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Mast Cells in Kidney Transplant Biopsies With Borderline T Cell-mediated Rejection and Their Relation to Chronicity

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Background. Mast cells are potential contributors to chronic changes in kidney transplants (KTx). Here, the role of mast cells (MCs) in KTx is investigated in patients with minimal inflammatory lesions. **Methods.** Fourty-seven KTx biopsies (2009–2018) with borderline pathological evidence for T cell-mediated rejection according to the Banff'17 Update were retrospectively included and corresponding clinical data was collected. Immunohistochemistry for tryptase was performed on formalin-fixed paraffin-embedded sections. Cortical MCs were counted and corrected for area (MC/mm^2). Interstitial fibrosis was assessed by Sirius Red staining and quantified using digital image analysis (QuPath). **Results.** Increased MC number was correlated to donor age (spearman's $r = 0.35$, $P = 0.022$), deceased donor kidneys (mean difference = 0.74, t [32.5] = 2.21, $P = 0.035$), and delayed graft function (MD = 0.78, t [33.9] = 2.43, $P = 0.020$). Increased MC number was also correlated to the amount of interstitial fibrosis ($r = 0.42$, $P = 0.003$) but did not correlate with transplant function over time ($r = -0.14$, $P = 0.36$). Additionally, transplant survival 2 y post-biopsy was not correlated to MC number (mean difference = −0.02, t [15.36] = −0.06, $P = 0.96$). **Conclusions.** MC number in suspicious (borderline) for acute T cell-mediated rejection is correlated to interstitial fibrosis and time post-transplantation, suggesting MCs to be a marker for cumulative burden of tissue injury. There was no association between MCs and transplant function over time or transplant survival 2 y post-biopsy. It remains unclear whether MCs are just a bystander or have pro-inflammatory or anti-inflammatory effects in the KTx with minimal lesions.

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The immune cell repertoire seen in acute T cell-mediated rejection (TCMR) is large, including cells as activated cytotoxic T-cells (CD8+), B-cells, plasma cells, NK-cells, macrophages, and mast cells (MCs). MCs are innate immune cells that possess immune-modulatory mediators, which can be released upon contact with pathogens, cellular/tissue injury, or interactions with other immune cells.[1-3]

Activated MCs primed with IgE interact with various T cells such as helper T cells, CD8+, and regulatory T cells. The crosstalk with T cells and, in particular, with CD8+ can result in the development of an acute TCMR in the kidney transplant (KTx) because it is known that CD8+ T cells are a main player in transplant rejection.[4,5] Research has shown that in the setting of acute KTx rejection, MCs are increased.[5,6] Additionally, in chronic transplant rejection there is also an increase in MCs number.[7-9]

According to the Banff Classification for Allograft Pathology, the diagnosis "suspicious (borderline) for TCMR (bTCMR)" is currently defined as foci of tubulitis (t1 or greater) with mild interstitial inflammation (i1), or mild (t1) tubulitis with moderate–severe interstitial inflammation (i2 or i3).[10] The term "bTCMR" can be interpreted in 2 ways: (1) it can imply that the diagnosis of TCMR is present, but it falls short of meeting the criteria established by the Banff diagnostic threshold or (2) it can indicate that the finding is unclear and may or may not be TCMR. Nankivell et al propose that bTCMR might be under-sampled form of TCMR and is considered an unfavorable diagnostic category, characterized by increased acute and chronic tubular injury, progressive nephron destruction, dnDSA generation, and new-onset antibody-mediated morphologic changes, allograft dysfunction, and ultimately transplant failure.[11] Multivariable predictors of functional recovery after bTCMR are indication biopsy, earlier post-transplantation (Tx) time, and delayed graft function (DGF). Failed functional recovery after bTCMR occurs in 25%–58% and was predicted by interstitial fibrosis and tubular atrophy, creatinine levels, and chronic vascular scores.[12,13] This suggests that bTCMR could also be a phase of rejection in which the trajectory is not set in stone or a histologic finding that is treated by some and not treated by others.

MCs are known to be related to the development of interstitial fibrosis in different settings and through different mechanisms, including allergic reactions and KTx immunological mechanisms.[3,7-9,14-20] Research on the relationship between MCs and fibrosis in KTx patients is limited, and of those, most identify a pro-fibrotic role of MCs.[8] Papadimitriou et al found a strong relationship between MCs and both interstitial fibrosis and time post-Tx, suggesting MCs to be a marker for cumulative burden of tissue injury.[21] Additionally, Mengel et al found a distinct pattern of inflammatory molecules, particularly MC-associated transcripts, in scarred areas in KTx biopsies, which correlated with poor outcomes.[22] Multiple studies in other solid organ transplants, such as the heart, have demonstrated that MCs also play a role in chronic inflammation and interstitial fibrosis.[23-25] In contrast, some studies suggest that MCs can also be mediators for allograft tolerance.[26-30] This paradoxical relationship between MCs and fibrosis could be related to intricate cascades of cytokines and proteinases. MCs are currently not mentioned in the Banff Classification, as the clinical impact of these cells in KTx remains unclear.[10]

This article reports the presence of MCs in the KTx biopsies classified as bTCMR, in particular in relation to interstitial fibrosis and transplant outcome. The presence of MCs and fibrosis in KTx biopsies was visualized by multiplex immunohistochemistry and correlated to graft function and outcome at 2 y post-biopsy.

MATERIALS AND METHODS

Patients

We performed a retrospective search in the database of the Department of Pathology, Erasmus Medical Center, Rotterdam, The Netherlands. All biopsies with the diagnosis bTCMR according to the Banff'17 Update between 2009 and 2018 were included. The exclusion criteria were BK viremia, biopsies with antibody-mediated rejection, and biopsies with a TCMR in the 3 mo before the indication biopsy. Patient characteristics and demographics were collected with a follow-up time of 2 y.

The status of the graft function after 2 y was noted (such as function, no function, death of the patient, or unknown). No function was defined as return to dialysis. DGF was defined as the need for renal replacement therapy within the first week after KTx.

This research has been performed following the Declaration of Helsinki.[31] All transplant procedures have been performed in accordance with the Declaration of Istanbul.[32] No transplants from prisoners have been used. Ethical approval of the inclusion of these patients was obtained from the Medical Ethical Review Committee MEC-2019-0308.

Materials

Formalin-fixed paraffin-embedded (FFPE) for-cause KTx biopsies, were obtained from the local archives of the Erasmus Medical Center Pathology department. Two-micrometer sections were stained by Hematoxylin and Eosin, Period Acid Schiff, Jones, Trichrome, and Elastin van Gieson techniques according to standard diagnostic protocol. Additionally, immunohistochemistry for C4d was performed on a 4-μm FFPE section. Cases were re-evaluated for the diagnosis of bTCMR (including scoring for the individual lesion "i" and "t") and assessed for adequacy according to the Banff'17 Update (J.P.D., M.R., and M.C.v.G.).[10,33,34] An extra 4-μm FFPE section containing tonsil tissue as a positive control and liver tissue as a negative control was included on each slide as a quality control of the multiplex immunohistochemical staining.

Sirius Red

To assess the amount of fibrosis, that is, collagen I and III depositions, we performed a Sirius Red staining. Four-micrometer FFPE sections were cut and put on glass slides and subsequently baked for 20 min at 60°C. Slides were de-paraffinized and rehydrated through a passage through decreasing ethanol series. The slides were then pre-differentiated for 5 min using 0.2% phosphomolybdic acid followed by 45 min of incubation with 0.1% Sirius Red solution.

Immunohistochemistry for Tryptase and CD34

Tryptase was used to detect MCs; CD34 staining, an endothelial cell marker, was used to visualize the glomerular and tubular interstitial compartments. The corresponding second section of each biopsy was stained by chromogenic multiplex staining: briefly, after de-paraffinization, CC1

(#950-124; Ventana Medical Systems, Tucson, AZ) antigen retrieval was performed for 64 min at 95°C, followed by incubation for 8 min with the Discovery inhibitor (#760-4840; Ventana Medical Systems). Primary antibody CD34 (#790-2927; Ventana Medical Systems) was incubated for 32 min at 37°C, followed by detection with OmniMap goat-anti-rabbit HRP (#760-4311; Ventana Medical Systems), and visualized using purple kit for 32 min. A CC2 (#950-123, Ventana Medical Systems) 100°C stripping step was performed for 8 min. Tryptase (#760-4276; Ventana Medical Systems) was incubated at 37°C for 32 min, followed by secondary antibody, OmniMap goat-anti-mouse HRP (#760-4310; Ventana Medical Systems) at 37°C for 24 min, and visualized with 3,3′-Diaminobenzidine (#760-229; Ventana Medical Systems) for 32 min. Finally, Hematoxylin II (#790-2208; Ventana Medical Systems) was used to counter stain for 8 min and then a blue coloring reagent (#760-2037; Ventana Medical Systems) for 4 min according to the manufactures instructions (Ventana Medical Systems).

Digital Quantification

After staining, the slides were scanned with a digital scanner (Hamamatsu NanoZoomer, Japan) under 40x enlargement for further analysis, resulting in a resolution of 0.2277 μm/pixel. The scanned slides were analyzed using QuPath: Quantitative Pathology and Bioimage analysis software, version 0.2.3.[35] Cortical areas were annotated by hand, with the following structures being excluded from the annotation: the outer renal capsule; all medium- and large-sized arteries, veins, arterioles, and venules bigger than the adjacent tubule; tissue artifacts that in any way could distort the analysis. A pixel thresholder was used to calculate the total area stained by Sirius Red. MCs were counted using the QuPath manual counting function.

Statistical Analysis

Statistical analysis was performed using SPSS software version 25 (IBM Corp., 2017). Mann-Whitney U tests were performed to compare clinical and biological data with graft function. Independent samples (Student's) t-tests and Welch's tests were performed to compare biopsy data with clinical outcome parameters. G-tests (log-likelihood ratio, a derivative of χ^2) were performed between categorical variables (donor-type, [no] DGF, and graft function/loss at t1). A 2-sided significance level (α) of 0.05 was used in all analyses.

RESULTS

Patient Characteristics

A total of 53 biopsies classified as bTCMR according to the 2017 Banff Update classification were included. Twenty-eight biopsies had no inflammation or <10% inflammation of unscarred cortical parenchyma (i0) and 25 biopsies had inflammation in 10%–25% of unscarred cortical parenchyma (i1). Five samples were excluded because of an inadequate amount of cortical tissue in accordance with the Banff Classification. Table 1 shows the demographic and Tx characteristics, including age, sex, graft type, human leukocyte antigen mismatch, presence of DGF, graft loss, and follow-up of serum creatinine. Patient characteristics in relation to graft outcome are depicted in Table 2. Donor age at Tx, DGF, and serum creatinine at the time of biopsy are correlated to inferior outcomes.

TABLE 1.

Patient demographics and Tx characteristics

Characteristic	n = 48 (%)
Recipient sex	
Male	36 (75)
Female	12 (25)
Recipient age at Tx	
Mean (SD), range (min to max)	49.02 (15.66), 18 to 78
Donor type	
Living	32 (66.7)
Circulatory death	10 (20.8)
Brain death	6 (12.5)
Donor age at Tx	
Mean (SD), range (min to max)	52.74 (13.57), 8 to 71
D since Tx	
Mean (SD), range (min to max)	193.69 (341.24), 4 to 1321
Status at y 2 (±3 mo)	
Functioning graft	36 (75)
Graft loss	8 (16.7)
Death	2 (4.2)
"Other"	2 (4.2)
HLA mismatch[a]	
0	4 (8.3)
1	1 (2.1)
2	5 (10.4)
3	14 (29.2)
4	9 (18.8)
5	9 (18.8)
6	6 (12.5)
Delayed graft function* (DGF)	
DGF	15 (25.5)
No DGF	32 (74.5)
Serum creatinine at biopsy	
Mean (SD), range (min to max)	201 (57.06), 125 to 400
Serum creatinine slope	
Mean (SD), range (min to max)	−0.93 (16.99), −31.32 to 53.76

[a]HLA mismatch was calculated by combining HLA-A, HLA-B, and HLA-DR mismatch scores.
*n = 47.
DGF, delayed graft function; Tx, transplant.

MCs

MCs were visualized in the cortical area of the biopsy by tryptase staining (the medulla was excluded from analysis). MCs were normalized for tissue area to MCs per mm² (MC/mm²). On average, 10.79 MCs were found per mm² in the cortical area of the biopsy, ranging from 0 MC/mm² to 76.55 MC/mm² (Table 3). Because MC/mm² was distributed logarithmically, lognormal conversion was applied ((ln)MC/mm²). One patient was removed from the analysis because of a count of zero MC/mm² (Z = −4.24).

MCs were primarily present in the interstitial compartment. Fewer MCs were present in or directly under the capsular tissue or in the area surrounding vascular tissue, especially on the border of inflammatory clusters. Figure 1 shows an example of an inflammatory cluster.

Almost no MCs were found in the glomerular or vascular compartment. No MCs were found in the lumen of arteries, veins, and capillaries. Therefore, no quantification of MCs was performed in these areas. Figure 2 shows examples of the interstitial compartment, the glomerular compartment, and medium-sized vessels in bTCMR biopsies.

TABLE 2.

Association between clinical and biological parameters and graft function.

Parameter (n = 48)	No graft failure, n = 37 (%)	Graft failure, n = 11 (%)	P
Recipient sex			
Male	27 (73)	8 (73)	0.987
Female	10 (27)	3 (27)	
Recipient age at Tx			
Mean (SD)	47 (16)	48 (14)	0.816
Donor age at Tx			
Mean (SD)	50 (15)	60 (8)	0.021
Donor type			
Living	26 (70)	8 (73)	0.876
Deceased	11 (30)	3 (27)	
D till first KTx biopsy			
Mean (SD)	236 (378)	116 (120)	0.922
Delayed graft function (DGF)			
Yes	11 (30)	8 (73)	0.007
No	26 (70)	3 (27)	
Serum creatinine at time of biopsy			
Mean (SD)	157 (43)	268 (160)	0.004
Serum creatinine slope			
Mean (SD)	0.04 (19.02)	−1.19 (11.68)	0.741

P Value was calculated using the Mann-Whitney U test.
DGF, delayed graft function; KTx, kidney transplant; Tx, transplantation.

TABLE 3.

Mast cells and interstitial fibrosis in the cortical area in bTCMR biopsies

Characteristic, n = 47		
MC/mm² (Mean [SD], range [min–max])	10.79 (13.8)	0–76.55
Area % SR (Mean [SD], range [min–max])	17.80 (6.5)	5.76–32.36

Area % SR, area percentage Sirius Red staining in cortical area of the biopsy; bTCMR, suspicious (borderline) for acute T cell-mediated rejection; MC/mm², mast cells found per mm² in the cortical area of the biopsy.

MCs and Patient Characteristics

MC/mm² was significantly correlated to the time post-Tx, with r = 0.67 and *P* < 0.001. The number of MC/mm² increased with the duration after Tx (Figure 3). Patients who had their biopsy taken >6 mo post-Tx had almost 3 times more MCs/mm² than patients who had their biopsy within 6 mo post-Tx (on average 21.01 and 7.96, respectively). Donor age was significantly correlated to the number of MC/mm² (r
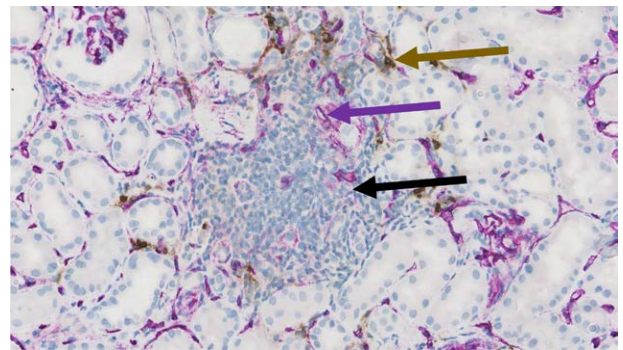

FIGURE 1. Renal cortex with an inflammatory area (black arrow), surrounded by MCs. Brown/3,3′-Diaminobenzidine: tryptase (MCs) (brown arrow); purple: CD34 (endothelium of blood vessels/capillaries) (purple arrow). MC, mast cell.

= 0.35, *P* = 0.022). Having a deceased donor type and DGF were also significantly correlated to the number of MC/mm² (respectively, *P* = 0.035 and *P* = 0.020).

MCs and Fibrosis

Area percentage Sirius Red staining in cortical area of the biopsy (area % SR), depicting collagen I and II depositions, varied between 5.76% and 32.36%, with an average of 17.80% (Table 3). Figure 4 shows examples of the presence of MCs and interstitial fibrosis. The number of (lognormal) MCs per mm² was significantly correlated to the area % SR staining in the biopsy, with r = 0.42 and *P* = 0.003. Here, a higher MC count was found in cases with a higher area % SR staining (Figure 5). Interestingly, there was no significant correlation between interstitial fibrosis and time post-Tx (r = 0.20, *P* = 0.15). MC/mm², area % SR and the individual Banff Lesion Score (i = interstitial inflammation) of all (n = 48) bTCMR biopsies are presented in the Supplementary Digital Content (**Table S1, SDC**, http://links.lww.com/TXD/A525).

MCs and Transplant Function

No significant relationship was found between MC/mm² and serum creatinine at the time of biopsy or creatinine slope after biopsy. Also, the MC/mm² did not correlate with the graft loss at 2 y after biopsy (Table 4).

DISCUSSION

In for-cause KTx biopsies classified as bTCMR, we investigated the presence of MCs and their relationship with fibrosis
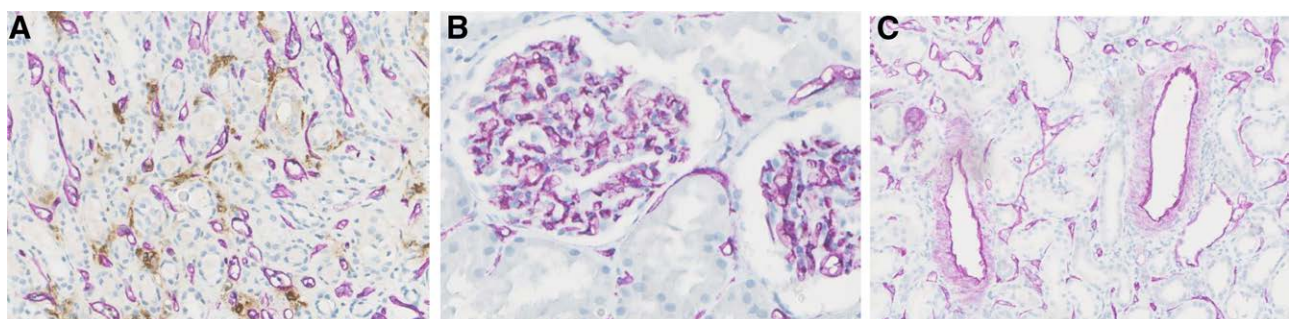

FIGURE 2. Examples of the presence of mast cells (MCs) in the renal cortex. (A) Interstitial compartment with multiple MCs. (B) Area with 2 glomeruli without MCs. (C) Medium-size vessels without MCs. Brown/3,3′-Diaminobenzidine: tryptase (MCs); purple: CD34 (endothelium of blood vessels/capillaries). MC, mast cell.
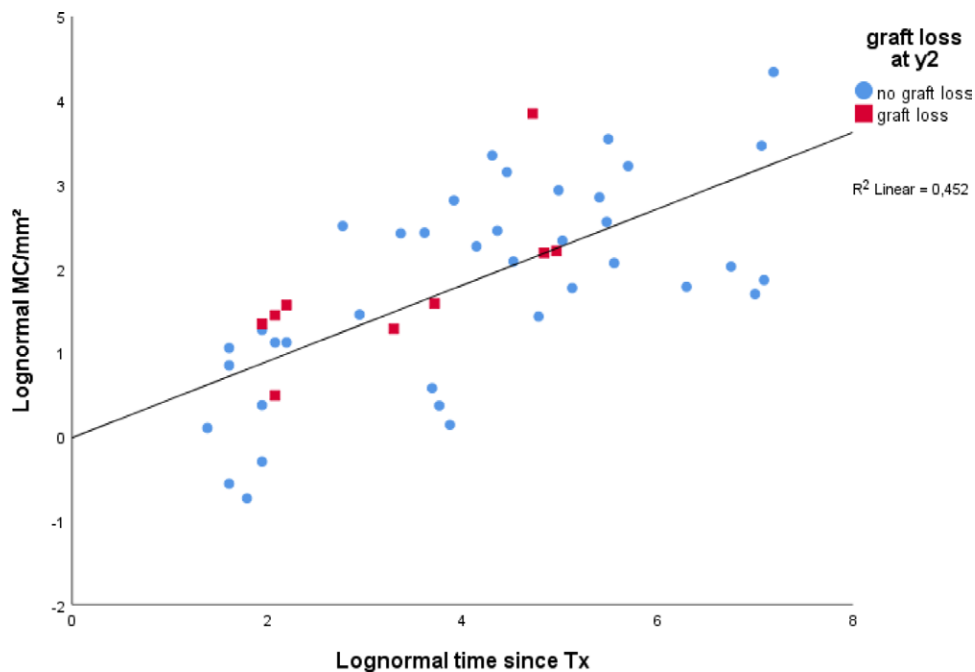
FIGURE 3. Time (mo) since Tx (lognormal) plotted against mast cells per mm² (lognormal). Subgroups represent donor type. There was a significant relationship between time since Tx and MC/mm², with higher MC counts in patients with a longer time between Tx and biopsy (r = 0.672, *P* < 0.001, black line). There were no significant between-group effects. MC, mast cell; Tx, transplant.

and subsequently transplant function 2 y after biopsy. The number of MCs in bTCMR biopsies was positively correlated to the amount of interstitial fibrosis. Interestingly, those KTx recipients with DGF had an increased number of MCs in their KTx biopsy classified as bTCMR compared with those without DGF.

Previous research showed a native normal kidney contains between 1.08 and 9.1 MC/mm² on average,[7,8,20,36] and up to 2.7–13.88 MC/mm² in KTx biopsies classified with rejection. For the samples with rejection, it is not stated whether these cases included transplant biopsies with minimal inflammatory cells as in bTCMR.[7,19,20] We found an average of 10 MC/mm² in for-cause KTx biopsies classified with bTCMR. This, however, had no apparent correlation to KTx outcome because many patients showed a slight improvement in function rather than loss at 2 y after biopsy. Time post-Tx was an important predictor for MCs/mm² at the time of for-cause biopsy because the number of MCs increased over time. This is in accordance with previous research showing a strong correlation between MCs and both interstitial fibrosis and time post-Tx, suggesting MCs to be a marker for cumulative burden of tissue injury.[21] Microarray analysis has shown that there is a positive relation between interstitial fibrosis and tubular atrophy and MCs in KTx biopsies, although the effect is rather late to implicate causation.[37] This suggests that MC influx is not the cause but rather the result of nephron loss and transplant damage.

Having DGF was positively correlated to MC/mm² within the biopsy, as was having a deceased donor. The role of MCs in ischemia and reperfusion injury in KTx patients is complex. Most studies on ischemia and reperfusion injury have been performed in animals, and data from human beings are lacking.[38] Multiple animal studies have indicated that MC-derived pro-inflammatory cytokines and chemotactic endothelial–cell interactions promote ischemia and reperfusion injury.[39-41]
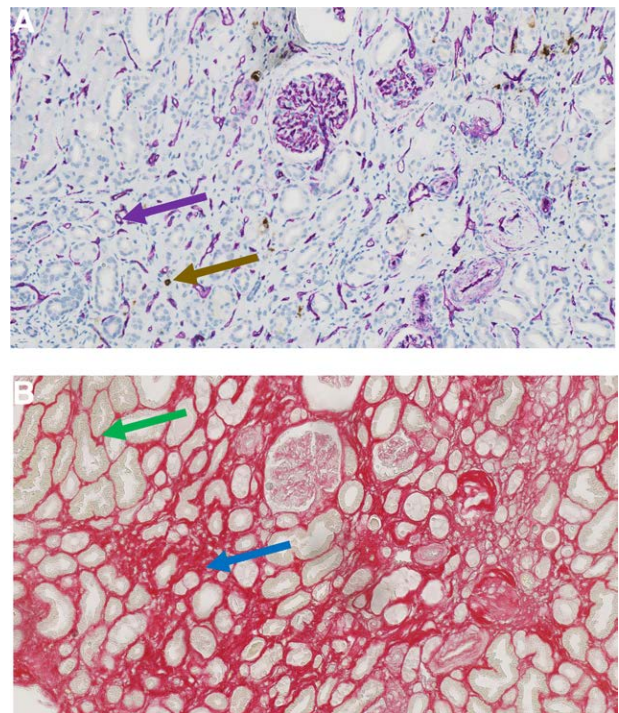
FIGURE 4. Examples of the presence of MCs and interstitial fibrosis. (A) Renal cortex with multiple MCs. The purple arrow depicts a small vessel, also notice the glomerulus with multiple capillaries. The brown arrow depicts an MC. (B) Area with dense interstitial fibrosis (blue arrow) and areas of loose or no fibrosis (green arrow). Brown/3,3′-Diaminobenzidine: tryptase (MCs); purple: CD34 (endothelium of blood vessels/capillaries). Red: Sirius Red staining (fibrosis). MC, mast cell.

More research on the effects of ischemia and reperfusion damage on the KTx is needed to gain more insights into their role in this process.

FIGURE 5. MCs in relation to fibrosis. Subgroups represent donor type (A) and delayed graft function (B). The dotted line represents the total correlation. The correlation between MC/mm² and area % SR staining was significant, with a higher MC count relating to a higher area % SR staining (r = 0.418, P = 0.003). Both MC/mm² and delayed graft function predicted area % SR independently (R² change = 0.12, P change = 0.01; β_{MC/mm^2} = 0.3, P = 0.028; β_{DGF} = 0.36,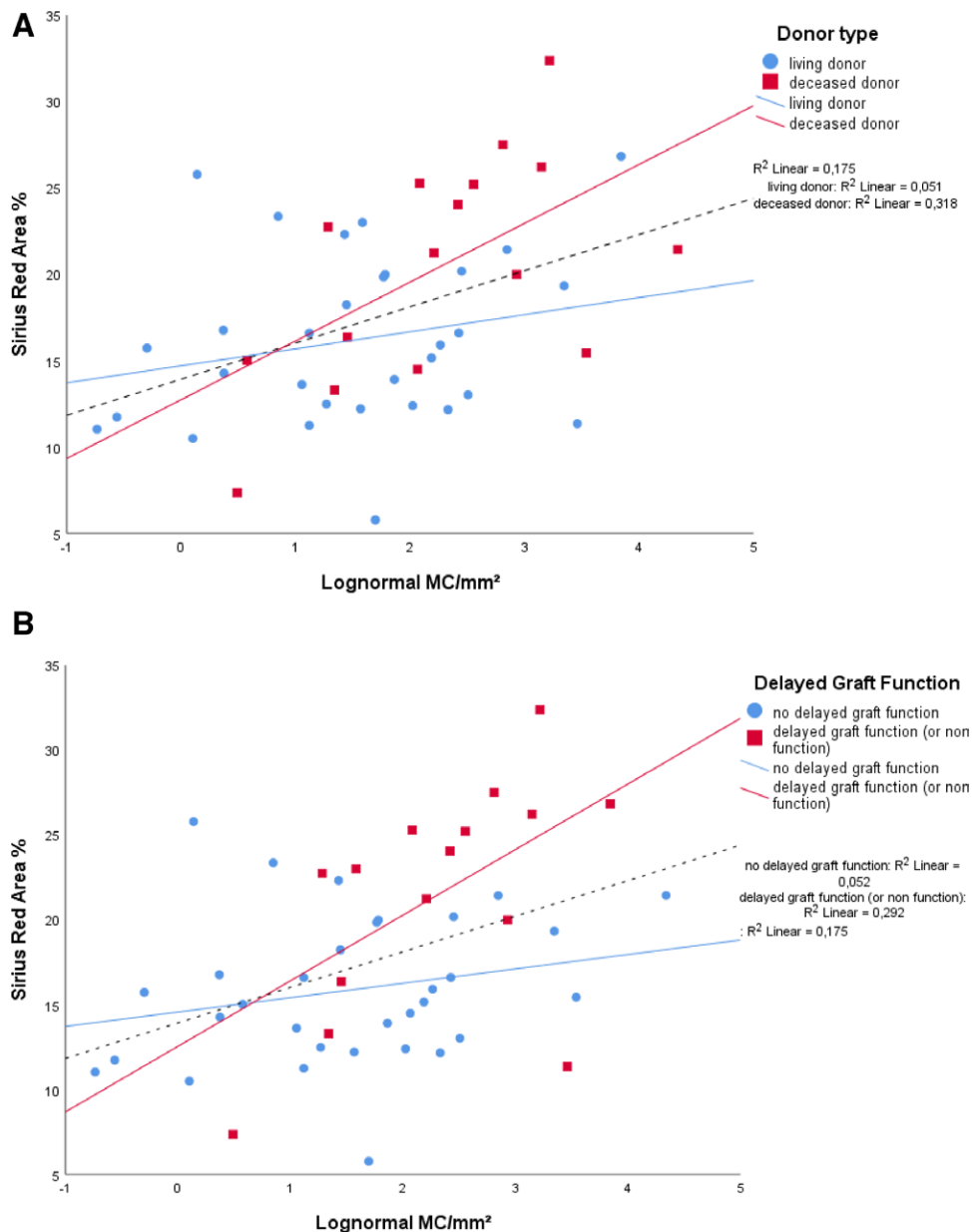 P = 0.01). Red line represents the relationship between MC/mm² and area % SR staining in patients with delayed graft function, the blue line represents the same for patients without delayed graft function. Area % SR, area percentage Sirius Red staining in cortical area of the biopsy; MC, mast cell.

Additional investigations focusing on MC phenotypes in KTs could be of interest as MCs mature in peripheral tissues and adjust their molecular expression profiles. In particular, the focus should be directed to the chymase-positive MC subtype as chymase is being a seemingly more potent protease.[7,8,42] Another interesting subtype is the tryptase- and chymase-positive MC. Ishida et al. found that increases in the number of total MCs and a higher ratio of the tryptase- and chymase-positive MC to total MC in early biopsy specimens were related to the decline of long-term graft function and fibrosis.[8,43]

The MC count in for-cause biopsies with bTCMR was not significantly correlated to serum creatinine at the time of biopsy or to long-term graft function or survival. Interestingly, many patients showed a nonsignificant trend toward improvement in function rather than loss at 2 y after biopsy. This could be in line with various studies suggesting a beneficial role of MCs during post-Tx period and in induction of T-cell tolerance.[44,45] Assuming that bTCMR could possibly be seen as a phase of rejection in which the trajectory is not set in stone, it would be interesting to investigate whether MCs contribute to the restoration of KTx function by extending the follow-up duration and explore the functions and complex cascades of MCs.

Our study has several limitations. Firstly, our study has a relatively short follow-up time of 2 y, so possibly too short to investigate the relationship between MC and long-term clinical outcome defined as graft function 2 y post-biopsy was not

TABLE 4.

Association between clinical and biological parameters and MC/mm²

Parameter (n = 47)	(ln)MC/mm²	
	Statistic	**P**
Recipient sex (1 = male)[a]	MD = 0.21, t (15.65) = 0.49	0.628
Recipient age at Tx[b]	r = −0.06	0.714
Donor age at Tx[b]*	r = 0.35	0.022
Donor type (1 = deceased)[a]	MD = 0.74, t (32.5) = 2.21	0.035
Delayed graft function (1 = DGF)[a]	MD = 0.78, t (33.86) = 2.43	0.020
Serum creatinine at t0[b]	r = 0.05	0.381
% SR[b]	r = 0.42	0.003
Serum creatinine slope[b]	r = −0.04	0.606
Graft loss at t1 (1 = graft loss)[a]	MD = −0.02, t (15.36) = −0.06	0.956
Total average	M = 1.65, SD = 1.48	

[a]Welch's test (*t*).
[b]Pearson's correlation (*r*).
*N = 42.
DGF, delayed graft function; MD, Mean Difference; M, Mean; MC/mm², mast cells found per mm² in the cortical area of the biopsy; % SR, percentage Sirius Red staining in cortical area of the biopsy; Tx, transplant.

found in our study. However, MC number was correlated to cortical fibrosis, a marker that is known to be related to inferior graft outcome.[46-48] Therefore, it would not be inconceivable that a significantly different outcome would emerge with a longer follow-up period. Although the sample size was relatively large for an exploratory study, our study consisted of only one group (being bTCMR), and the number of patients with graft loss was relatively low (9 of 48), resulting in a lack of statistical power.

In conclusion, we have shown that there is a positive correlation between MCs and both interstitial fibrosis and time post-Tx in for-cause biopsies classified as bTCMR, suggesting MCs to be a marker for cumulative burden of tissue injury. MC count was also correlated to having a deceased donor and DGF, suggesting that MCs could play a role in ischemia and reperfusion injury. In multiple studies, MCs are described as immunoregulators with both pro- and anti-inflammatory functions and potentially both positive and detrimental effects on long-term transplant function and tissue remodeling depending on the milieu and complex cascade. It remains unclear whether MCs are just a bystander or might have a pro- or anti-inflammatory effect in KTx biopsies classified as bTCMR.

ACKNOWLEDGMENTS

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