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Presence of CD163^{+} macrophages in DCD kidneys with high DGF reduces the risk for acute cellular rejection in 6 months after kidney transplantation

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ABSTRACT

Acute cellular rejection (ACR) occurs in 10% of renal allograft recipients and is characterized by leukocyte infiltration as observed in needle biopsies. ACR onset is subject to several risk factors, including delayed graft function (DGF). As the impact of DGF on the etiology of ACR remains unclear, this study analyzed the association between presence of leukocyte subsets and ACR onset, in DCD kidney biopsies with extensive DGF following transplantation. Immunohistochemical analysis of protocol biopsies taken 10 days after kidney transplantation revealed that patients with high levels of renal CD163^{+} macrophages have a decreased risk (OR = 0.021, P = 0.008) for ACR in the first 6 months after transplantation. In pre-transplant biopsies of a comparable DCD cohort, with >80% DGF, presence of donor CD163+ macrophages showed no effect on ACR risk. Therefore, leukocyte infiltrate present during the inflammatory response at the time of DGF may contain anti-inflammatory macrophages that exert a protective effect against ACR development.

1. Introduction

Because of the worldwide shortage of donor organs, the criteria used to select donor organs for transplantation are continuously extended [1]. This development has led to the use of kidneys from donation after circulatory death (DCD) donors [2]. These kidneys often suffer from delayed graft function (DGF) after transplantation [3], which is a form of acute kidney injury thought to have a basis in ischemic and immunologic causes [3] but its exact etiology remains unclear. The occurrence of DGF has been associated with increased rates of acute rejection and worse long-term outcomes such as decreased graft survival [4–7].

On average, acute rejection (AR) still affects almost 10% of KTx patients [8], but this rate can be higher in subpopulations. Diagnosis of acute cellular rejection (ACR) includes analysis of leukocyte presence in the kidney biopsy. Therefore, when a patient presents with clinical suspicion of ACR, a needle biopsies of the transplanted kidney is the golden standard to diagnose ACR [9].

On a cellular level, leukocytes are the main effector cells in acute cellular rejection (ACR), and for ACR diagnosis the presence of leukocytes is included in the Banff criteria measuring tubulitis and interstitial inflammation. However, identifying infiltrating leukocyte subtypes such as pro- or anti-inflammatory macrophages using molecular markers is not taken into account during the diagnostic process [10]. Therefore, in early stages after kidney transplantation it may be challenging to differentiate between the leukocyte influx in a kidney biopsy caused by ACR or the repair response in the phase of DGF, as leukocyte infiltration is common in both ACR and DGF [3,11].

In recent years, not only the presence of leukocytes, but also the cellular composition of infiltrating leukocytes has been fundamentally studied in kidney biopsies. Particularly, macrophages and dendritic cells have emerged as important mediators of the innate immune system, contributing to the inflammatory and immunological response in both ACR [12,13] and DGF [14]. Within these cell populations, there are additional distinctive subsets contributing to a pro- or anti-inflammatory response, which has a significant effect on the onset and repair response of kidney injury after transplantation [13,15]. Especially CD163+ macrophages, FoxP3+ T-cells and DC-SIGN+ dendritic cells have been associated with anti-inflammatory properties [16–18]. Anti-

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inflammatory macrophages have been associated with both early and late-stage repair processes [19]. Further study of these populations might prove useful in characterizing the etiology of leukocytes presence in DGF, and subsequent onset of ACR.

1.1. Objective

The aim of this paper is to study the relation between the presence of different leukocyte subsets in kidney biopsies and onset of ACR, in a cohort of patients receiving a DCD kidney with a high level of DGF, where inflammatory reactions are common [4]. To this end, the presence of $CD3^+$ and $FoxP3^+$ T-cells, $CD68^+$ and $CD163^+$ macrophages, and DC-$SIGN^+$ dendritic cells is determined in protocol biopsies taken during the DGF period, at day 10 after KTx. Together with clinical data these will be used to assess the relationship between the presence of these subsets and the risk of ACR occurrence within 6 months after KTx, in a DGF setting.

2. Materials and methods

2.1. Selection of patients

The present study analyses samples and data obtained from patients in two separate cohorts. All donor kidneys were matched to recipients via the Eurotransplant Kidney Allocation System. Day 10 allograft biopsies were obtained from donor kidney recipients participating in the PROTECT study in the Leiden University Medical Center [20]. All patients provided informed consent for use of their material for scientific research. Pre-Tx biopsies were obtained from patients who underwent KTx between 2013 and 2016 in the LUMC. The current study using biopsy material from these patients was approved by the ethical committee at the Department of Pathology at the LUMC. The collection of the original patient material and data was performed in accordance with all relevant (inter-)national relevant guidelines and regulations regarding informed consent, where next of kin approved for use of donor kidney material for scientific research.

2.1.1. Cohort of allograft biopsies at 10 days after KTx

The PROTECT study was approved by the medical ethics committee of the Leiden University Medical Center (LUMC), and written informed consent was obtained from all patients. The in- and exclusion criteria, as well as the (standard of care) treatment regimen these patients underwent is described by Aydin et al. [20] Briefly, the exclusion criteria encompassed panel-reactive antibodies over 60% at the time of transplantation, a donor serum creatinine over 150 mol/L, a prolonged first warm ischemic time of >45 min or a cold ischemic time over 24 h. Recipients received anti-CD25 induction therapy and triple therapy including cyclosporine A, steroids and mycophenolate mofetil as maintenance therapy. All patients received a kidney biopsy at 10 days after transplantation.

We have followed the functional definition of DGF, which constitutes failure of the serum creatinine to decrease by at least 10% daily on 3 successive days during the first 7 days after transplantation, irrespective of dialysis requirement. This is similar to the definition used in the original paper published on this specific patient cohort [20].

2.1.2. Pre-Tx cohort

The Pre-Tx cohort was composed of all patients who suffered from ACR after receiving a DCD kidney in the LUMC between 2013 and 2016. In this period, ACR occurred in 7 DCD recipients, who were matched to 9 DCD recipients who did not develop ACR, based on time of transplantation. A total of 16 patients were included. Pre-transplantation biopsies were taken from the donor kidney as part of protocol care. None of the patients objected to the inclusion of their donor material in this study. Recipients were treated according to the standard of care similar to the Day 10 cohort.

2.2. Immunohistochemical staining

Immunohistochemical stainings with CD68, CD163, CD3, FoxP3, or DC-SIGN antibodies (anti CD68, clone 514H12 from BioRad; anti CD163, clone 3D4 from LifeSpan BioSciences; Anti-CD3 clone SP7 from Abcam; anti DC-SIGN, clone 120,507 from R&D Systems; anti FoxP3, clone 236A/E7 from Abcam) were performed on 4 μm paraffin sections after heat induced antigen retrieval using a citrate buffer. These primary antibody stainings were matched with appropriate isotype controls, followed by a secondary antibody (either a goat-anti-rabbit HRP conjugated antibody or a goat-anti mouse-HRP conjugated antibody from DAKO/Agilent, or in the case of FoxP3 a Mouse EnVision kit from Agilent). Visualization was achieved using 3,3′-diaminobenzidine tetrahydrochloride hydrate (DAB) (Sigma-Aldrich), followed by a Mayer's hematoxylin (Merck, NJ, USA) nuclear counterstain.

2.2.1. Quantification

Immunohistochemically stained biopsies were digitalized using the Panoramic MIDI slide scanner. Per staining, the threshold for DAB positivity was determined on images from 5 separate biopsies by two independent observers. The average threshold for positivity from these 5 biopsies was used for automated quantification of positive signal in the full-size biopsy sections from all available biopsies. The area% of positive staining was measured at 200× zoom via the QuantCenter 2.2 image analysis platform using the HistoQuant plugin (all from 3D Histech, Hungary [21]). The presence of acute tubular necrosis, and the Banff classification was scored by a renal pathologist at the LUMC using the extended Banff classification from the Banff 2009 working classification for renal allograft pathology, as previously reported by Bank et al. [22] Diagnostic ACR biopsies were scored using the 2018 Banff classification [10].

2.2.2. Multivariate logistic regression

Data from several variables were split into tertiles of equal participant sizes, to allow use of these skewed variables in a multivariate logistic regression analysis. In the Day 10 cohort, the tertiles for the CD68 stained samples ranged from 0 to <0.5, ≥0.5 to <0.9, and ≥ 0.9 area%, while CD163 stained samples were slit into the following categories; 0 to <4, ≥4 to <8, and ≥ 8 area%. The levels of the calcineurin inhibitors AUC were classified as low beneath 2800 μg/L (cyclosporine) or 80 μg/L (tacrolimus), normal from ≥2800 μg/L and < 4500 μg/L (cyclosporine) or ≥ 80 μg/L and < 120 μg/L (tacrolimus), and high when they exceeded 4500 μg/L (cyclosporin) or 120 μg/L (tacrolimus).

In the PreTx cohort, the $CD3^+$ cell presence variable is divided into ≤0.04 area%, >0.08 and ≤ 0.18 area%, and > 0.18 area%. The $CD68^+$ cell presence variable is divided into ≤0.08 area%, >0.04 and ≤ 0.11 area%, and > 0.11 area%.

2.3. Statistical analysis

Statistical comparisons were performed using Mann-Whitney U for non-parametrical data. Multivariate logistic regression analysis were used to determine the odds ratio on the occurrence of ACR within 6 months after KTx. For the variables split into tertiles, the lowest range was used as indicator, or dummy variable. $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using SPSS Statistics 24 (IBM, USA).

3. Results

The risk associated with the presence of different leukocyte subsets in allograft biopsies on the occurrence of ACR within 6 months after KTx was determined in a cohort of patients who participated in the PROTECT study [20]. In the PROTECT study, KTx recipients were treated with short-course high-dose erythropoietin (EPO) in a randomized fashion, and its effect on incidence of primary non-function and DGF was studied.

Table 1
Baseline characteristics of patients in the day 10 cohort.

	Day 10
Included patients (N)	64
Transplant	
Donor age (years mean ± SD)	45,8 ± 16,1
Recipient age (years mean ± SD)	54,6 ± 11,3
Recipient gender (Male, %)	28,1
HLA class I mismatch (Mean ± SD)	1,9 ± 1,0
HLA class II mismatch (Mean ± SD)	0,8 ± 0,6
Panel reactive antibodies	5,8 ± 9,8
DCD (%)	100
1st WIT (Min Mean ± SD)	17,8 ± 5,8
2nd WIT (Min Mean ± SD)	29,7 ± 7,3
KIT (Hours Mean ± SD)	17,2 ± 3,9
Post-transplant	
Induction therapy*	100%
Calcineurin inhibitor AUC (μg/l x h, Mean ± SD)	6121,4 ± 1740,9
ACR within 6 months (%)	13 (20%)
Days to ACR (Mean ± SD)	74,0 ± 43,7
DGF (>7 days) (%)	51 (80%)
EPO treatment (%)	28 (43.8%)

* Induction therapy consisted of an Il-2 receptor inhibitor; either Basiliximab or Zenapax.

From the 92 patients included into the PROTECT study, 28 samples were excluded due to the following ineligibility criteria; 7 biopsies were not collected, 8 biopsies were not available in the pathology archives, 2 kidneys were unavailable due to nephrectomy before day 10, 5 biopsies were not representative for cortical tissue, 2 kidneys suffered from primary non-function, and 4 patients suffered from clinical ACR prior to day 10. Based on these criteria, the current cohort included 64 patients. These recipients were predominantly female (71.9%), with a mean age of 54.6 ± 11.3 years. All patients received organs from donors after cardiac death (DCD), with limited HLA class I (HLA-A,-B) and II (HLA-DR) mismatches and short warm and cold ischaemia times (Table 1). Nearly half of the recipients were treated with EPO according to the protocol of the PROTECT study [20] and 80% suffered from DGF longer than 7 days. Within the first 6 months after KTx ACR occurred in 13 patients (20.3%). ACR was defined as an episode of treatment for acute rejection (solumedrol 1000 mg for 3 consecutive days) that was administered based on clinical suspicion, with or without a confirmed histological diagnosis of ACR. Biopsy material was available for 7 patients, who were diagnosed with borderline rejection (i1t1; $N = 3$), TCMR grade 1B (i3t3; $N = 3$) and TCMR grade 1A (i1t3; $N = 1$) according to the 2018 Banff criteria [10].

3.1. High levels of anti-inflammatory macrophages at 10 days after KTx decrease the risk of developing ACR within 6 months after KTx

Patient biopsies taken 10 days after KTx (Fig. 1a) were scored according to the extended Banff criteria and immunohistochemically

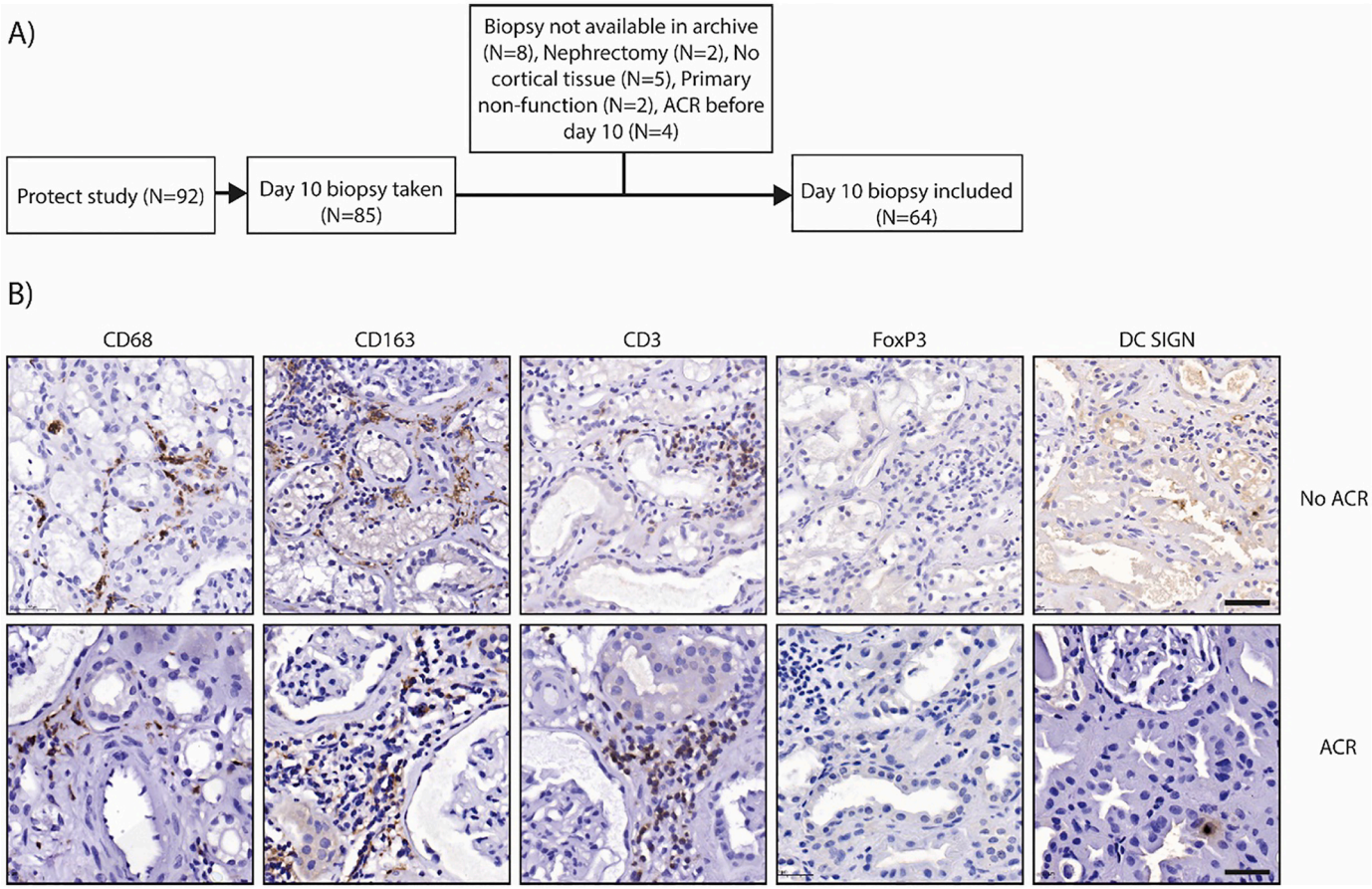

Fig. 1. Selection and immunohistochemical staining of the day 10 cohort. A) 64 biopsies where selected from the PROTECT cohort and were B) immunohistochemically stained for the presence of $CD68^+$ macrophages, $CD163^+$ macrophages, $CD3^+$ T-cells, $FoxP3^+$ T-cells, and DC $SIGN^+$ dendritic cells. Antibody positivity is visualized with DAB (Brown), and counterstained with HE. Representative images of the immunohistochemical stains at 400× magnification (scale bar of 50 μm) in patient samples that progressed and did not progress to ACR. Adobe Illustrator CC 2018 was used to create this figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Biopsy values of leukocytes present at day 10.

Variables	Day 10			
	$n = 64$	$n = 51$	$n = 13$	
	Mean	no ACR	ACR	*P*-value
CD3 (area% Mean ± SD)	0,22 ± 0,35	0,25 ± 0,39	0,14 ± 0,11	0,927
FoxP3 (area% Mean ± SD)	0,008 ± 0,01	0,01 ± 0,01	0,01 ± 0,02	0,531
CD68 (area% Mean ± SD)	0,83 ± 0,86	0,92 ± 0,93	0,47 ± 0,40	**0,048**
CD163 (area% Mean ± SD)	7,24 ± 4,38	7,89 ± 4,40	4,69 ± 3,36	**0,005**
DC-SIGN(area% Mean ± SD)	0,27 ± 0,35	0,28 ± 0,37	0,23 ± 0,24	0,874

Table 3
Regression analysis to determine the risk of variables present at day 10 on the occurrence of ACR within 6 months after KTx.

	Day 10		
	Exp(B)	95% CI	P-value
CD163 (area%) medium	0.133	0.016–1.117	0.063
CD163 (area%) high	**0.021**	**0.001–0.365**	**0.008**
HLA class I mismatch	2.409	0.718–8.086	0.155
HLA class II mismatch	1.136	0.221–5.831	0.879
EPO treatment	0.411	0.061–2.770	0.361
First warm ischemia time	0.942	0.785–1.131	0.524
Second warm ischemia time	1.091	0.966–1.232	0.162
AUC Calcineurin inhibitor (Week 6) - HIGH	0.573	0.090–3.654	0.556
Days of DGF	0.957	0.863–1.062	0.407

stained to analyze the presence of $CD3^+$, $FoxP3^+$, $CD68^+$, $CD163^+$ and DC-$SIGN^+$ leukocyte subsets (Fig. 1b). Automated analysis of the percentage positive area of immunohistochemical staining revealed that most subsets were present in low numbers (under 1% area percentage), except $CD163^+$ macrophages which exceeded 7% area percentage (Table 2). At this timepoint, univariate analysis showed that both $CD68^+$ and $CD163^+$ staining was significantly different between biopsies from patients who develop ACR within 6 months, compared to biopsies from patients who did not develop ACR (Table 2).

To determine the effect of presence of $CD68^+$ and $CD163^+$ macrophages on the risk of ACR within 6 months after KTx, a multivariate regression analysis was performed. In addition to either the $CD68^+$ and $CD163^+$ macrophages variables, using the lowest tertile as an indicator, the following potential confounders were entered into the model: acceptor age and gender, mismatch of HLA class I and II, EPO treatment status from the PROTECT study, first and second warm ischemia times (WIT), trough levels of calcineurin inhibitors during the first week after KTx, and the duration of DGF [5,20,23–25]. The multivariate regression analysis performed with presence of $CD163^+$ macrophages revealed that high values (≥8 area%, compared to the group average of 7,24 ± 4,38%; mean ± SD) of $CD163^+$ macrophages decrease the risk on the occurrence of ACR (OR 0.021, $P = 0.008$) (Table 3). In this analysis, none of the other variables showed a significantly altered risk for ACR occurrence. Using Pearson's correlation coefficient, no correlation between the presence of $CD163^+$ macrophages and acute tubular necrosis and Banff criteria tubulitis, tubulo-interstitial inflammation was observed. Presence of $CD68^+$ macrophages had no significant effect on the risk of ACR occurrence (OR = 4.67 (0.334–65.334, 95% confidence interval), $P = 0.25$), as this relation was affected by several confounders.

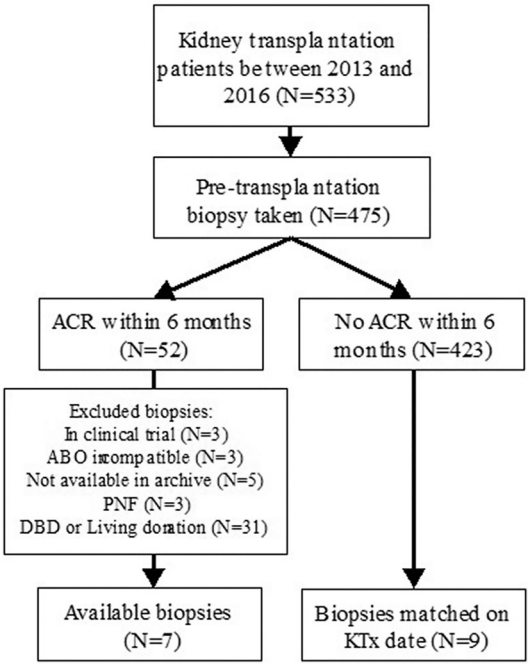

Fig. 2. Selection of the PreTx cohort. The preTx cohort was composed of recipients who received a kidney transplantation in the Leiden University Medical Center in the Netherlands between 2013 and 2016 from a DCD donor. After exclusion of biopsies that were either unavailable or ineligible 16 biopsies where immunohistochemically stained for the presence of $CD68^+$ macrophages, $CD163^+$ macrophages, $CD3^+$ T-cells, $FoxP3^+$ T-cells, and DC $SIGN^+$ dendritic cells. ACR = acute cellular rejection, PNF = primary non-function, DBD = Donation after Brain Death. Adobe Illustrator CC 2018 was used to create this figure.

Table 4
Baseline characteristics of patients in the preTx cohort.

	PreTx
Included patients (N)	16
Transplant	
Donor age (years mean ± SD)	55,8 ± 12,9
Recipient age (years) (Mean ± SD)	62,4 ± 9,7
Recipient gender (Male, %)	75
HLA class I mismatch (Mean ± SD)	2,0 ± 1,0
HLA class II mismatch (Mean ± SD)	1,1 ± 0,6
DCD (%)	100
Post-transplant	
Induction therapy*	100%
Tacrolimus AUC (μg/l x h, Mean ± SD, $N = 13$)	207,9 ± 132,9
ACR within 6 months (N)	7
DGF (>7 days) (%)	13 (81,3%)

* Induction therapy consisted of an Il-2 receptor inhibitor; either Basiliximab or Zenapax.

3.2. *High levels of $CD163^+$ macrophages in pre-transplantation biopsies do not affect the risk for developing ACR*

The observation of a protective effect of high levels of CD163+ macrophages in the day 10 biopsy on the risk to ACR, raised the question whether a protective effect of CD163+ macrophages could also be observed in pre-transplantation biopsies. Using an unrelated cohort of pre-transplantation biopsies, a selection was made for patients transplanted between 2013 and 2016 in the Leiden University Medical Center, who received kidney allografts from DCD donors, and who experienced an episode of rejection within 6 months after transplantation ($N = 7$, Fig. 2). This selection of patients was matched to patients who did not have an episode of rejection but received their

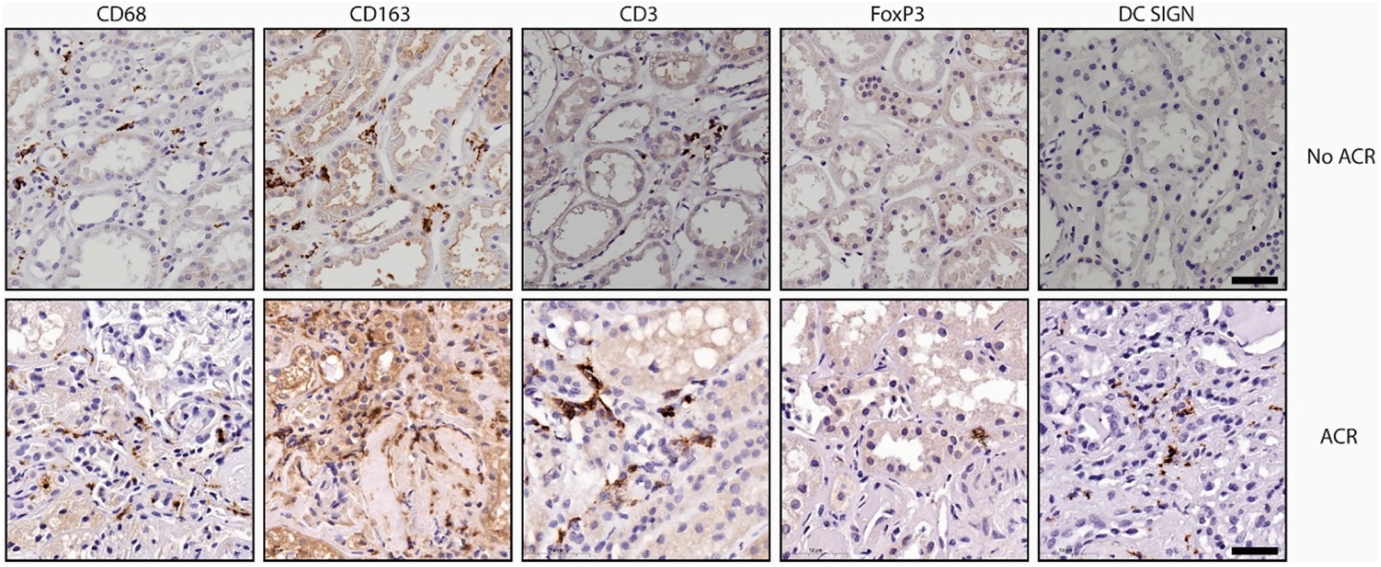

Fig. 3. Immunohistochemical staining of the pre-Tx cohort. 16 biopsies where immunohistochemically stained for the presence of $CD68^+$ macrophages, $CD163^+$ macrophages, $CD3^+$ T-cells, $FoxP3^+$ T-cells, and DC $SIGN^+$ dendritic cells. Antibody positivity is visualized with DAB (Brown), and counterstained with HE. Representative images of the immunohistochemical stains at 400× magnification (scale bar of 50 μm) in patient samples that progressed and did not progress to ACR. Adobe Illustrator CC 2018 was used to create this figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Biopsy values of leukocytes present in pre-Tx biopsies.

	Pre-Tx			
Variables	*n* = 16	n = 9	n = 7	
	Mean	no ACR	ACR	*P*-value
CD3 (area% Mean ± SD)	0,12 ± 0,18	0,05 ± 0,03	0,22 ± 0,25	0,012
FoxP3 (area% Mean ± SD)	0,02 ± 0,01	0,02 ± 0,01	0,02 ± 0,00	0,758
CD68 (area% Mean ± SD)	0,20 ± 0,17	0,14 ± 0,08	0,27 ± 0,22	0,408
CD163 (area% Mean ± SD)	2,86 ± 1,50	2,24 ± 1,14	3,66 ± 1,60	0,091
DC-SIGN(area% Mean ± SD)	0,11 ± 0,10	0,09 ± 0,07	0,13 ± 0,14	0,681

kidney transplant in the same time period, and received comparable treatment within this period ($N = 9$), which included induction therapy at time of transplantation and maintenance on triple therapy with low-dose glucocorticoids, calcineurin inhibitors and mycophenolate mofetil. This treatment was also comparable to the treatment received by patients in the day 10 cohort. In the 16 patients of this pre-transplantation (preTx) cohort, 75% was male, with an average age of 52,8 years (Table 4).

Immunohistochemical staining of $CD3^+$, $FoxP3^+$, $CD68^+$, $CD163^+$ and DC-$SIGN^+$ (Fig. 3) revealed low presence of all cell types including $CD163^+$ cells (2,86 ± 1,5 area%). No difference in expression of $CD163^+$ cells was observed between the ACR and no ACR groups after univariate analysis (Table 5). A differential expression of $CD3^+$ cells was observed between ACR and no ACR groups, but after correction for age and sex using a logistic regression, this significance was lost.

4. Discussion

Upon assessing the different leukocyte subsets present in day 10 biopsies from a cohort with a high background of functional DGF [26,27], an increased presence of $CD163^+$ macrophages was observed. Traditionally, an influx of leukocytes in the kidney biopsy at day 10 will be interpreted as a pro-inflammatory response, according to the Banff criteria. However, when analyzing the influx of specific leukocyte subsets at this timepoint, we observed an opposite effect, as high levels of $CD163^+$ macrophages decreased the risk of developing ACR, indicating an anti-inflammatory profile in these day 10 DCD kidney biopsies.

In a recent study on kidney donor biopsies at a pre-Tx timepoint, a difference in DGF phenotype between DCD donors and donations after brain death was observed, where DCD donor kidneys were postulated to be more resilient to the impact of DGF [28]. Ingenuity Pathway Analysis revealed a suppression of pro-inflammatory pathways in DCD organs, but no specific cell populations were analyzed. Additionally, in a direct comparison between DCD and DBD kidneys post-transplantion, DCD kidneys showed less upregulation of inflammatory and injury genes [29], which may be the reason why recipients of DCD kidneys might experience a milder course of DGF. In literature, mainly pro-inflammatory macrophages have been associated with DGF, as the pro-inflammatory environment in DGF can activate macrophages and downstream cascades towards acute rejection [4]. Little research is available on the role of anti-inflammatory macrophages in DGF both in a pre-Tx and post-Tx setting. CD163 is thought to exert anti-inflammatory effects through directly stimulation production of anti-inflammatory cytokines, and inducing macrophages to take up heme, which in turn also has strong anti-inflammatory properties [30]. The currently observed high levels of $CD163^+$ macrophages at 10 days after transplantation further supports the hypothesis that DCD donors are resilient to the impact of DGF.

The macrophages in the allograft biopsies, obtained at 10 days after KTx, most-likely originate from the recipient, as the presence of these non-proliferative cells is nearly tripled at day 10 when compared to the pre-Tx cohort. Based on this observation, it could be hypothesized that recipient monocytes might be recruited in response to the inflammatory signals and polarize locally into a repair-oriented $CD163^+$ phenotype. In support of the latter hypothesis, monocyte/macrophage polarization has been shown to shift to anti-inflammatory CD163+ macrophages upon exposure to tacrolimus [31]. In both cases, this will be part of natural chimerism which has also been observed in endothelial cells of transplanted kidneys [32–34]. Immunologically, both donor and recipient macrophages will have their specific role in regulating direct and

indirect alloreactivity respectively. To further study the origin and subsequent fate of these anti-inflammatory cells, additional research is required using either animal models or human biopsy studies with multiple timepoints.

The relation between macrophages and ACR has been studied more extensively, as several studies have reported the importance of macrophages and dendritic cells as potential biomarkers before and during ACR, where they positively correlated with graft loss [12,35]. In those studies, infiltrating cells including Antigen Presenting Cells (APCs) are mostly studied as a part of the pro-inflammatory cascade during ACR.

Also during ACR, macrophage populations have been shown to exert a regulatory role [36]. mRNA analysis of AR biopsies revealed that increased levels S100 calcium-binding protein S100A8 and S100A9, produced by $CD68^+$ macrophages, have a protective effect on progression to chronic allograft nephropathy. Even in a therapeutic setting, APCs are being investigated for their tolerogenic and regulatory role as immunomodulatory cell therapy after kidney transplantation [37]. Particularly, the ONE study showed that cell therapy using regulatory macrophages could safely be used as part of the immunosuppressive therapy [38]. The current data therefore supports and fits the concept of anti-inflammatory polarized macrophages as immunomodulators, as the risk of developing ACR is reduced when high levels of $CD163^+$ macrophages are present at day 10 after transplantation of a DCD kidney. These macrophage subtypes would therefore be a fitting target for future research on therapeutic immunomodulation. Especially in the field of machine perfusion of donor organs a unique opportunity for organ-specific intervention is available [39], where anti-inflammatory modulation via $CD163^+$ macrophages supply can be studied.

In order to interpret these findings accurately, the limitations of this study need to be taken into account. As a study performed in a single center, the number of samples that fit our eligibility criteria is limited, which in turn has an impact on the statistical power of the analyses that were performed. Especially the pre-transplantation cohort was small, leading to loss of power in the statistical test to analyze the protective effect of $CD163^+$ macrophages, present prior to occurrence of DGF, on ACR development. Additionally, in order to accurately analyze the impact of potential confounding variables, larger sample sizes are needed. Nonetheless, variables which have been shown in previous studies to have an impact on ACR risks have been included in this analysis. In addition to limits on statistical analysis, extrapolation of these data need to be carefully considered. Due to logistic reasons or high-risk circumstances, golden standard histological diagnosis of ACR was not available for all included patients. As the study is performed on a unique set of biopsies in the context of DGF in DCD transplant recipients, the applications for conclusions and hypothesis postulated are limited to a specific population. In addition, based on a different timing of biopsy (pre-transplantation or day 10 post-transplantation), we should be careful in the direct comparison between the two cohorts. The obtained results do however, open up several interesting avenues for further exploration.

Furthermore, the immunohistochemical staining performed made use of cell surface markers, except in the identification of regulatory T-cells where FoxP3 was stained, which is a nuclear marker. This discrepancy can cause an underestimation of regulatory T-cell presence. In literature presence of FoxP3 in kidney transplant biopsies has been associated with suppressive effects on rejection [40].

In conclusion, the data presented in this study support a role for the $CD163^+$ macrophage population in decreasing the ACR risk in patients who received a DCD kidney. To further elucidate the origin and fate of these cells, and possibly identify targets for immunomodulation therapy, additional timepoints after transplantation need to be studied.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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