Chapter 5

The NOX2-mediated ROS producing capacity of recipient cells is associated with reduced T cell infiltrate in an experimental model of chronic renal allograft inflammation

Marina D. Kraaij[a], Karin M. Koekkoek[a], Kyra A. Gelderman[a,b], and Cees van Kooten[a]

[a]Department of Nephrology, Leiden University Medical Center, Leiden, the Netherlands
[b]Department of Pathology, VU University Medical Center, Amsterdam, the Netherlands

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Abstract

We previously showed that anti-inflammatory Mph (Mph2) can both *in vitro* and *in vivo* induce regulatory T cells (Tregs) in a reactive oxygen species (ROS)-dependent fashion. As influx of Mph is an important characteristic of chronic inflammatory responses, we investigated the impact of NOX2-mediated ROS production by recipient cells in an experimental model of chronic allograft inflammation. We used a kidney transplantation (Tx) model with Lewis (Lew) rats as donor and congenic DA.*Ncf1*$^{DA/DA}$ (low ROS) and DA.*Ncf1*$^{E3/E3}$ (normal ROS) rats as recipients. At day 7 the contralateral kidney was removed, and the animals were sacrificed four weeks after Tx. Renal function and injury was monitored in serum and urine and the composition of the infiltrate was analyzed by immunohistochemistry. Four weeks after Tx, large leukocyte clusters were observed in the allograft, in which signs of ROS production could be demonstrated. These clusters showed no difference regarding composition of myeloid cells or the number of FoxP3 positive cells. However, T cell infiltrate was significantly reduced in the DA.*Ncf1*$^{E3/E3}$ recipients having normal ROS production. Therefore, this study suggests a regulatory effect of ROS on T cell infiltration, but no effect on other inflammatory cells in the allograft.

Introduction

Loss of renal function will ultimately result in the need for a renal transplantation. Despite significant improvement, especially in the early post-transplantation period, the occurrence of allograft rejection remains a problem, which negatively affects the function and survival of transplanted organs. Allograft rejection is characterized by the influx of recipient immune and inflammatory cells in the transplanted organ [1;2]. Although initial attention focused on T cell infiltrates, it has become clear that also other cells, including B cells, macrophages, dendritic cells are part of this infiltrate. However, not all infiltrating cells actively contribute to the rejection process, and there is accumulating evidence that some of these cells have regulatory functions [3]. Macrophages appear in different subsets like the pro-inflammatory Mph (Mph1) which maintain inflammation, and the anti-inflammatory Mph (Mph2) that will clear up cell debris and repair the tissue [4]. Regulatory T cells (Tregs) can also be part of the infiltrate, and have been considered a protective biomarker of graft outcome [5-7]. Therefore, it is of great importance to characterize cellular infiltrates and the local inflammatory conditions, since these will contribute to the composition of the infiltrate within the graft.

The inflammatory response at time of rejection is characterized by the presence of reactive oxygen species (ROS), which are generated amongst others by the Mph [8;9]. Next to oxidative stress, also a role for reactive oxygen species (ROS) in immune regulation has been observed [10;11]. Recently we showed that ROS produced by type 2 Mph contribute to the generation of Tregs [12].

Mph from chronic granulomatous disease (CGD) patients, who have a deficient ROS production due to mutations in the phagocytic NADPH oxidase (NOX2) complex, were shown to be less efficient in the induction of Tregs. Considering that CGD patients are characterized by chronic inflammation, these findings point towards a role for ROS in dampening inflammation. In line with this, rats and mice with a decreased ROS production have increased T cell activation and more severe arthritis, which, in the mouse, was shown to be dependent on the ROS producing capacity of Mph [13;14].
Here we studied the impact of NOX2-mediated ROS production by recipient cells on the inflammatory response in the transplanted kidney. For this we used the model of Lewis-to-DA kidney transplantation, resulting in a model of chronic inflammation. As recipients we used the congenic rat strains DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$genotypes, which only differ in their phagocytic NOX2-derived ROS-producing capacity. In this way, we were able to investigate the role of ROS produced by infiltrating inflammatory cells, in chronically inflamed kidneys. When sacrificed at 4 weeks, we observed a decreased T cell infiltration in grafts derived from recipients with a normal ROS production (DA.*Ncf1*$^{E3/E3}$), compared with the congenic DA.*Ncf1*$^{DA/DA}$ recipients.

Objective
To study the impact of NOX2-mediated ROS production by infiltrating cells on the inflammatory response in the transplanted kidney.

Materials and Methods

Animals
Littermates (DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$) were obtained by intercrossing F1 animals from a DA (Harlan, Horst, The Netherlands) x DA.*Ncf1*$^{E3/E3}$ cross (DA.*Ncf1*$^{E3/E3}$ founders originating from Medical Inflammation Research, Karolinska Institute, Stockholm, Sweden) [15]. Lewis rats were purchased from Harlan (Horst, The Netherlands). The animals were housed in IVC cages and had free access to water and standard rat chow. Animal care and experimentation were performed in accordance with the Dutch law and the local committee of animal experiments of the Leiden University Medical Center.

Kidney transplantation model
Lewis rats were used as donors and DA.*Ncf1*$^{E3/E3}$ (N=5) and DA.*Ncf1*$^{DA/DA}$ (N=5) rats (190–350 g) were used as recipients. Kidney transplantations were performed under isoflurane anesthesia and 0.02 mg/kg buprenophine (Temgesic®, Schering-Plough). The left kidney from the donor was perfused using cold ringer (Fresenius Kabi) with 400 U/ml Heparin (LEO, Pharma BV) and kept on ice. The left kidney from the recipient was removed and the donor kidney was transplanted in the abdominal cavity from the recipient. The donor renal artery and vein were anastomosed end-to-site to respectively the

recipient aorta and vena cava using running sutures. The donor urether was anastomosed end-to-end to the urether of the recipient using lose stitches. Postoperatively, 10 mg/kg of cyclosporine (Sandimmune®, Novartis) was given daily s.c. and provided up till day 7. Seven days after Tx the contralateral kidney was removed. Blood samples were collected at several time points after Tx by tail vein puncture. The rats were twice a week placed in metabolic cages to collect urine samples. The animals were sacrificed four weeks after Tx. Creatinine and urea in serum samples were measured using standard autoanalyzer methods by our hospital diagnostic services. In addition, rocket immuno-electrophoresis (protocol modified from [16]) was used to quantify albumin levels in urine.

Immunohistochemistry

Snap frozen rat kidney sections (4 µm) were air dried and acetone fixed. Sections were stained for OX-42 (anti-CD11b/c, kindly provided by Dr. P. Kuppen, LUMC, Leiden)), ED-1 (anti-CD68; kindly provided by Dr. C.D. Dijkstra, VU, Amsterdam), ED-2 (anti-CD163), R73 (anti-TCR; both kindly provided by Dr. E. de Heer, LUMC, Leiden), and CD45 (BD Biosciences), followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse (Jackson). Goat anti-rat KIM-1 (TIM-1; R&D) staining was assessed by HRP-conjugated rabbit anti-goat (DAKO) as secondary antibody. In addition, the sections were incubated with Tyramide-fluorescein isothiocyanate (FITC). FoxP3 (eBioscience) staining was followed by secondary rabbit anti-fluorescein HRP antibody (Abcam). All stainings were visualized by 3,3'-Diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma), followed by nuclear counterstaining with Mayer's hematoxylin (Merck). Quantification of immunohistochemistry was performed by assessing 10 consecutive fields on each section. Using image J software, the positive area in each image (expressed in area fraction) was quantified, except for the FoxP3 staining where analysis was performed by counting the number of positive cells.

Immunofluorescence

Snap frozen rat kidney sections (10 µm) were air dried. Sections were stained with 10 µM dihydroethidium (DHE; Invitrogen) for 30 min at 37°C in the dark. After washing the sections were incubated with 1.6 µM Hoechst (Invitrogen) for 1 min. Quantification was performed by assessing 15 consecutive fields on each section. The positivity of the staining was semi-quantified by assigning 1 (weak), 2 (positive), or 3 (strong) to each field. The average of all fields was takes as measurement of positivity per section.

Statistical analysis

All data were presented as mean ± standard error of the mean (SEM) and subjected to statistical analysis with one- or two-way ANOVA and the Mann-Whitney U-test using GraphPad Prism software. A value of $p<0.05$ was considered statistically significant.

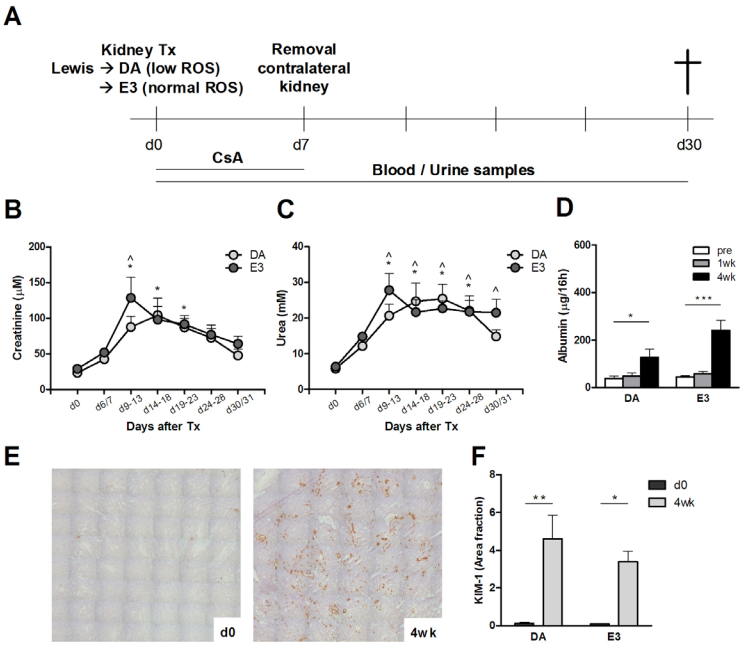

Figure 1: The ROS producing capacity of recipients does not affect the injury of chronically inflamed transplanted kidneys. (***A***) Experimental model with donor Lewis kidney transplanted into either DA.*Ncf1*$^{DA/DA}$ (DA) or DA.*Ncf1*$^{E3/E3}$ (E3) rats. Creatinine (***B***) and urea (***C***) levels measured in serum from DA rats (N=5) and E3 rats (N=5) at different time points after Tx. * p<0.05 compared to d0 for DA, ^ p<0.05 compared to d0 for E3. (***D***) Albumin levels measured in urine and correlated with urine out-put before, at one week and four weeks after Tx. (***E***) KIM-1 staining in kidney tissue of control kidney (d0) and four weeks after Tx (4wk). (***F***) Analysis of KIM-1 staining in cortex at d0 and 4wk. * p<0.05, ** p<0.01, *** p<0.001.

Results

Dysfunctional and damaged kidney four weeks after Tx

In an experimental rat model we transplanted Lewis kidneys into either a DA.*Ncf1*$^{DA/DA}$ (low ROS) or DA.*Ncf1*$^{E3/E3}$ (normal ROS) recipient (Fig. 1A). To assess renal function, we measured serum creatinine and urea levels and observed that both slightly increased at day 6/7 after Tx, but showed a steep increase following removal of the remaining native kidney (Fig. 1 B,C). Until sacrifice at week 4, a descending trend was observed with creatinine, but urea levels remained significantly elevated compared with the pretransplant situation. For both parameters, there was no difference between DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$ recipients.

Albumin levels in urine were measured as a sign of injury and were significantly increased four weeks after Tx, but no difference was observed between DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$ recipients (Fig. 1D). It should be noted that the albuminuria in this model is low compared to other strain combinations, where 8-10 mg/24h have been measured [17;18]. In addition, damage to the kidney

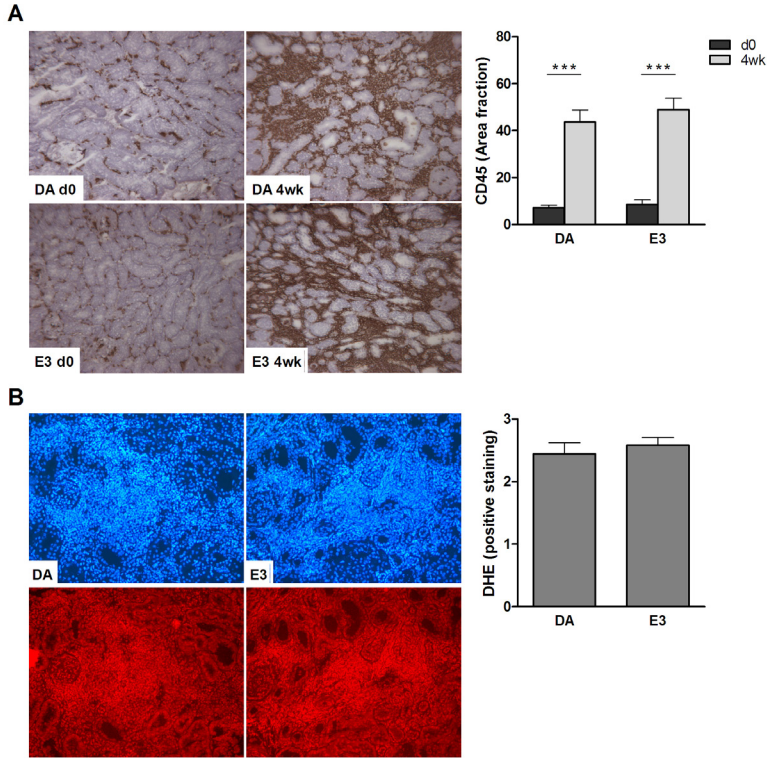

Figure 2: Chronically inflamed kidneys are characterized by leukocyte clusters and presence of ROS. (***A***) Representative picture from CD45 staining for both a DA and E3 rat at d0 and 4wk (left). Digital image analysis of CD45 staining in of the cortex at d0 and 4wk (right). (***B***) DHE (upper) and Hoechst (lower) staining of kidney tissue from a representative DA and E3 rat at 4wk (left), and semi-quantative analysis of DHE staining at 4wk (right).* $p<0.05$, ** $p<0.01$, *** $p<0.001$.

four weeks after Tx was confirmed by strong expression of the tubular the injury marker KIM-1, but again no difference was observed between DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$ recipients (Fig. 1E,F).

Leukocyte clusters and ROS present in infiltrate

We next investigated the number of infiltrating cells by staining with the leukocyte marker CD45. In the cortex of control kidneys, small numbers of CD45 positive cells were observed located in the peritubular space, most likely representing resident myeloid cells. In contrast, four weeks after Tx the cortex was characterized by a strong increase of CD45 positive cells, observed in large leukocyte clusters (Fig. 2A). Both in distribution and quantity, no difference was observed between DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$ recipients.

To monitor the contribution of ROS in the graft inflammation, we stained the tissue with the ROS marker dihydroethidium (DHE). DHE staining was observed, and most prominently in the leukocyte clusters as indicated by the high cell density observed with Hoechst staining, although there was no difference

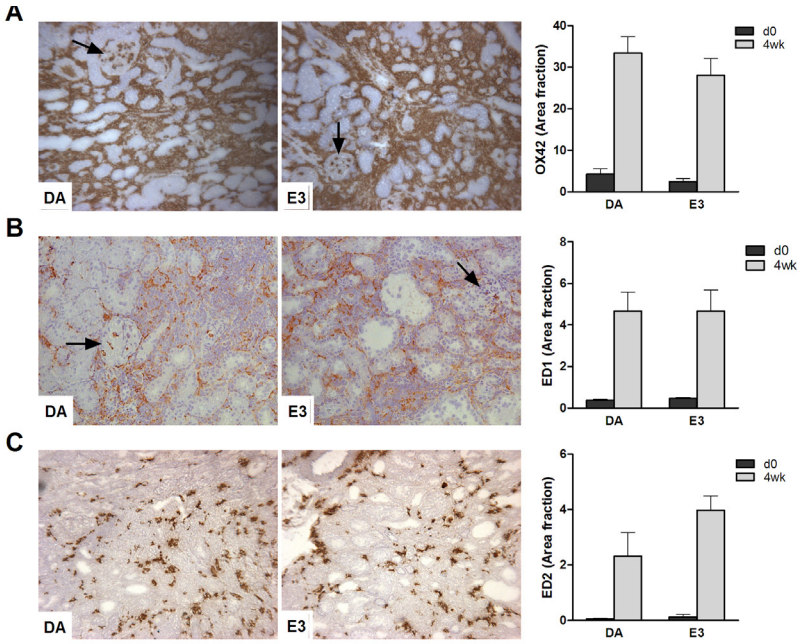

Figure 3: The ROS producing capacity of recipients does not affect the composition of myeloid cell populations. (***A***) OX42, (***B***) ED-1, (***C***) and ED-2 staining in the cortex at d0 and 4wk after Tx. On the left side a representative picture of the cortex at 4wk from both a DA and E3 rat, and quantification of positive area fraction on the tissue on the right side. Arrows indicate positive staining in glomeruli.

between the rats (Fig. 2B).

Similar composition of myeloid cell populations

As Mph have an important role in graft rejection [4] and are a major cell type expressing NOX2, we investigated the presence of myeloid cells in the kidney tissue. A high number of OX42 (CD11b/c) positive cells were seen distributed throughout the entire cortex (Fig. 3A). ED-1 (CD68) positive Mph were observed both in leukocyte clusters and the peritubular area, whereas ED-2 (CD163) positive Mph, most likely representing Mph2, were mostly located in the leukocyte clusters (Fig. 3B,C). However, the quantity of ED1 and ED2 positive cells was lower compared with the OX42 staining.
We also observed OX42 and ED1 positive cells in the glomeruli (indicated by arrows), which has been shown before [19]. No difference in DA.*Ncf1*$^{E3/E3}$ and DA.*Ncf1*$^{DA/DA}$ rats was shown regarding the number of myeloid cells, although a trend towards higher numbers of ED2 positive Mph was observed in recipients with normal ROS-producing cells.

Decreased T cell infiltrate with ROS-producing cells

T cells are an essential cell involved in graft rejection [2]. Therefore we stained the kidney with R73, a pan T cell marker specifically detecting the TCR. T cells

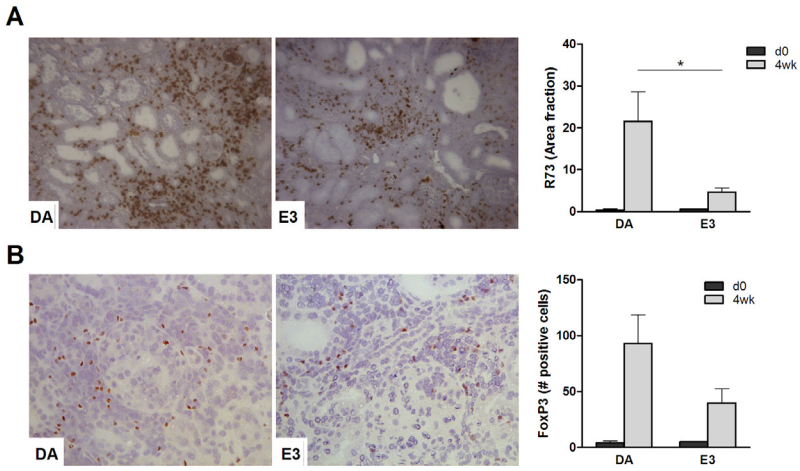

Figure 4: Decreased T cell infiltrate in recipients with a normal ROS-producing capacity. (***A***) A representative picture of the cortex 4wk after Tx (left) and analysis of R73 staining at d0 and 4wk (right). (***B***) FoxP3 positive cells observed in the cortex at d0 and 4wk after Tx. On the left side a representative picture of the cortex at 4wk from a DA and E3 rat, and quantification of the tissue on the right side. * $p<0.05$

were mostly observed in the leukocyte clusters throughout the cortex, but some positive cells were also located in the interstitium (Fig. 4A). The number of T cells was significantly lower in DA.*Ncf1*$^{E3/E3}$ rats compared to DA.*Ncf1*$^{DA/DA}$ rats (Fig. 4A).

Since the presence of Tregs can be beneficial for graft outcome [5], we investigated the presence of these cells in the kidney tissue. We observed FoxP3 staining especially in the leukocyte clusters in the cortex, with a trend towards lower numbers observed in DA.*Ncf1*$^{E3/E3}$ recipients compared to DA.*Ncf1*$^{DA/DA}$ rats (Fig. 4B). In view of the reduced T cell infiltrate, the relative amount of FoxP3 positive cells (ratio FoxP3: R73 cells) appeared higher in DA.*Ncf1*$^{E3/E3}$ rats (43 positive cells/ 5 % positive area fraction) compared to DA.*Ncf1*$^{DA/DA}$ rats (22 positive cells/ 5 % positive area fraction).

Discussion

In this study we investigated the contribution of NOX2-derived ROS in a model of chronic renal inflammation. Lewis rat kidneys were transplanted into recipients with either normal or reduced NOX2-derived ROS, making use of the congenic DA rat strain DA.*Ncf1*$^{E3/E3}$ and DA.*Ncf1*$^{DA/DA}$. Four weeks after Tx, the allografts were still dysfunctional and damage to the kidney tissue was observed. Moreover, large leukocyte clusters were observed in the kidney, characterized by CD45 and CD11b/c expression. For these parameters, there was no difference between DA.*Ncf1*$^{E3/E3}$ and DA.*Ncf1*$^{DA/DA}$ recipients. However, a decreased number of T cells infiltrated the Tx kidney when recipients had a

normal ROS producing capacity.
The transplant model using DA rats as donor and Lewis rats as recipient has been more widely described in the literature [17;20]. In this study we wanted to specifically address the contribution of ROS by infiltrating inflammatory cells. Therefore we used the congenic DA rats as recipients. We observed ROS staining in the kidney tissue, especially in the leukocyte clusters. Making use of DHE staining, we observed that also DA.*Ncf1*$^{DA/DA}$ rats still have ROS producing capacity [12;21], indicating that next to NOX2, also other sources of ROS are present. ROS produced during oxidative stress (high levels) has been implicated in chronic kidney diseases or kidney Tx in a negative way [22;23]. In contrast, it has been shown that ROS produced in the immunological synapse or produced in lower amounts can inhibit T cell activation and has been indicated in various signaling pathways as well [24-27].
Mph2 have been shown to induce Tregs via the production of ROS [12]. In the current experiments we found no difference in absolute numbers of FoxP3 positive cells in the cortex of transplanted kidneys between DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$ recipients. However, the reduction in Tregs was not as prominent as the reduction in total T cells. Therefore it is tempting to speculate that in DA.*Ncf1*$^{E3/E3}$ recipients the relative amount of FoxP3 positive cells was higher, thereby tilting the balance towards more regulation. This relative increase may be important since it has been assumed that the ratio between effector T cells and Tregs is a main determinant in allograft outcome [28]. *In vitro*, Tregs can steer monocyte differentiation towards CD163 positive Mph [29], whereas *in vivo* Tregs expansion increased the number of CD163 positive Mph in the infiltrate and decreased pro-inflammatory cytokine production [30]. This further indicates that Tregs might exert an immune regulatory role on the infiltrating cells [31]. However, in the current study this altered balance did not affect any of the other inflammatory, injury or functional markers.
When investigating the renal tissue for different myeloid cells, we observed OX42 staining throughout the entire cortex located both in the leukocyte clusters and between the tubuli. There was considerably less ED1 and ED2 staining in the cortex compared with OX42. This might suggest that apart from Mph also granulocytes (which abundantly express OX42) were present in the kidney tissue four weeks after Tx. However, the presence of neutrophils at this late stage after Tx is unlikely, although a constant influx of these cells due to chronic inflammation is possible. An alternative explanation is that OX42 identifies another myeloid population not recognized by ED1 or ED2, or that the OX42 expression is higher and therefore more easily detected in immunohistochemistry. Similarly, the abundant expression of OX42 can explain why reduced amount of T cell infiltrate (as shown by R73 staining) was not reflected by a difference in CD45 staining.
ED1 is considered to be a general Mph marker, whereas ED2 is more specific for Mph2. Since, compared to ED1, there was less ED2 staining present in the infiltrate, it is likely that also Mph1 were present. This indicates that both pro-inflammatory mechanisms and tissue repair were simultaneously taking place at this stage after Tx. Since no difference was observed between DA.*Ncf1*$^{E3/E3}$

and DA.*Ncf1*$^{DA/DA}$ rats, this suggests that the ROS producing capacity of the recipient's immune cells is not involved in the active process of Mph recruitment and function after Tx. However a trend towards more Mph2 was observed in the grafts from DA.*Ncf1*$^{E3/E3}$ recipients. The presence of the diverse immune cells in the lymphoid clusters would allow interactions between these cells and therefore also allow for the possibility of ROS signaling within the graft. Nevertheless, diminished numbers of R73 positive T cells were observed in the cortex of the Lew kidney with DA.*Ncf1*$^{E3/E3}$ rats as recipients. By performing the renal transplantation and analyzing the tissue of this chronically inflamed allograft (with infiltrating macrophages having a difference in ROS producing capacity), we hypothesized to pick up differences in the 'local' inflammatory response (ie in the graft). However, we cannot exclude that part of the difference we ultimately found has its origin at other locations (like in the lymphoid organs). This will be the place where most T cell activation will be initiated, and also here signaling through ROS might take place. Since these molecules only act on a short distance it could be hypothesized that the immunological synapse could be important for this process. The reduced T cell infiltrate could potentially hint towards an immunosuppressive role of NOX2-derived ROS. In a collagen-induced arthritis model Mph-derived ROS was clearly shown to be beneficial in suppressing T cell responses [13]. It most likely that the subtle ROS producing capacity by Mph in our model was insufficient to overcome the high inflammatory state in the kidney after Tx, which only showed a decreased T cell infiltrate. Due to the nature of our model, we believe that the amount of ROS produced here by Mph was insufficient to have an impact on the inflammatory response in the allograft. We recently showed that inhibition of ROS can under some conditions also suppress T cell proliferation [32]. In that study we used a human *in vitro* model of monocyte-derived macrophages and used the inhibitor DPI for blocking ROS-mediated effects. Unfortunately, DPI is not specific for NOX2, and therefore this can explain the inhibitory effects of DPI on T cell activation. In the current study the difference is based on a genetic polymorphism in one of the NOX2 gene, and therefore can specifically address differences in NOX2-mediated ROS. A partially protective role of ROS under such conditions is in line with various auto-immune diseases where Mph-derived ROS has been shown to be essential for protection [13;14].

In conclusion, in the Lew-to-DA renal Tx model we observed leukocyte clusters four weeks after Tx. In these clusters the presence of myeloid cells and FoxP3 positive Tregs were observed, but no difference was shown between DA.*Ncf1*$^{E3/E3}$ and DA.*Ncf1*$^{DA/DA}$ rats. Although a decreased number of infiltrating T cells was shown in allografts of DA.*Ncf1*$^{E3/E3}$ recipients having a normal ROS-producing capacity, in this experimental model no effect was observed on other inflammatory cells or signs of injury.

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