Transplant Immunology 30 (2014) 65–70

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

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,1], Karin M. Koekkoek [a,2], Kyra A. Gelderman [a,b,3], Cees van Kooten [a,*,4]

[a] Dept. of Nephrology, Leiden University Medical Center, Leiden, The Netherlands
[b] Dept. of Pathology, VU University Medical Center, Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 13 October 2013
Received in revised form 13 December 2013
Accepted 13 December 2013
Available online 21 December 2013

Keywords:
Macrophage
ROS
T cell
Transplantation

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 were 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.

1. 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, and 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

Abbreviations: CGD, Chronic granulomatous disease; DA, Dark Agouti; DHE, Dihydroethidium; Lew, Lewis; Mph, Macrophage; NOX2, NADPH oxidase 2; ROS, Reactive oxygen species; Tx, Transplantation; Tregs, Regulatory T cells.

* Corresponding author at: LUMC, Nephrology, C7-Q, Albinusdreef 2, 2333 ZA Leiden, The Netherlands. Tel.: +31 71 5262148; fax: +31 71 5266868.
E-mail address: kooten@lumc.nl (C. van Kooten).

[1] Performed experiments and wrote the paper.
[2] Performed experiments.
[3] Designed research and wrote the paper.
[4] Participated in research design and wrote the paper.

0966-3274/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.trim.2013.12.001

the congenic rat strains DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$genotype, 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.

2. Objective

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

3. Materials and methods

3.1. Animals

Littermates (DA.*Ncf1*$^{DA/DA}$ and DA.*Ncf1*$^{E3/E3}$) were obtained by intercrossing F1 animals from a DA (Harlan, Horst, The Netherlands) × 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.

3.2. 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 buprenorphine (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.

3.3. 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.

3.4. 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 taken as measurement of positivity per section.

3.5. 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.

4. Results

4.1. 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/24 h have been measured [17,18]. In addition, damage to the kidney 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).

4.2. 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 between the rats (Fig. 2B).

4.3. 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.

4.4. 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 were mostly