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Efficient Renal Recruitment of Macrophages and T Cells in Mice Lacking the Duffy Antigen/Receptor for Chemokines

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The Duffy antigen/receptor for chemokines (DARC) is a chemokine-binding protein that is expressed on erythrocytes and renal endothelial cells. DARC-mediated endothelial transcytosis of chemokines may facilitate the renal recruitment of macrophages and T cells, as has been suggested for neutrophils. We studied the role of *Darc* in two mouse models of prolonged renal inflammation, one that primarily involves the tubulointerstitium (unilateral ureteral obstruction), and one that requires an adaptive immune response that leads to glomerulonephritis (accelerated nephrotoxic nephritis). Renal expression of *Darc* and its ligands was increased in both models. Leukocytes effectively infiltrated obstructed kidneys in *Darc*-deficient mice with pronounced T-cell infiltration at early time points. Development of interstitial fibrosis was comparable in both genotypes. Nephrotoxic nephritis was inducible in *Darc*-deficient mice, with both an increased humoral immune response and functional impairment during the early phase of disease. Leukocytes efficiently infiltrated kidneys of *Darc*-deficient mice, with increased cell numbers at early but not late time points. Taken together, renal inflammation developed more rapidly in DARC-deficient mice, without affecting the extent of renal

injury at later time points. Thus, genetic elimination of *Darc* in mice does not prevent the development of renal infiltrates and may even enhance such development during the early phases of interstitial and glomerular diseases in mouse models of prolonged renal inflammation. (*Am J Pathol* 2009, 175:119–131; DOI: 10.2353/ajpath.2009.080590)

Chemokines orchestrate the recruitment of inflammatory cells to specific microenvironments in both lymphoid tissue and the sites of inflammation, like the injured kidney.^{1–3} The Duffy antigen/receptor for chemokines (DARC) is a seven-transmembrane-spanning protein, which binds various inflammatory chemokines of different chemokine subgroups.^{1,4,5} It was initially identified as a blood group, the Duffy antigen, involved in rare transfusion reactions. In addition DARC serves as the cellular receptor for invasion of erythrocytes by the malaria parasite *Plasmodium vivax*.^{4,6} DARC is also expressed on endothelium of high endothelial venules in lymphoid tissue and peritubular capillaries in the kidney, where recirculation and extravasation of inflammatory cells takes place.^{7,8} The expression of DARC on endothelial cells can be regulated as we demonstrated an increased number of DARC-positive peritubular vessels in biopsies from various forms of human allograft rejection and from glomerulonephritis.^{9–11} Furthermore, the number of CCR5 positive cells (a receptor for the chemokine CCL5/RANTES) correlated positively with the number of DARC positive vessels, suggesting a potential role of DARC in inflammatory cell recruitment.¹¹

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However, a functional role for DARC in renal inflammation remains hypothetical, and might depend on the time point and exact inflammatory microenvironment. DARC could function as a chemokine sink on erythrocytes. *Darc* deficiency might result in an increased inflammatory response, as it has been demonstrated in *Darc*-deficient mice.^{6,12} On the other hand, on endothelial cells DARC is involved in chemokine transcytosis and apical retention, and therefore it might have pro-inflammatory functions.^{5,13–15} Zarbock et al¹⁶ used two neutrophil-dependent models, ie, ischemia-reperfusion and lipopolysaccharide (LPS)-induced acute renal injury to examine the role of *Darc*. *Darc*-deficient mice were protected from renal injury in both models with no renal dysfunction during ischemia-reperfusion injury and only mild renal dysfunction in LPS-induced injury.¹⁶ Neutrophil recruitment to the postischemic kidney was significantly reduced.¹⁶

Currently, there is no published evidence for DARC being involved in macrophage or T cell recruitment into diseased kidneys. These cell types are involved in progressive renal diseases. We therefore used two models of renal injury characterized by prominent renal macrophage and T cell accumulation to investigate a potential role of *Darc* for mediating their renal recruitment. In obstructive nephropathy induced by unilateral ureteral obstruction (UO) infiltrating macrophages have been identified as mediators of interstitial injury and fibrosis.^{17,18} In the nephrotoxic nephritis (NTN) model a T-cell-dependent adaptive immune response leads to immune complex-mediated glomerular and secondary tubulointerstitial injury. This is driven by both macrophages and T cells accumulating in nephritic kidneys.^{19–21} Here, we show that in both models macrophages and T cells efficiently infiltrate the injured renal tissue in a *Darc*-independent manner, arguing against a major role for *Darc* in T cell and monocyte extravasation in these models. Moreover, we demonstrate that renal inflammation developed more rapidly in *Darc*-deficient mice, without affecting the extent of renal injury at later time points.

Materials and Methods

Mouse Experiments

Homozygous *Darc*-deficient mice backcrossed for more than 10 generations onto a C57BL/6 genetic background were provided by Antal Rot (Novartis-Institute, Vienna). Obstructive nephropathy was induced by unilateral ureteral ligation in mice as previously described.²² In brief, the left distal ureter was ligated with a 2/0 Mersilene suture through a low midline abdominal incision in anesthetized female mice aged 8 to 10 weeks. Unobstructed contralateral kidneys served as controls. Initially, two series of experiments were pooled with a total of 13 C57BL/6 wild-type and 11 *Darc*-deficient (*Darc*^{−/−}) mice analyzed at day 10 after UO. In addition, 8 to 11 mice of each genotype were analyzed at day 5 of UO. At 5 and 10 days, respectively, mice were sacrificed in deep anesthesia by cervical dislocation.

NTN was induced in 7- to 9-week-old C57/BL6 wild-type mice ($n = 27$) or *Darc*-deficient mice ($n = 18$) essentially as previously described.²³ In brief, nephrotoxic serum was prepared by immunizing rabbits with mouse glomerular basement membrane preparations. NTN was induced in male mice following subcutaneous immunization in both flanks with 0.2 mg of rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) in Freund's complete adjuvant (Sigma-Aldrich, Deisenhofen, Germany). Three days later, mice were injected intravenously with 75- μ l heat-inactivated, filter-sterilized nephrotoxic rabbit serum. Spot urine samples were collected at weekly intervals. At days 7, 21, or 42 mice were sacrificed in deep anesthesia by cervical dislocation. Both kidneys were harvested for flow cytometry and histological analysis. All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local governmental authorities.

Functional Assessment of Renal Injury in NTN

Peripheral blood was collected at necropsy to obtain serum and whole blood samples. Serum values for creatinine, urea, protein, and cholesterol were measured with an Olympus AU-640 autoanalyzer, and hematological parameters were obtained with a Sysmex XE-2100 autoanalyzer at Synlab.vet (Augsburg, Germany). Urinary albumin concentrations in spot urine samples were determined by enzyme-linked immunosorbent assay as previously described.²⁴ Urinary creatinine levels were measured by the alkaline picric acid method with a commercially available kit (DiaSys Diagnostic Systems, Holzheim, Germany).

Humoral Immune Response to Rabbit Antiserum in NTN

Circulating mouse anti-rabbit IgG antibodies were measured by enzyme-linked immunosorbent assay in serum collected at the end of the NTN study.²³ Assays were performed using 96-well microtiter plates coated with rabbit IgG (Jackson ImmunoResearch Laboratories) at a concentration of 100 μ g/ml, and bound mouse IgG was detected using fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) as the detecting antibody at a dilution of 1:250. The fluorescence activity was measured at 530 nm by a fluorescence plate reader. Serum from each mouse was tested in serial dilutions from 1:100 to 1:800, and results are given in arbitrary units.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

To quantify the mRNA expression of *Darc*, *Ccl2/Mcp-1*, *Ccl3/Mip-1 α* , *Ccl5/RANTES*, *Cxcl10/Ip-10* and *Cx3cl1/fractalkine*, we used real-time RT-PCR as described.²⁵ In brief, real-time RT-PCR was performed on a TaqMan ABI

7700 sequence detection system (Applied Biosystems, Darmstadt, Germany) using heat-activated TaqDNA polymerase (Amplitaq Gold, Applied Biosystems). Quantification of the given templates was performed according to the standard curve method. Commercially available predeveloped TaqMan reagents were used for murine Ccl2/Mcp-1 (NM_011333.3), Ccl3/Mip-1 α (NM_011337), Ccl5/RANTES (NM_013653.1), Cxcl10/Ip-10 (NM_021274.1), and Cx3cl1/fractalkine (NM_009142.3) (all from Applied Biosystems). For murine Darc (NM_010045.2) the following oligonucleotide primers (300 nmol/L) and probe (100 nmol/L) were used: sense primer 5'-GGCTGAA-GATAGCACTGAGCAA-3', anti-sense primer 5'-TGGAC-CTCACCAAGCATCA-3', fluorescence-labeled probe (FAM) 5'-TCTGGTTCATTTCTGGTGGCCTCATG-3'.¹² The expression of candidate genes was normalized to 18S rRNA. All measurements were performed in duplicates. Controls consisting of bidistilled H₂O were negative in all runs.

Flow Cytometry of Peripheral Blood and Renal Leukocyte Subsets

Single-cell suspensions from individual kidneys were prepared essentially as previously described.²⁶ In brief, mechanically disaggregated tissue was incubated for 20 minutes in 5 ml of 1X Hanks' balanced salt solution containing 1 mg/ml collagenase type I and 0.1 mg/ml deoxyribonuclease type I (both from Sigma-Aldrich) at 37°C. After incubation in 5 ml 2 mmol/L EDTA in 1X Hanks' balanced salt solution (without calcium and magnesium) for 20 minutes at 37°C the supernatant containing isolated cells was removed and kept on ice. In a second enzyme step the remaining pellet was incubated in 5 ml of 1 mg/ml collagenase I in 1X Hanks' balanced salt solution for 20 minutes at 37°C. The resulting suspension was subsequently passed through a 19-gauge, 27-gauge, and 30-gauge needle, and pooled with the first supernatant from the EDTA incubation. Cells were filtered through a 70- μ m cell strainer (Becton Dickinson, Heidelberg, Germany), and washed twice in Dulbecco's phosphate-buffered saline. The obtained single-cell suspension contained infiltrating leukocytes and intrinsic renal cells, the largest proportion being tubular epithelial cells.

Peripheral blood leukocyte subsets and infiltrating renal leukocytes were quantified by 4-color flow cytometry using the following antibodies: phycoerythrin-conjugated anti-mouse CD45 (clone 30-F11, Becton Dickinson) as a pan-leukocyte marker, fluorescein isothiocyanate-conjugated anti-mouse CD3 ϵ (clone 145-2C11, Becton Dickinson) as a T cell marker, allophycocyanin-labeled anti-mouse CD4 (clone RM4-5, Becton Dickinson), phycoerythrin-Cy5-labeled anti-mouse CD8 (clone 53-6.7, Becton Dickinson) for peripheral blood and renal cell suspensions, or phycoerythrin-conjugated CD45 and fluorescein isothiocyanate-conjugated anti-mouse F4/80 (clone CL:A3-1, Serotec, Oxford, UK) as a macrophage/dendritic cell (DC) marker for renal cell suspensions. Monocytes and granulocytes in peripheral blood were

identified by their characteristic light scatter properties. The amount of positively stained cells was expressed as percentage of CD45 positive leukocytes (peripheral blood) or total renal cells (kidneys).

Histology and Immunohistochemistry

Hematoxylin and eosin, periodic acid-Schiff, and silver stains were performed following routine protocols. Immunohistochemistry for the macrophage/DC marker F4/80 (Serotec), for CD3 (Serotec), and for smooth muscle actin (SMA, DAKO, Hamburg, Germany) was performed on formalin-fixed, paraffin-embedded tissue as previously described.²⁷⁻³⁰ 3- μ m tissue sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. After subsequent washing steps in phosphate-buffered saline, endogenous peroxidases were blocked by incubation in 3% hydrogen peroxide and endogenous biotin using the avidin/biotin blocking kit (Vector, Burlingame, CA). Antigen retrieval was performed in an autoclave oven (for SMA, CD3, and neutrophils) or by protease digestion (for F4/80). After subsequent washing steps, tissues were incubated by secondary biotinylated antibodies (Vector). For F4/80 staining an additional biotinylated antibody against the secondary reagent was used for signal amplification. This was followed by the ABC reagent (Vector), a washing step with Tris buffered saline and signal development with DAB combined with nickel chloride to produce a black color product. For counterstaining we used a brief incubation in methyl green.

Image Analysis

Morphometric quantification was performed on 15 consecutive high-power fields (original $\times 400$) or on 15 glomeruli by the use of Qwin software (Leica, Bensheim, Germany) in five to eight mice per group and time point.²⁷ The area of positive signal was measured by an observer blinded to experimental groups and expressed as fraction of the area of the high-power field. The number of CD3 positive T cells and neutrophils were counted per high-power field ($\times 400$).

Point counting was used for the quantification of interstitial matrix area in UUO.²² Digital pictures were taken from 10 consecutive cortical areas from both wild-type ($n = 5-7$) and Darc-deficient ($n = 5-7$) kidneys per time point (original $\times 250$). A grid of 48 points was superimposed and the points over interstitial matrix were expressed as matrix score.

Statistical Analysis

For the comparison of means the nonparametric Mann-Whitney *U* and Dunn's multiple comparisons test were used (InStat software, version 3.05, Intuitive Software for Science, San Diego, CA). $P < 0.05$ was considered to be statistically significant. Values are given as means $\pm$ SEM.

Figure 1. Expression of Darc, Ccl2/Mcp-1, Ccl5/RANTES, Ccl3/Mip-1 α , Cxcl10/Ip-10, and Cx3cl1/fractalkine mRNA in ligated kidneys 5 and 10 days after UUO. Expression of Darc was studied in non-ligated contralateral and ligated kidneys by quantitative real-time RT-PCR. In ligated kidneys of wild-type mice Darc expression was significantly induced at days 5 and 10 after UUO compared with contralateral control kidneys (A). In addition, mRNA expression of Ccl2/Mcp-1 and Ccl5/RANTES progressively increased in ligated wild-type kidneys, and a similar increase was present in ligated kidneys of Darc-deficient mice (B, C). Expression of Ccl3/Mip-1 α , Cxcl10/Ip-10, and Cx3cl1/fractalkine, which do not bind to Darc, was increased to comparable extent in ligated kidneys of wild-type and Darc-deficient mice (D-F). Values given are normalized to 18S mRNA and are shown as means $\pm$ SEM; $n = 6-8$ per group. n.s., not significant.

Results

Expression of Darc and Chemokine Ligands Correlates with Interstitial Leukocyte Infiltration and Fibrosis in UUO

Expression of Darc mRNA significantly increased in ligated kidneys of wild-type mice at days 5 and 10 compared with contralateral controls (Figure 1A). Moreover, mRNA expression of the Darc ligands Ccl2/Mcp-1 and Ccl5/RANTES was progressively up-regulated in UUO kidneys (Figure 1, B and C), consistent with our own previous reports.³¹ Furthermore, we evaluated three chemokines that might be involved in the recruitment of macrophages and T cells, which do not bind to Darc. Ccl3/Mip-1 α is a ligand of the chemokine receptor CCR1, which has been shown to be functional relevant in the UUO model.²² Cxcl10/Ip-10 is a T cell chemoattractant

and Cx3cl1/fractalkine has broad leukocyte chemoattraction. For all these three chemokines mRNAs were significantly induced in UUO kidneys of wild-type animals, as compared with non-ligated contralateral control kidneys (Figure 1, D-F).

Silver methenamine-stained sections revealed severe tubular injury and interstitial fibrosis in UUO kidneys with progression from day 5 to day 10. The contralateral non-ligated kidneys demonstrated normal morphology as expected (Figure 2, A and B; Figure 3A). Renal leukocyte infiltrates were assessed by flow cytometry of whole kidney single cell suspensions and immunohistochemistry. At days 5 and 10 after UUO, a prominent accumulation of F4/80 positive macrophages/dendritic cells and CD3 positive T cells was present in the interstitium of wild-type mice, compared with non-ligated contralateral control kidneys (Figure 2, E, F, I, J; Figure 3, B and C). As a cellular marker of fibrosis staining of SMA positive myo-

Figure 2. Renal injury and leukocyte infiltrates in wild-type and *Darc*-deficient mice at day 10 of UUO. Renal injury was studied on routine histological stainings (silver methenamine, **A–D**; original magnification, $\times 400$). The non-ligated kidneys demonstrated a normal appearance of the tubulointerstitium with the tubuli back to back (arrow in **A**) and normal tubular epithelial cells (arrowhead in **C**). In contrast in the ureter-ligated kidneys the interstitium was widened with deposition of interstitial matrix (arrows in **B** and **D**), and signs of severe tubular injury (arrowhead in **B**). Leukocyte infiltrates in non-ligated contralateral and ligated kidneys of wild-type and *Darc*-deficient mice were analyzed by immunohistochemistry for F4/80 (**E–H**, original magnification, $\times 200$; inset original magnification, $\times 1000$) and CD3 (**I–L**, original magnification, $\times 200$; inset original magnification, $\times 1000$) and CD3 positive myofibroblasts (**M–P**, original magnification, $\times 200$; inset original magnification, $\times 1000$) was performed to assess the extent of interstitial fibrosis. The inset illustrates the cellular source of the staining. In comparison with contralateral non-ligated kidneys numbers of F4/80 positive macrophages/DCs and CD3 positive T cells significantly increased in the cortex of UUO kidneys of wild-type and *Darc*-deficient mice in a similar way. Renal injury and interstitial accumulation of myofibroblasts was also comparable in both groups, indicating a similar extent of interstitial inflammation and fibrosis in wild-type and *Darc*-deficient mice.

fibroblasts was assessed by immunohistochemistry. In wild-type UUO kidneys a progressive interstitial accumulation of myofibroblasts was found from days 5 to 10, compared with contralateral control kidneys (Figure 2, M and N; Figure 3D).

UUO in *Darc*-Deficient Mice

To examine the potential impact of *Darc* on renal leukocyte infiltration and tissue injury, we induced UUO in *Darc*-deficient mice. Renal mRNA expression of the chemokines Ccl2/Mcp-1 and Ccl5/RANTES was not different in *Darc*^{−/−} mice as compared with wild-type controls (Figure 1, B–D). If at all, there was a nonsignificant trend toward increased expression of Ccl2/Mcp-1 in UUO kidneys of *Darc*-deficient mice. Expression of Ccl3/Mip-1 α , Cxcl10/Ip-10, and Cx3Cl1/fractalkine was also not significantly different between wild-type and *Darc*-deficient

mice at days 5 and 10 after UUO (Figure 1, D–F). These chemokines do not bind to Darc.

When total renal cell suspensions were analyzed by flow cytometry at day 5 after UUO numbers of CD45 positive leukocytes, F4/80 positive macrophages/dendritic cells and CD3 positive T cells, including CD4 and CD8 T cell subsets, were not significantly different between ligated kidneys of wild-type and *Darc*-deficient mice (data not shown). Immunohistochemistry of cortical kidney sections demonstrated similar numbers of F4/80 positive leukocytes in ligated kidneys of both groups of mice (Figure 2, F–H; Figure 3B). However, immunohistochemistry revealed an increased cortical accumulation of CD3 positive T cells in ligated kidneys of *Darc*-deficient mice at day 5 after UUO (Figure 2, K and L; Figure 3C). At day 10 a trend persisted for higher T cell numbers in *Darc*-deficient mice, but did not quite reach statistical significance (Figure 3C). Interestingly, by immunohisto-

Figure 3. Morphometric analysis of renal injury, leukocyte infiltrates and myofibroblast accumulation in UUO kidneys of wild-type and *Darc*-deficient mice at days 5 and 10. Interstitial matrix deposition (A), accumulation of F4/80 positive macrophages/DCs (B), CD3 positive T cells (C), and SMA positive myofibroblasts (D) were quantified as described in *Materials and Methods*. Compared with non-ligated control kidneys a progressive accumulation of interstitial matrix occurred at days 5 and 10 after UUO (A). This was paralleled by a significant influx of macrophages/DCs and T cells in UUO kidneys at days 5 and 10 (B, C), which was accompanied by an accumulation of interstitial myofibroblasts (D). Note a significantly enhanced T cell infiltration into UUO kidneys of *Darc*-deficient mice at day 5 compared with wild type, with a similar nonsignificant trend at day 10 (C). No significant differences of infiltrating macrophages/DCs and SMA positive fibroblasts were present in ligated kidneys of wild-type and *Darc*-deficient mice, consistent with the comparable extent of fibrosis in both genotypes following UUO (8–12 per group were analyzed; data are given as means $\pm$ SEM).

chemistry we also found a significant increase in CD3 positive leukocyte numbers in the unligated contralateral kidneys of *Darc*-deficient mice at day 5 but not day 10 after UUO, which may indicate the loss of erythrocyte scavenging of chemokines in the *Darc*^{-/-} mice. The numbers of F4/80 positive leukocytes were comparable in contralateral kidneys of wild-type and *Darc*-deficient mice.

No differences in the severity of tubulointerstitial injury and fibrosis was found between wild-type and *Darc*-deficient mice at both time points, as revealed by silver staining and point counting of the interstitial matrix area (Figure 2, B and D; Figure 3A). Moreover, a similar extent of SMA positive fibroblast accumulation was present in UUO kidneys of both genotypes (Figure 2, N–P; Figure 3D). Unligated contralateral kidneys of *Darc*-deficient mice demonstrated normal morphology.

Induction of NTN in Wild-Type and *Darc*-Deficient Mice

Although interstitial T cell infiltrates are a prominent feature in ligated kidneys with UUO, previous studies suggested a predominant pathogenic role of macrophages/

DCs in the UUO model.^{17,18} To investigate the potential role of *Darc* in a T cell-dependent form of nephritis we induced accelerated NTN in wild-type and *Darc*-deficient mice.^{19–21} This immune complex glomerulonephritis leads to secondary interstitial nephritis within 21 days, characterized by a prominent infiltration of macrophages and T cells into glomeruli and the tubulointerstitium.²¹ Functionally, these changes correlate with the development of progressive proteinuria and impairment of renal function.

In kidneys with NTN the mRNA expression of renal *Darc* was increased especially at day 21 in wild-type mice (Figure 4A). In addition, expression of the chemokines Ccl2/Mcp-1 and Ccl5/RANTES was induced in NTN kidneys of wild-type animals. Interestingly, renal mRNA levels of these chemokines was significantly higher in *Darc*-deficient mice at day 21 of NTN (Figure 4, B and C). There was also a non-significant trend toward increased renal mRNA levels for the non-*Darc* ligands Ccl3/Mip-1 α , Cxcl10/Ip-10, and Cx3cl1/fractalkine in *Darc*-deficient mice at day 21 of NTN (Figure 4, D–F).

As shown in Figure 5, wild-type mice subjected to NTN developed progressive albuminuria from day 7 to day 42 (Figure 5A). This was accompanied by elevated serum

Figure 4. Expression of *Darc*, *Ccl2/Mcp-1*, *Ccl5/RANTES*, *Ccl3/Mip-1 α* , *Cxcl10/Ip-10*, and *Cx3cl1/fractalkine* mRNAs in nephritic kidneys at days 7 and 21 of NTN. Expression of *Darc* was studied in NTN kidneys by quantitative TaqMan PCR. An abundant expression of *Darc* was present in wild-type mice especially at day 21 of NTN, compared with untreated control kidneys (dotted line) (A). This was paralleled by a progressively induced mRNA expression of *Ccl2/Mcp-1* and *Ccl5/RANTES* from days 7 to 21, which was significantly enhanced in NTN kidneys of *Darc*-deficient mice at day 21 compared with wild-type (B, C). There was also a nonsignificant trend toward an increased renal mRNA expression of the non-*Darc* ligands *Ccl3/Mip-1 α* , *Cxcl10/Ip-10*, and *Cx3cl1/fractalkine* at day 21 of NTN in *Darc*-deficient mice (D–F). The dotted lines represent respective expression levels in untreated control kidneys. Values given are normalized to 18S mRNA and are means $\pm$ SEM; $n = 6$ or 7 per group.

levels of urea at days 21 and 42, indicating the development of renal insufficiency (Figure 5B). Moreover, serum protein levels were slightly decreased and cholesterol values were increased in wild-type mice with NTN (Figure 5B). This is consistent with their chronic proteinuric state. Urinary albumin excretion and serum parameters were most severely elevated at day 21, with a subsequent decrease, but not normalization, through day 42 of NTN.

Darc-deficient mice with NTN also developed persistent albuminuria (Figure 5A). The degree of albuminuria in *Darc*^{-/-} mice was more pronounced after 7 days of NTN, but was comparable with wild-type mice with NTN at 14, 21 and 42 days. Renal functional impairment of *Darc*-deficient mice was also more severe at day 7 of NTN, but at later time points, values of urea were comparable in wild-type and *Darc*-deficient mice (Figure 5B). The degree of hypoproteinemia was similar in both groups, but hypercholesterolemia was more pronounced in *Darc*-deficient mice throughout the study (Figure 5B).

Hematological parameters commonly altered in chronic inflammatory states like hemoglobin levels, leukocyte counts, and the number of platelets were not different between wild-type and *Darc*-deficient mice at all time points analyzed, ie, days 7, 21, and 42. An exception was an increased peripheral leukocyte count in *Darc*-deficient mice at day 42 (Figure 6A).

Differential blood counts in wild-type and *Darc*-deficient mice at the time of necropsy revealed increased relative numbers of monocytes, decreased granulocytes, and a nonsignificant trend toward higher T cell numbers in the peripheral blood at day 7 of NTN (Figure 6B). At later time points (days 21 and 42) relative numbers were equal for monocytes, granulocytes, lym-

phocytes, and T cells in wild-type and *Darc*-deficient mice (Figure 6B).

Formation of autologous antibodies against the deposited rabbit IgG was not impaired in *Darc*-deficient animals. Notably, at day 7 serum titers of murine anti-rabbit IgG were significantly higher in *Darc*-deficient mice than in wild-type mice (Figure 6C). At later time points antibody titers decreased in both genotypes, being significantly lower in *Darc*-deficient mice at day 42 (Figure 6C).

Leukocyte Infiltrates in NTN Kidneys of Wild-Type and *Darc*-Deficient Mice

To directly assess the potential role of *Darc* in leukocyte recruitment into NTN kidneys, renal leukocyte accumulation was characterized both by flow cytometry (Figure 7) and immunohistochemistry (Figures 8 and 9) at days 7, 21, and 42 of NTN. Flow cytometric analysis of single cell suspensions prepared from whole NTN kidneys revealed a significant 1.6-fold increase in renal CD3 positive T cells, with a 1.8-fold increase in CD8 T cells in *Darc*-deficient mice at day 7 of NTN. The overall numbers of infiltrating renal CD45 positive leukocytes or F4/80 positive macrophages/DCs were not significantly different in wild-type and *Darc*-deficient mice at this time point (Figure 7A). At day 21 after NTN, CD45 positive leukocyte infiltrates in nephritic kidneys of *Darc*-deficient mice were increased by 1.7-fold compared with wild-type, mainly due to an increased influx of F4/80 positive macrophages/DCs (2.1-fold compared with wild-type controls). In addition, a 2.0-fold increase in CD8 positive T cells

Figure 5. Functional parameters of renal injury in wild-type and *Darc*-deficient mice with NTN. Wild-type mice developed persisting albuminuria (A), elevated serum urea levels, hypoalbuminemia and hypercholesterolemia (B) from days 7 to 42 of NTN. Urinary albumin excretion and serum parameters were most severely elevated at day 21, with a subsequent decrease but not normalization at day 42. At day 7, but not days 21 and 42, of NTN albuminuria (A) and increases in urea (B) were more pronounced in *Darc*-deficient mice than in wild-type controls. Cholesterol levels remained higher in *Darc*-deficient mice at all time points (B). Albuminuria was evaluated at indicated days after injection of nephrotoxic serum, and results given are pooled data from independent experiments terminated at 7, 21, and 42 days of NTN ($n = 6$ to 14 per group). Serum levels for urea, protein, cholesterol and hematological parameters were determined at days 7, 21, and 42 at the respective end of the experiments ($n = 6$ –12 per group). The dotted lines indicate baseline values observed in normal wild-type mice. Data represent means $\pm$ SEM; $\#P < 0.05$.

persisted in *Darc*-deficient mice, although the absolute number of the latter cells remained low (Figure 7B). A progressive accumulation of renal T cells was noted in prolonged NTN at day 42, but no quantitative differences were found between leukocyte subpopulations infiltrating kidneys of wild-type and *Darc*-deficient mice (Figure 7C). Analysis by immunohistochemistry for F4/80 and CD3 confirmed their accumulation in NTN kidneys from both wild-type and *Darc*-/- mice (Figure 8, C–F; Figure 9, A and B). There was a trend toward higher T cell numbers at day 7 and F4/80 positive macrophages/DCs at day 21, corresponding to the flow cytometric data. The different results obtained by flow cytometry and immunohistochemistry may be due to differences in sensitivity and analysis of different renal compartments by these two methods (whole kidney including cortex and medulla versus cortex alone, respectively).

Consistent with the rapid inducibility of NTN and the efficient renal leukocyte recruitment in *Darc*-deficient mice, a similar extent of glomerular and tubulointerstitial injury developed in these mice compared with wild-type, as demonstrated by silver methenamine staining (Figure 8, A and B). Moreover, the numbers of interstitial SMA positive myofibroblasts were not significantly different in wild-type and *Darc*-deficient mice throughout the study, indicating a similar extent of tubulointerstitial fibrosis present in both genotypes (Figure 8, G and H; Figure 9C).

Together, the results clearly indicate an efficient recruitment of renal macrophages/DCs and T cells into NTN kidneys of mice lacking *Darc*, leading to a similar extent of renal injury and fibrosis as in wild-type controls. Notably, during the induction phase of NTN development of the systemic immune response against the planted antigen, early leukocyte infiltration and development of renal functional impairment was accelerated in *Darc*-/-

mice as compared with wild-type mice. This accelerated onset of disease, however, did not result in worse renal injury at later time points.

Discussion

The functional role of DARC in chronic renal inflammation is unclear. DARC is expressed on erythrocytes and on endothelial cells of peritubular capillaries in the renal interstitium.^{5,9–11} Erythrocyte-expressed DARC may function as an abundant chemokine binding protein, that quenches some chemokines, thereby preventing broad activation of circulating leukocytes. Furthermore, this may modulate leukocyte infiltration into inflamed tissue.^{6,12} This sink hypothesis for DARC expressed on red blood cells, is supported by studies on early neutrophil trafficking in *Darc*-deficient mice. Dawson and colleagues investigated inflammatory infiltrates at an early time point (2 hours) after LPS injection, which resulted in increased myeloperoxidase activity as a reflection of granulocyte recruitment in lung and liver of *Darc*-deficient mice.¹² Consistent with these findings, Luo et al reported increased myeloperoxidase activities in intestine and lungs of *Darc*-deficient mice challenged with LPS (author's correction).^{12,32} Besides the suggested function as a chemokine sink, erythrocyte DARC may also serve as a chemokine reservoir, prolonging the half-life of chemokines in the circulation. Injected chemokines disappeared more rapidly from blood in *Darc*-deficient mice as compared with wild-type controls.³³ Serum chemokine levels were also lower in volunteers of African descent exposed to LPS, who were DARC-deficient on red blood cells but not on endothelial cells.³⁴

Figure 6. Hematological parameters and humoral immune response in wild-type and *Darc*-deficient mice with NTN at days 7, 21, and 42. Hematologic parameters that are commonly altered by chronic inflammatory responses including hemoglobin levels, leukocyte counts and platelets were not different in nephritic wild-type and *Darc*-deficient mice at days 7, 21, and 42 with the exception of an increased peripheral leukocyte count in *Darc*-deficient mice at day 42 (A). However, flow cytometric analysis of whole blood revealed a significant increase in circulating monocytes, a decrease in granulocytes and a nonsignificant trend toward higher T cell numbers in *Darc*-deficient mice compared with wild-type at day 7, but not days 21 and 42, of NTN (B). Circulating autologous mouse anti-rabbit IgG levels were significantly increased in *Darc*-deficient mice at day 7, without significant differences at day 21 of NTN. In contrast, at day 42 autologous IgG levels were significantly lower in *Darc*-deficient mice than in wild-type controls (C). Antibody titers in serial dilutions of serum are expressed in arbitrary units. Data given are means $\pm$ SEM of 6–12 mice per group.

The expression of DARC on a defined subset of endothelial cells (ie, high endothelial venules in lymphoid tissue, peritubular capillaries in the kidney) has been interpreted as pointing toward a direct role of DARC in inflammatory cell recruitment across the endothelium.³⁵ We recently demonstrated that DARC expressed by endothelial cells is involved in transcellular chemokine transport and enhanced binding of chemokines at the endothelial apical surface.¹⁵ This might increase endothelial chemokines and the recruitment of inflammatory cells *in vivo*. Zarbock et al¹⁶ used two neutrophil dependent models of renal injury to study the role of *Darc* in kidney disease, ie, renal ischemia-reperfusion injury and LPS injection.¹⁶ In contrast to the above-mentioned earlier studies, which described an increased inflammatory response in *Darc*-deficient mice, the authors demonstrated a dramatic improvement in renal function and decreased renal myeloperoxidase activity in ischemia-reperfusion injury.¹⁶ After LPS challenge overall myeloperoxidase activity did not change, but the number of

extravascular neutrophils was reduced.¹⁶ Furthermore, CXCL1/MIP-2 staining on renal endothelial cells was significantly reduced in mice lacking *Darc*,¹⁶ potentially reflecting a decreased capacity of *Darc*-deficient endothelial cells to present this chemokine to circulating leukocytes in peritubular capillaries. The latter study supports a proinflammatory role for endothelial Darc in the recruitment of neutrophils in two acute, neutrophil-dependent models of renal injury.

It remains unknown whether DARC-mediated transendothelial transport of chemokines plays a role in the recruitment of macrophages and T cells in more chronic kidney injury. To explore this possibility we investigated two well established models of progressive kidney injury characterized by renal accumulation of macrophages/DCs and T lymphocytes. In the UUO model a pathogenic role of macrophages in mediating interstitial inflammation and fibrosis has been established.¹⁷ A functional role for T cells could not be demonstrated so far, despite their prominent interstitial accumulation in obstructed kid-

Figure 7. Renal leukocyte infiltrates in nephritic kidneys of wild-type and *Darc*-deficient mice at days 7, 21, and 42 of NTN analyzed by flow cytometry. Quantitative analysis of single cell suspensions prepared from whole kidneys revealed a significant 1.6-fold increase in renal CD3 positive T cells, and a 1.8-fold increase in CD8 T cells in *Darc*-deficient mice at day 7 of NTN, compared with wild-type (A). At day 21, a 1.7-fold increase in CD45 positive leukocytes was present in nephritic kidneys of *Darc*-deficient mice. This was mainly due to a 2.1-fold greater number of F4/80 positive macrophages/DCs in these mice compared with wild-type controls. In addition, numbers of renal CD8 positive T cells increased by twofold in *Darc*-deficient mice (B). At day 42, a particularly prominent infiltration of renal T cells was present in NTN kidneys, without any significant differences in renal leukocyte subpopulations between both genotypes (C). Data given are means $\pm$ SEM of 5 or 6 mice per group.

neys.¹⁸ In the NTN model of immune complex-mediated crescentic glomerulonephritis, a T helper type 1 response drives the inflammatory process in the kidney leading to glomerular and secondary tubulointerstitial injury, with T cells and macrophages being essential for this process.^{19–21}

In both disease models the functional contribution of DARC ligands like CCL2/MCP-1 and CCL5/RANTES

Figure 8. Renal injury and leukocyte infiltrates in NTN kidneys of wild-type and *Darc*-deficient mice at day 21. Renal injury and interstitial fibrosis was assessed by silver methenamine staining (A, B). Leukocyte infiltrates in NTN kidneys of wild-type and *Darc*-deficient mice were analyzed by immunohistochemistry for F4/80 (C, D) and CD3 (E, F). Immunohistochemistry for SMA positive myofibroblasts was used as a surrogate for renal fibrosis (G, H). Glomerular and interstitial injury was similar in both genotypes (A, B), as was the accumulation of SMA positive myofibroblasts in the interstitium (G, H). At day 21 of NTN a prominent infiltration of F4/80 positive macrophages/DCs and CD3 T cells was present in the cortex of nephritic kidneys, both in wild-type and *Darc*-deficient mice (C–F, all original magnifications, $\times 200$. WT, wild-type; *Darc*^{-/-}, *Darc*-deficient mice.

has been demonstrated. We and others have previously shown that Ccl2/MCP-1 and Ccl5/RANTES are induced in UUO kidneys, which correlated with increased expression of the consecutive chemokine receptors CCR1, CCR2 and CCR5 on the infiltrating cells.³¹ Blocking CCR1 or CCR2 substantially reduced renal leukocyte recruitment and ameliorated fibrosis in UUO.^{22,36} Similarly, blockade of Ccl5/RANTES using the antagonist Met-RANTES decreased renal leukocyte infiltration and proteinuria in murine NTN.³⁷ Thus, we hypothesized that reducing renal endothelial transcytosis and apical binding of these chemokines by genetic elimination of DARC could be of potential benefit for the development of the chronic inflammation, which contributes to the progressive decline in renal function.

Figure 9. Morphometric analysis of renal leukocyte infiltrates and myofibroblast accumulation in NTN kidneys of wild-type and *Darc*-deficient mice. Accumulation of F4/80 positive macrophages/DCs (**A**), CD3 positive T cells (**B**), and SMA positive myofibroblasts (**C**) in the cortex of nephritic kidneys was quantified as described in *Materials and Methods*. A progressive infiltration of macrophages/DCs and T cells was present in NTN kidneys from days 7 to 42. By immunohistochemistry this leukocyte influx was similar in wild-type and *Darc*-deficient mice at days 7, 21, and 42 (**A**, **B**). Interstitial myofibroblasts accumulated in NTN kidneys of both genotypes, without quantitative differences between the two genotypes at all time points (**C**). Data are given as means $\pm$ SEM; 6–14 mice per group were analyzed.

Unexpectedly, we found an efficient accumulation of macrophages/DCs and T cells in kidneys of *Darc*-deficient mice subjected to UUO and NTN. Notably, in both models *Darc* deficiency resulted in a moderately increased infiltration of T cells into kidneys during early

phases of the disease. In the NTN model this was associated with a more rapid onset of renal functional impairment at day 7, and a transient increase in numbers of infiltrating renal macrophages/DCs as compared with wild-type mice at day 21. In line with these results we detected an increased expression of the proinflammatory chemokines Ccl2/Mcp-1 and Ccl5/RANTES in NTN kidneys of *Darc*-deficient as compared with wild-type mice. Presumably, the increased number of infiltrating mononuclear leukocytes contributed to the enhanced renal expression of chemokines in *Darc*-deficient mice.

Darc-deficient mice also had a more vigorous immune response against the planted rabbit IgG in the early phase of NTN, which may have contributed to the accelerated development of nephritis. However, the increased T cell infiltration at day 5 of the UUO model cannot be explained by altered systemic immunity, as this model is induced by mechanical obstruction of the kidney. Thus *Darc* elimination resulted in an enhanced inflammatory response as previously reported in the endotoxin model. Potentially this points toward a loss of chemokine binding and thereby scavenging by erythrocytes as a contributing mechanism to the worsening of disease in the *Darc* knockout mice. This might result in lower rates of chemokine removal from the site of injury and an enhanced initial inflammatory response. This hypothesis would be compatible with the accelerated onset of renal inflammation in *Darc*-deficient mice, while the more chronic phase after renal leukocyte infiltration had occurred, would no longer be influenced by this mechanism.

DARC binds a particular subset of inflammatory chemokines like CXCL1/Gro- α , CXCL5/ENA-78, CCL2/MCP-1, CCL5/RANTES and CCL7/MCP-3 both on endothelial and red blood cells. As renal leukocyte recruitment was not impaired in *Darc*-deficient mice, it could be hypothesized that additional chemokines, which do not interact with DARC, might contribute in a compensatory manner to renal leukocyte infiltration in *Darc*-deficient mice. Therefore, we measured the expression of three inflammatory chemokines not bound by DARC ie, Ccl3/Mip-1 α , Cxcl10/Ip-10, and Cx3cl1/fractalkine. None of these were significantly up-regulated in UUO kidneys of *Darc*-deficient as compared with wild-type mice, arguing against that hypothetical explanation.

Taken together, our data indicate that the genetic deficiency of *Darc* enhances the initial inflammatory infiltrate in two different models of renal injury, obstructive nephropathy and NTN. In the more chronic phase of either disease model, *Darc* elimination had no influence on the progressive disease phase. These results would be compatible with the hypothesis that Darc on erythrocytes may serve as a sink for locally generated chemokines, thereby containing the acute inflammatory response. Once the cell infiltrate has occurred, such an effect of erythrocyte Darc would play a lesser role. Our data do not provide evidence for DARC coupled renal endothelial transcytosis of chemokines is contributing in a major way to the interstitial inflammatory infiltrate of UUO or NTN. However, given the potential opposing effects resulting from

the loss of chemokine scavenging by *DARC*-deficient erythrocytes, we cannot at present completely eliminate this possibility. The systemic blockade of *DARC*, although potentially beneficial in acute neutrophil-dependent renal injury, is not a promising therapeutic approach in chronic renal disease.

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