success of transplantation largely depends on the effectiveness of immunosuppressive therapies that maintain the balance between graft rejection and long-term adverse effects. Advances in immunosuppressive therapies have led to a reduction in the incidence of T cell-mediated rejection, with an increasing focus now on the suppression of antibody-mediated rejection (AMR) [3–6]. Immunosuppressive therapy remains critical for this purpose, with reports indicating that blood concentrations of tacrolimus can influence the development of de novo donor-specific antibodies (DSA) [7].

Microvascular inflammation (MVI) refers to inflammation of the microvasculature within the kidney graft, particularly in the capillaries and small arteries. MVI, characterized by the combined assessment of the Banff g score and ptc score (g+ptc) [8], used to be considered one of the findings associated with AMR. However, in the Banff 2022 report, a new classification was proposed: MVI, DSA-negative, and C4d-negative [9]. Possible causes for "MVI, DSA-negative and C4d-negative" include T cell-mediated rejection, natural killer (NK) cell activation, infections, ischemia-reperfusion injury, anti-non-human leukocyte antigen (HLA) antibodies, and thrombotic microangiopathy [10–13]. However, the precise causes and mechanisms remain unclear. Furthermore, there are conflicting reports regarding the prognosis of cases histopathologically diagnosed as AMR but negative for DSA or C4d. Some studies suggest a favorable prognosis [14, 15], whereas others report that MVI itself, irrespective of antibody dependence, affects graft survival [16, 17].

This study assessed the prognosis and underlying risk factors associated with MVI in kidney transplant recipients who were negative for DSA and lacked C4d deposition in the peritubular capillaries. By focusing on cases in which conventional indicators of AMR were absent, this study aimed to identify specific patient and clinical characteristics that may predispose patients to MVI and elucidate their impact on long-term graft survival and function. This investigation aimed to advance the understanding of MVI pathogenesis in DSA- and C4d-negative contexts, thereby contributing to improved post-transplant management strategies.

## 2 | Methods

### 2.1 | Ethics Statements

The study protocol was approved by the Research Ethics Committee of our institution (approval number: 21374) and was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent.

### 2.2 | Study Design and Population

This prospective observational study included 268 DSA-negative living kidney transplant recipients, excluding cases with preformed DSA-positive kidney transplants or those with de novo DSA or C4d deposition in peritubular capillaries, from the cases performed between 2013 and 2022, at the Department of Urology, Osaka Graduate School of Medicine. We did not exclude cases of C4d deposition in peritubular capillaries in ABO-incompatible kidney transplantation. The recipients received induction therapy with basiliximab and immunosuppressive therapy comprising extended-release tacrolimus, mycophenolate mofetil (MMF), and/or everolimus, with or without steroids. In cases of ABO-incompatible kidney transplantation, plasma exchange, and rituximab were also administered. The recipients were divided into two groups: MVI+DSA-C4d- ($n$ = 31) and MVI-DSA-C4d- ($n$ = 237).

For ABO blood-type compatible kidney transplantation, tacrolimus was started at a dose of 0.15 mg/kg/d 4 days before transplantation, and trough levels were adjusted to 5–8 ng/mL after transplantation. MMF was started at a dose of 1000 mg/d 4 days before transplantation and was adjusted to 2000 mg/d for 2 weeks post-transplantation, 1500 mg/d during post-transplantation week 2–4, and 1000 mg/d after 4 weeks post-transplantation. Everolimus was initiated at a dose of 3 mg after transplantation and adjusted to achieve a trough level of 3–8 ng/mL. Steroids were discontinued 22 days after kidney transplantation. For ABO blood type-incompatible kidney transplantation, MMF was started 14 days before transplantation, and tacrolimus was started 7 days before transplantation and adjusted after transplantation, as in blood type-compatible cases. Patients who underwent ABO-incompatible kidney transplantation received rituximab infusion and plasma exchange. No recipients had donor-specific anti-HLA antibodies.

Graft biopsies were routinely performed 3, 12, 36, and 60 months after kidney transplantation. Additionally, biopsies were performed in patients with elevated serum creatinine levels. The pathological diagnosis was conducted according to the Banff 2022 guidelines, with MVI defined using a threshold of g+ptc≥2 [10]. All biopsy specimens were re-evaluated retrospectively in accordance with the Banff 2022 to ensure consistent pathological assessment. Two independent transplant pathologists, blinded to clinical outcomes, reviewed all available biopsy specimens. Discrepancies were resolved through consensus. In cases of ABO-incompatible kidney transplantation, if C4d deposition was present but DSA was negative, it was considered C4d-negative.

### 2.3 | Definitions

Graft failure was defined as the return to dialysis. Mortality was defined as death owing to any cause. The estimated glomerular filtration rate (eGFR) was calculated using a modified Japanese equation [18]. Recipients undergoing treatment for dyslipidemia were defined as having dyslipidemia. Clinical data, including laboratory data, were collected monthly after kidney transplantation.

### 2.4 | Statistical Analysis

Data are presented as means with standard deviation and frequency (percentage). A $t$-test was used to analyze continuous

**TABLE 1** | Patient background characteristics.

| Variable | MVI+DSA-C4d- | MVI-DSA-C4d- | *p*-value |
|---|---|---|---|
| Recipient | | | |
| Age (years) | 50.68±13.70 | 50.18±13.67 | 0.848 |
| Female sex (%) | 34.6 | 35.5 | 1.000 |
| Body mass index (kg/m$^2$) | 22.33 ± 3.45 | 22.12 ± 4.11 | 0.791 |
| Duration of dialysis (IQR) | 2.73 (0.86-3.19) | 4.55 (0.87-5.05) | 0.20 |
| Cause of ESRD (%) | | | 0.870 |
| Chronic glomerulonephritis | 25.8 | 16.0 | |
| IgA nephropathy | 19.4 | 16.9 | |
| Diabetic nephropathy | 19.4 | 21.1 | |
| Polycystic kidney disease | 3.2 | 2.5 | |
| FSGS | 3.2 | 6.8 | |
| Nephrosclerosis | 3.2 | 5.5 | |
| Lupus nephritis | 3.2 | 2.5 | |
| Others | 22.6 | 28.7 | |
| HLA mismatch | | | |
| Class I | 3.56 ± 1.36 | 3.14 ± 1.57 | 0.239 |
| Class II | 2.28 ± 1.21 | 1.81 ± 1.18 | 0.064 |
| Everolimus (%) | 74.2 | 75.1 | 1.000 |
| Steroid off (%) | 51.6 | 32.9 | 0.047 |
| ABO incompatible (%) | 25.8 | 37.1 | 0.239 |
| Observation period | 6.02 ± 2.17 | 5.25 ± 2.31 | 0.081 |
| Donor | | | |
| Age (y) | 59.90 ± 9.92 | 60.12 ± 11.26 | 0.920 |
| Female sex (%) | 67.7 | 92.8 | 0.693 |
| Body mass index (kg/m$^2$) | | | |

Abbreviations: ESRD, end-stage renal disease; FSGS, focal segmental glomerulosclerosis; HLA, human leukocyte antigen; IgA, immunoglobulin A; IQR, interquartile range.

parameters with skewed normal distributions. Non-normally distributed variables were compared between groups using the Mann–Whitney *U* test; the findings are presented as medians with interquartile ranges (25%–75%). The $\chi^2$-test or Fisher's exact was used to compare differences in the proportions of nominal-level variables. Multivariate logistic regression analysis was performed to identify independent risk factors for MVI+DSA-C4d-. All data were analyzed using the REDCap electronic registration software (Vanderbilt University, Nashville, TN, USA), and all statistical analyses were performed using R software (version 4.3.1; The R Project for Statistical Computing Vienna, Austria). Statistical significance was set at a two-tailed $p$ value $< 0.05$.

## 3 | Results

### 3.1 | Patient Demographics

Baseline characteristics exhibited no statistically significant differences between the MVI+DSA-C4d- and MVI-DSA-C4d cohorts (Table 1). Variables such as recipient age (50.68 ± 13.70 vs. 50.18 ± 13.67 years, $p = 0.848$), body mass index (22.33 ± 3.45 vs. 22.12 ± 4.11 kg/m$^2$, $p = 0.791$), and dialysis duration (median 2.73 vs. 4.55 years, $p = 0.20$) demonstrated equivalent distributions. End-stage renal disease etiologies and HLA mismatch frequencies were similar across the groups. Notably, steroid withdrawal was significantly more prevalent in the MVI+DSA-C4d- group (51.6% vs. 32.9%, $p = 0.047$), indicating a potential correlation with the incidence of MVI+DSA-C4d-.

### 3.2 | The Details of MVI Scores

The g scores and ptc scores at the time of diagnosis and the final follow-up biopsies (87.1% conducted at 60 months and the remaining at 36 months) are shown in Table 2. At the time of diagnosis, 22 cases (71.0%) were classified as g1+ptc1, five cases (16.1%) as g1+ptc2, two cases (6.5%) as g2+ptc2, and one case each (3.2%) as g1+ptc3 and g3+ptc3. In the follow-up biopsies, 17 cases (54.8%) showed resolution of MVI, while three cases (9.7%)

**TABLE 2** | The details of MVI scores at the time of diagnosis and at the final follow-up biopsies.

| | Diagnosis | Follow-up |
|---|---|---|
| g score | | |
| 0 | 0% | 64.5% |
| 1 | 90.3% | 29.0% |
| 2 | 6.5% | 6.5% |
| 3 | 3.2% | 0% |
| ptc score | | |
| 0 | 0% | 64.5% |
| 1 | 70.9% | 25.8% |
| 2 | 22.6% | 9.7% |
| 3 | 6.5% | 0% |

showed resolution of ptc but persistence of g, and another three cases (9.7%) showed persistence of ptc only. MVI persisted in eight cases (25.8%). In all cases where MVI persisted, the MVI scores remained stable or showed a trend toward improvement. Transplant glomerulopathy was observed in only one case. The proportion of g1+ptc1 was 69.6% in ABO-compatible kidney transplantation and 75.0% in ABO-incompatible cases, with no indication that ABO-incompatible cases had higher MVI scores. No cases showed DSA or C4d deposition during the observation period.

### 3.3 | Graft and Patient Survival

Graft survival rates were not statistically different between groups, with a 5-year survival rate of 95.5% in the MVI+DSA-C4d- group versus 96.6% in the MVI-DSA-C4d- group ($p = 0.772$, Figure 1a). Additionally, patient survival at 5 years showed no significant differences between the groups (95.7% vs. 95.9%, $p = 0.735$, Figure 1b). Furthermore, we compared the groups with positive and negative g status among those negative for DSA and C4d; however, no significant differences were observed in graft or patient survival rates. Similar results were observed for the ptc status.

### 3.4 | Immunosuppressive Drug Concentrations

Low trough concentrations of tacrolimus and everolimus were significantly associated with a higher rate of antibody-independent MVI. Among those negative for DSA and C4d, comparisons between groups with positive and negative g status, as well as ptc status, revealed no significant differences in graft or patient survival rates. Tacrolimus levels in the MVI+DSA-C4d- group averaged 3.10 ± 0.94 ng/mL, which was significantly lower than the average of 5.25 ± 1.54 ng/mL in the MVI-DSA-C4d- group. Everolimus concentrations were also lower in the MVI+DSA-C4d group (2.96 ± 1.16 ng/mL vs. 4.37 ± 1.38 ng/mL). The odds ratios (ORs) were 0.169 (95% confidence intervals [CI], 0.055–0.515; $p = 0.002$) for tacrolimus and 0.386 (95% CI, 0.171–0.874; $p = 0.022$) for everolimus. Conversely, mycophenolic acid (MPA) levels and (OR, 0.994; 95% CI, 0.554–1.780; $p = 0.984$) steroid withdrawal (OR, 1.980; 95% CI, 0.318–12.000; $p = 0.470$) exhibited no significant associations with MVI+DSA-C4d- risk. Both the g+DSA-C4d and ptc+DSA-C4d groups had lower trough concentrations of these drugs than their respective negative counterparts. Multivariate models further substantiated that lower trough concentrations of tacrolimus (OR, 0.169; 95% CI, 0.056–0.515; $p = 0.0018$) and everolimus (OR, 0.386; 95% CI, 0.171–0.874; $p = 0.0224$) independently correlated with MVI+DSA-C4d- onset (Table 3). In contrast, MPA levels and steroid discontinuation were not statistically significant predictors of MVI+DSA-C4d- in either univariate or multivariate analyses.

### 3.5 | Graft Function

Graft function, indicated by creatinine levels, eGFR, and urinary protein levels, did not show statistically significant differences between the MVI+DSA-C4d- and MVI-DSA-C4d- groups. The mean creatinine level was 1.30 ± 0.43 mg/dL in the MVI+DSA-C4d- group and 1.38 ± 0.46 mg/dL in the MVI-DSA-C4d- group ($p = 0.370$). Similarly, the eGFR was comparable between the groups (41.54 ± 19.04 mL/min/1.73 $m^2$ in the MVI+DSA-C4d- group and 43.66 ± 15.05 mL/min/1.73 $m^2$ in the MVI-DSA-C4d- group; $p = 0.566$). Urinary protein levels, measured as both mg/dL and g/Cr, did not differ significantly between the groups, with $p$ values of 0.117 and 0.427, respectively. These results suggest that the preservation of graft function was similar in both groups, regardless of the MVI+DSA-C4d- status.

### 3.6 | Clinical Complications and Additional Variables

The incidences of viral infections, cardiovascular disease, hypertension, and dyslipidemia were statistically comparable between the groups. Similarly, the occurrence rates of new-onset diabetes mellitus post-transplantation and de novo DSA did not differ significantly, suggesting that MVI+DSA-C4d- status did not exert a notable influence on these post-transplantation complications (Table 4).

## 4 | Discussion

This study provides new insights into the effects of immunosuppressive drug concentrations on antibody-independent MVI in kidney transplant patients and highlights the potential of maintaining optimal tacrolimus and everolimus levels to reduce the incidence of MVI without the need for steroids.

MVI in kidney transplant recipients is characterized by inflammation of small blood vessels in the transplanted kidney [8]. The Banff 2022 meeting introduced significant updates to the classification of AMR and MVI in kidney transplant pathology [9]. One of the key additions was the recognition of a specific phenotype of MVI that occurs in the absence of DSA and without C4d deposition in peritubular capillaries. Such cases are often grouped into broader categories that do not specifically account for their unique pathological features. This sometimes leads to confusion during diagnosis and treatment

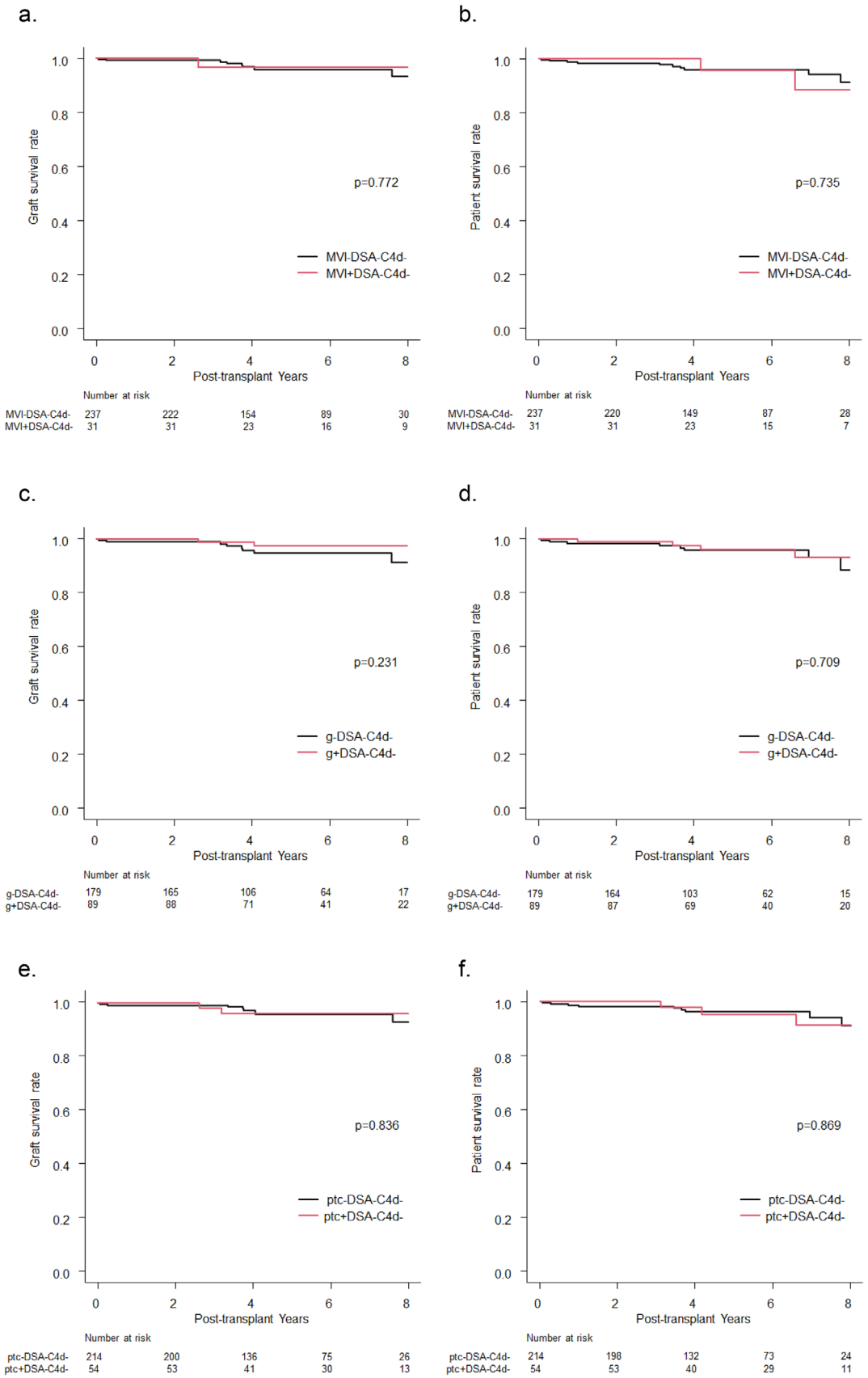


**FIGURE 1** | Patient and graft outcomes. (a) Death-censored graft survival and (b) patient survival in the MVI+DSA-C4d- and MVI-DSA-C4d- groups. (c) Death-censored graft survival and (d) patient survival in the g+DSA-C4d- and g-DSA-C4d- groups. (e) Death-censored graft survival and (f) patient survival in the ptc+DSA-C4d- and ptc-DSA-C4d- groups. DSA, donor-specific antibodies; MVI, microvascular inflammation.

**TABLE 3** | Univariate and multivariate analyses of MVI+DSA-C4d.

| | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| | **Odds ratio** | **95% CI** | ***p* value** | **Odds ratio** | **95% CI** | ***p* value** |
| Tacrolimus | 0.191 | 0.098–0.371 | <0.001 | 0.169 | 0.056–0.515 | 0.002 |
| Everolimus | 0.42 | 0.267–0.668 | <0.001 | 0.386 | 0.171–0.874 | 0.022 |
| MPA | 0.784 | 0.533–1.150 | 0.216 | 0.994 | 0.554–1.780 | 0.984 |
| Steroid off | 2.170 | 1.020–4.620 | 0.043 | 1.950 | 0.378–12.00 | 0.470 |

Abbreviations: CI, confidence interval; DSA, donor-specific antibodies; MVI, microvascular inflammation.

**TABLE 4** | Graft function and incidence of complications in the MVI+DSA-C4d- and MVI-DSA-C4d-groups.

| **Variable** | **MVI+DSA-C4d- (n=31)** | **MVI-DSA-C4d- (n=237)** | **p-value** |
|---|---|---|---|
| Graft function | | | |
| Creatinine level (mg/dL) | 1.30 ± 0.43 | 1.38 ± 0.46 | 0.370 |
| Estimated GFR (ml/min/1.73 $m^2$) | 41.54 ± 19.04 | 43.66 ± 15.05 | 0.566 |
| Urinary protein level (mg/dL) (IQR) | 44.65 (7.0–50.0) | 28.4 (8.0–30.0) | 0.117 |
| Urinary protein level (g/Cr) (IQR) | 0.11 (0.07–0.11) | 0.24 (0.07–0.21) | 0.427 |
| Complications (%) | | | |
| Cytomegalovirus infection | 6.5 | 11.4 | 0.578 |
| COVID-19 infection | 29.0 | 24.5 | 0.659 |
| Cardiovascular disease | 6.5 | 7.6 | 1.000 |
| NODAT | 6.5 | 3.4 | 0.325 |
| Hypertension | 54.8 | 58.6 | 0.702 |
| Dyslipidemia | 45.2 | 38.8 | 0.559 |
| de novo DSA | 9.7 | 8.0 | 0.728 |

Abbreviations: COVID-19, coronavirus disease; DSA, donor-specific antibodies; GFR, glomerular filtration rate; IQR, interquartile range; MVI, microvascular inflammation; NODAT, new-onset diabetes mellitus after transplantation.

because the mechanisms underlying these conditions are not well understood. Inflammation without the presence of DSA and C4d deposition in the peritubular capillaries suggests a different mechanism compared with typical antibody-mediated rejection [10–13]. The absence of DSA indicates that the inflammation is not caused by antibodies targeting donor cells, and the lack of C4d deposition further supports this as it indicates no activation of the complement pathway. The Banff 2022 classification will facilitate more precise diagnostic and therapeutic approaches, encouraging further research into the underlying causes and optimal management of MVI in the absence of DSA and C4d.

Recent insights into MVI have reshaped the conventional view that antibodies and complement are the sole contributors to MVI development. These findings reveal that MVI can arise independent of antibody involvement. Antibody-independent MVI appears to be driven by the activation of NK cells via a "missing self" mechanism, a mismatch between donor HLA and recipient inhibitory killer-cell immunoglobulin-like receptors that ultimately disrupts the immune equilibrium. The resulting NK cell activation inflicts damage on graft endothelial cells, affecting graft survival. Survival analyses show that patients with MVI (both MVI+DSA+ and MVI+DSA-) experience significantly poorer outcomes compared with those without MVI (MVI-DSA-) [16].

However, in our study, the presence or absence of MVI, DSA-negative status, and C4d-negative status did not significantly affect patient prognosis. Several factors may be responsible for this discrepancy. First, cases classified as MVI, DSA-negative, and C4d-negative may include instances where mechanisms unrelated to the "missing self", such as ischemia-reperfusion injury, non-HLA antibody involvement, or other immune interactions, are at play. This reflects the complexity and diversity of post-transplantation immune interactions. Second, once MVI is identified in kidney transplant biopsies in clinical practice, timely modifications are made to the immunosuppressive therapy, including dose increases or delays in reduction. These responsive adjustments may contribute to mitigating the adverse effects typically associated with MVI and could account for the improved outcomes observed in our analysis. Furthermore, all cases of MVI in this study were identified through protocol biopsies, which may explain the lack of adverse outcomes associated with MVI+DSA-C4d-. Protocol biopsies enable the early detection of subclinical inflammation and allow timely adjustments to immunosuppressive regimens, potentially mitigating the

progression of MVI and preserving graft function. In contrast, MVI identified in for-cause biopsies, often performed in response to graft dysfunction, may reflect more severe immune injury and could be associated with worse outcomes. This distinction underscores the importance of routine protocol biopsies in identifying and managing MVI before it manifests as clinically significant graft dysfunction. These findings highlight the need for ongoing exploration of MVI pathogenesis and its impact on graft survival, particularly in DSA-negative and C4d-negative cases, as well as the role of protocol biopsies in mitigating these effects.

These findings underscore the need for ongoing exploration of the influence of MVI on transplant prognosis, especially with the emergence of new therapeutic strategies. In particular, the discovery that NK cell activation by the "missing self" mechanism is mediated via the mTORC1 pathway highlights a promising therapeutic target [16]. In preclinical studies, the mTOR inhibitor rapamycin has shown efficacy in curbing the progression of chronic vascular rejection associated with the "missing self" mechanism. In our study, we observed a correlation between blood everolimus levels and MVI, DSA-negative status, and C4d-negative status, suggesting a potential protective effect. Everolimus has been reported to inhibit both mTORC1 and mTORC2 more effectively than sirolimus, with the inhibition of mTORC2 specifically suppressing endothelial cell functional changes following HLA class I cross-linking, which are implicated in chronic rejection [19]. However, its effects on NK cells remain unclear, warranting further investigation.

Multiple studies have established a relationship between tacrolimus blood concentration and the emergence of de novo DSA and AMR [7, 20]. Although our findings indicate that tacrolimus may be correlated with MVI, DSA-negative status, and C4d-negative status, this is likely due to its effect on weak antigen-antibody responses or T cell-mediated reactions. These findings imply that although both everolimus and tacrolimus appear to reduce the incidence of MVI and DSA-negative- and C4d-negative cases, their mechanisms of action may differ fundamentally. Although the utility of regimens combining tacrolimus and everolimus has been previously reported [21], our findings provide valuable insights for further investigations regarding optimal immunosuppressive strategies to address antibody-independent MVI and improve overall transplant outcomes.

The univariate analysis showed a statistically significant association between steroid withdrawal and MVI+DSA-C4d- incidence, but this association was not observed in the multivariate analysis. This discrepancy suggests that steroid withdrawal may not independently predict MVI risk and that other factors, such as tacrolimus and everolimus levels, play a more significant role. Although our findings emphasize the importance of maintaining optimal tacrolimus and everolimus levels, the role of steroids in mitigating MVI cannot be entirely excluded and warrants further investigation in larger cohorts.

This study has several limitations, including its small sample size, single-center design, observational nature restricting causality assessment, short observation period, clinical heterogeneity, and the potential influence of confounding factors. Additionally, this study exclusively focused on living donor kidney transplant recipients, which limits the generalizability of the findings to deceased donor transplant. Differences in immunological risk profiles, ischemia-reperfusion injury, and graft survival outcomes between living and deceased donor transplants may influence the incidence and clinical relevance of MVI. Furthermore, this study relied on trough concentrations of immunosuppressive agents as a surrogate for total exposure, which is a recognized limitation. Trough levels, although practical for routine clinical use, may not fully capture total drug exposure, particularly for MMF, whose area under the curve (AUC) may better correlate with its pharmacodynamic effects. The lack of association between MMF levels and MVI+DSA-D4d in our study may partly be attributed to this limitation. Further studies employing pharmacokinetic modeling or AUC measurements could provide more robust insights into the relationship between drug exposure and MVI risk. Nevertheless, this study provides valuable new insights into DSA-negative- and C4d-negative MVI and highlights the impact of immunosuppressive drug concentrations on the incidence of MVI. These findings provide an important foundation for future multicenter studies and long-term prospective trials aimed at obtaining comprehensive and reliable data to refine immunosuppressive management strategies. It is important to note that this study excluded cases with preformed DSA. Early postoperative DSA-negative and D4d-negative MVI in recipients with preformed DSA may require careful attention, as it could pose a risk of progressing to AMR.

In conclusion, the current study underscores the significance of maintaining appropriate tacrolimus and everolimus trough levels to manage DSA-negative- and C4d-negative MVI, thereby providing insights into the nuanced effects of immunosuppression beyond conventional antibody-mediated pathways. These findings suggest that maintaining optimal levels of these drugs may help prevent adverse graft outcomes associated with antibody-independent MVI. Future studies should investigate the molecular mechanisms by which tacrolimus and everolimus modulate immune responses in kidney transplantation. This may involve exploring the roles of individual drug metabolism, patient genetic profiles, and interactions with NK cell pathways, potentially paving the way for a more personalized and effective approach to immunosuppressive therapy in kidney transplantation.

### Author Contributions

Yoichi Kakuta contributed to the research design, writing of the manuscript, research performance, and data analysis. Yoko Maegawa-Higa contributed to the research design. Soichi Matsumura contributed to the research design and data analysis. Shota Fukae contributed to the research design and data analysis. Ryo Tanaka contributed to the research design and data analysis. Hiroaki Yonishi contributed to the research design, investigation, and data analysis. Shigeaki Nakazawa contributed to the research design, investigation, and data analysis. K.Y. contributed to the research design, investigation, and data analysis. Tomoko Namba-Hamano contributed to the research design and pathological diagnosis. Yoshitaka Isaka contributed to the research design and investigation. Norio Nonomura contributed to the research design and investigation.

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The authors have nothing to report.

## Conflicts of Interest

The authors declare no conflicts of interest.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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