The Duffy antigen receptor for chemokines in acute renal failure: A facilitator of renal chemokine presentation

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Objective: **Acute renal failure remains a major challenge in critical care medicine. Both neutrophils and chemokines have been proposed as key components in the development of acute renal failure. Although the Duffy antigen receptor for chemokines (DARC) is present in several tissues and a highly specific ligand for various chemokines, its exact role *in vivo* remains unclear.**

Design: **Prospective, controlled experimental study.**

Setting: **University-based research laboratory.**

Subjects: **C57BL/6 wild-type and DARC gene-deficient mice ($DARC^{-/-}$).**

Interventions: **To unravel the functional relevance of DARC *in vivo*, we compared wild-type and $DARC^{-/-}$ using neutrophil-dependent models of acute renal failure, induced by either local (renal ischemia-reperfusion) or systemic (endotoxemia, lipopolysaccharide) injury.**

Measurements and Main Results: **Plasma creatinine and blood urea nitrogen concentrations served as indicators of renal function or dysfunction. Enzyme-linked immunosorbent assays were used to measure tissue and plasma chemokine concentrations. We also performed immunostaining to localize chemokine expression and flow cytometry to evaluate neutrophil recruitment into the kidney. Following renal injury, wild-type mice developed moderate renal ischemia-reperfusion(lipopolysaccharide, 300% increase in plasma creatinine concentrations) to severe acute renal failure (renal ischemia-reperfusion, 40% mortality) as well as extensive renal neutrophil recruitment. $DARC^{-/-}$ mice exhibited no renal dysfunction (renal ischemia-reperfusion) or only very mild renal dysfunction (lipopolysaccharide, 20% increase in serum creatinine concentrations). $DARC^{-/-}$ mice showed no postischemic neutrophil infiltration. Although $DARC^{-/-}$ and wild-type mice exhibited similar global renal neutrophil-recruitment during endotoxemia, $DARC^{-/-}$ mice showed significantly impaired neutrophil extravasation. Total renal concentrations of the chemokine macrophage inflammatory protein 2, which has been shown to bind to DARC and to be crucial in postischemic acute renal failure, were either identical (lipopolysaccharide) or only moderately different (renal ischemia-reperfusion) between wild-type and $DARC^{-/-}$ mice. Immunostaining revealed an absence of macrophage inflammatory protein-2 in renal endothelial cells of $DARC^{-/-}$ mice.**

Conclusions: **We suggest that DARC predominantly exerts its effects by controlling spatial chemokine distribution, which in turn regulates neutrophil recruitment and subsequent acute renal failure. (Crit Care Med 2007; 35:2156–2163)**

KEY WORDS: acute renal failure; chemokines; Duffy antigen; neutrophils

Acute renal failure (ARF) remains a major challenge in clinical practice, especially in critically ill patients (1), as it is still associated with high morbidity and mortality (2). Sepsis and ischemia are among the leading causes of ARF. Despite the need for better preventive and therapeutic measures, the precise mechanisms of ARF are still unknown.

Various subtypes of leukocytes, including neutrophils, have been implicated as key components in the development of ARF (3, 4). Leukocyte recruitment during inflammation occurs in a cascade-like fashion and includes several distinct steps: capture, rolling, activation, firm adhesion, and transmigration (5–7).

Chemoattractive cytokines (chemokines) play a pivotal role in leukocyte activation and subsequent firm adhesion (8, 9). Chemokines are highly basic proteins and consist of 70–125 amino acids (10). They are classified according to the number of amino acids between the first and second cysteine residue at NH_2-terminal end (C, CC, CXC, and CX_3C chemokines). Chemokines are expressed constitutively but can also be up-regulated during inflammation. Keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2, two chemokines critical for inflammatory neutrophil recruitment, are among those expressed during endotoxemia (11) and renal ischemia-reperfusion (RIR). Blockade of either chemokine-reduced neutrophil infiltration and improved renal function (11, 12).

Originally described as a blood group antigen and receptor for Plasmodium vivax on red blood cells (RBCs) (13), the Duffy antigen has emerged as a highly specific binding site for both CC and CXC chemokines (Duffy antigen receptor for chemokines, DARC) (14–17). DARC is expressed in postcapillary venular endothelial cells, which are the primary site of leukocyte transmigration in most organs.

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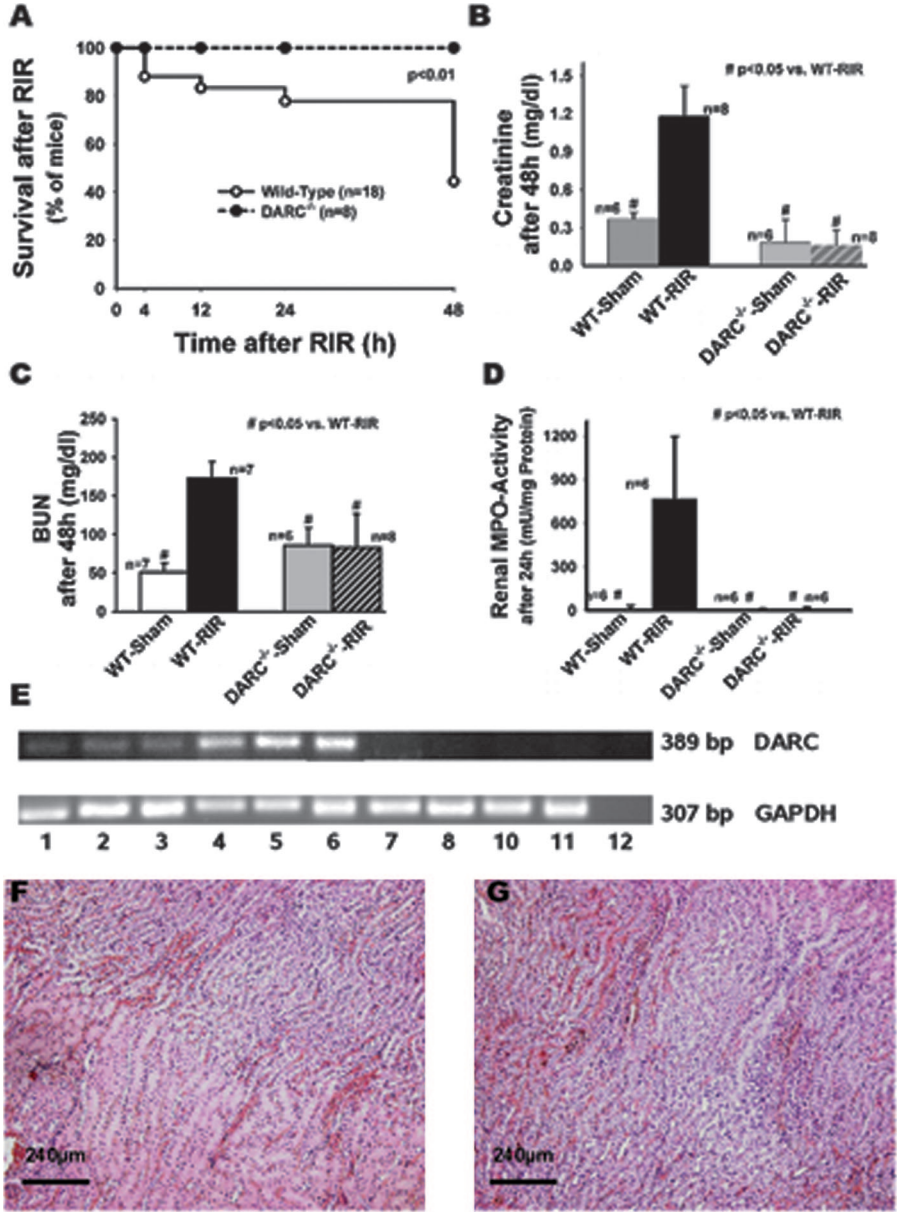

Figure 1. Duffy antigen receptor for chemokines (*DARC*) is expressed in the murine kidney and its deficiency provides vital protection from postischemic acute renal failure (*ARF*). DARC$^{-/-}$ mice displayed a significantly higher survival rate at 48 hrs after renal ischemia-reperfusion (*RIR*) than wild-type (*WT*) mice (*A*). In contrast to DARC$^{-/-}$ mice, WT mice exhibited a significant increase in plasma creatinine and blood urea nitrogen (*BUN*) concentrations at 48 hrs after RIR (*B* and *C*). As indicated by myeloperoxidase (*MPO*), RIR caused a massive influx of neutrophil into the kidney in WT mice but not in DARC$^{-/-}$ mice (*D*). Reverse transcriptase polymerase chain reaction analysis of renal DARC messenger RNA (mRNA) expression (*E*): RIR and lipopolysaccharide (LPS) administration gave rise to a strong up-regulation of DARC mRNA expression: Native WT mice (*lane 1*), WT mice 12 and 24 hrs after sham surgery (*lanes 2 and 3*), WT mice 12 and 24 hrs after RIR (*lanes 4 and 5*), WT mice 4 hrs after LPS (*lane 6*), native DARC$^{-/-}$ mice (*lane 7*), DARC$^{-/-}$ mice 12 and 24 hrs after RIR (*lanes 8 and 9*), DARC$^{-/-}$ mice 4 hrs after LPS administration (*lane 10*), and water (*lane 11*). Postischemic renal histology (*F* and *G*): Corticomedullary sections 48 hrs after RIR demonstrated massive tubular edema and loss of tubular epithelial cells in WT but not in DARC$^{-/-}$ mice. *GAPDH*, glyceraldehyde phosphate dehydrogenase.

Even individuals who do not carry the Duffy antigen on red blood cells express DARC on endothelial cells (18–20). DARC belongs to the family of rhodopsin-like seven helix transmembrane proteins (14, 21). DARC is not G-protein coupled and has no known signaling mechanism (15, 22). Recent studies have proposed two hypotheses for the role of DARC. First, endothelial DARC may be involved in transcellular transport of chemokines across endothelial barriers (23). Second, erythrocytic DARC may function as a regulator of chemokine levels in the blood (24), that is, chemokine sink. However, as current experimental data are unclear or sometimes even contradictory, the exact role of DARC *in vivo* remains unknown (23–25). Data regarding renal expression of DARC are also confusing.

To this end, we have used two previously established animal models of ARF to further unravel the role of DARC *in vivo*. We show that DARC, which is expressed in the murine kidney, is a critical modulator of ARF following either local (ischemia-reperfusion) or systemic (endotoxemia) renal injury. DARC appears to mediate its effects by controlling quantitative and spatial expression of neutrophil-specific chemokines within the kidney.

MATERIALS AND METHODS

Animals. We used adult C57BL/6 wild-type mice (WT mice, 2–3 months old, 20–32 g of body weight, Charles River Laboratories, Wilmington, DE) and corresponding mice with a null mutation in the DARC gene (DARC$^{-/-}$ mice, courtesy of Nobuyo Maeda, University of North Carolina). Mouse colonies were maintained under specific pathogen-free conditions. All experiments were approved by local government authorities and were in agreement with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Reagents. If not stated otherwise, all reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany).

RIR to Induce ARF—Local Injury. Our neutrophil-dependent model of RIR has been extensively described elsewhere (26, 27). It is characterized by a 50% mortality rate over 72 hrs and extensive tubular necrosis in untreated WT mice (28). Renal neutrophil infiltration, as measured by renal myeloperoxidase activities, and plasma creatinine/blood urea nitrogen (BUN) concentrations peak between 24 and 48 hrs (26, 27). Briefly, mice were anesthetized with intraperitoneal injections of ketamine, xylazine, and atropine sulfate and were placed on a heating pad to maintain body temperature. In animals undergoing RIR, both renal pedicles were clamped off for 32 mins with hemostatic microclips. Kidneys were inspected for immediate color change, indicating successful clamping. After clamp removal, kidneys were checked for a change in color within 3 mins to ensure reperfusion. In animals subjected to sham operation, the surgical procedure was identical except that no clamps were applied. Incisions were closed in two layers and animals were allowed to recover. Postoperative analgesia was provided by repeated subcutaneous injections of buprenorphine (2 µg/g body weight) diluted with lactated Ringer's solution (20 µL/g) to provide

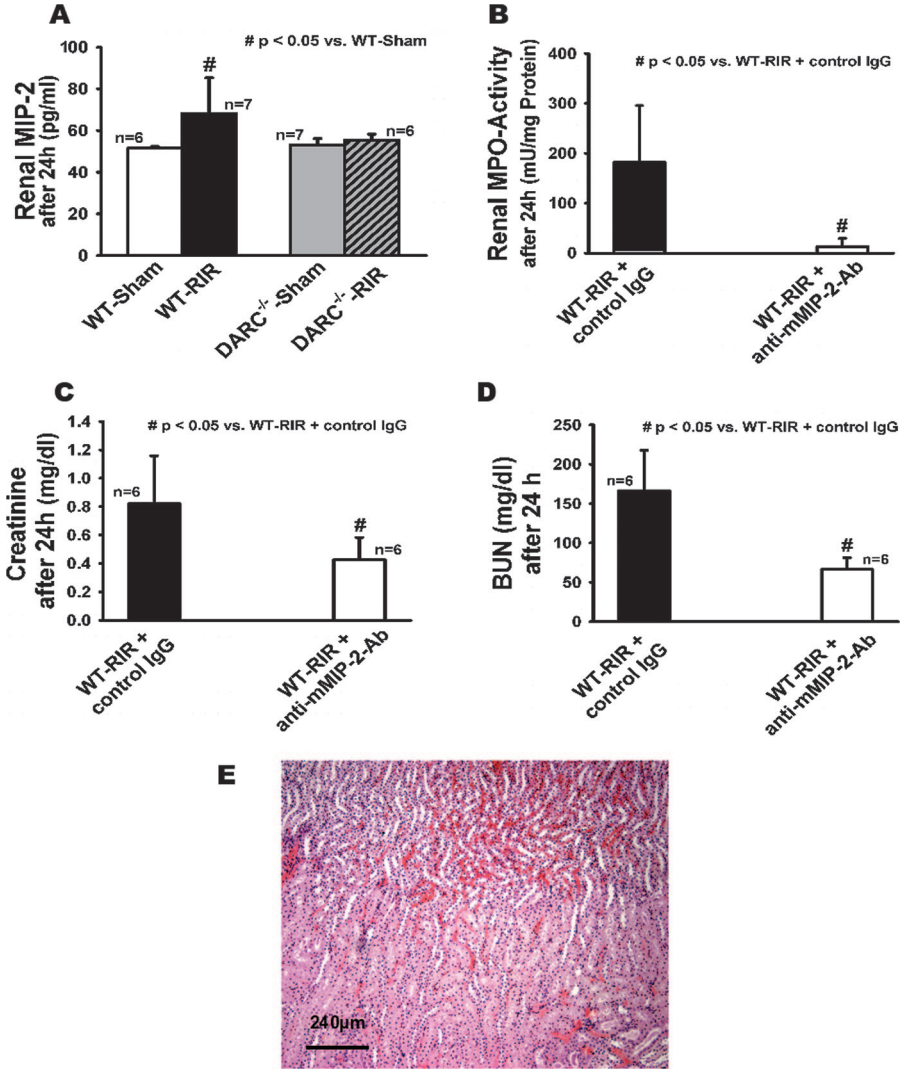

Figure 2. Neutralization of macrophage inflammatory protein (*MIP*)-2 protects from postischemic acute renal failure. Wild-type (*WT*) mice, but not Duffy antigen receptor for chemokines (*DARC*)$^{-/-}$ mice, exhibited a significant increase in renal MIP-2 concentrations after renal ischemia-reperfusion (*RIR*) (*A*). Only mice that had received a blocking polyclonal antibody against MIP-2 displayed a significant reduction in renal neutrophil-infiltration (myeloperoxidase, *MPO*) (*B*) as well as in serum creatinine (*C*) and blood urea nitrogen (*BUN*) concentrations (*D*). Mice pretreated with an anti-MIP-2 antibody did also not show any evidence of severe morphologic damage at 24 hrs after RIR, that is, no tubular edema or loss of tubular epithelial cells (*E*). *Ig*, immunoglobulin.

volume resuscitation. Based on our previous findings (26, 27), mice were euthanized in order to collect blood samples and to harvest both kidneys for further analyses after 24 hrs (peak of postischemic neutrophil infiltration) or 48 hrs of reperfusion (maximum postischemic plasma concentrations of creatinine and BUN).

Lipopolysaccharide (LPS) Injection to Induce ARF—Systemic Injury. As previously described in detail (29), intraperitoneal LPS injection leads to neutrophil-dependent acute renal failure in untreated WT mice. Here, mice received an intraperitoneal injection of LPS 10 µg/g of body weight (*Escherichia coli* O111: B4) as well as subcutaneous injection of 20 µL/g lactated Ringer's solution for fluid resuscitation. At 2, 4, 12, and 24 hrs after LPS injections, mice were anesthetized to harvest both kidneys and to collect blood samples. Untreated, genotype-matched mice served as controls (0 hrs). In this nonlethal model of endotoxemic ARF, peak concentrations of plasma creatinine and BUN are reached after 24 hrs. Renal myeloperoxidase activities reached their maximum 4 hrs after LPS injection and declined thereafter.

Renal Function and Structure. Plasma creatinine and BUN concentrations were measured using commercially available kits (Diazyme, San Diego, and Sigma-Aldrich, Taufkirchen, Germany, respectively). Harvested kidney tissue was fixed in a solution of 10% formaldehyde and 1.5% methanol and paraffin embedded. Next, 4-µm sections were stained with hematoxylin and eosin.

Myeloperoxidase (MPO) Activity. Renal MPO was used as an indicator of renal neutrophil content. The excellent correlation between global renal neutrophil content and renal MPO in these two models of ARF has been shown and discussed before (4, 26, 27, 29). MPO was measured according to our previously published protocol (26, 27). Briefly, samples were homogenized in ice-cold KPO_4 buffer. After removing 17,000 g supernatants, pellets were resuspended in ice-cold KPO_4 buffer, followed by two additional spins. Then 0.5% (w/v) hexacyltrimethylammonium bromide 10 mM EDTA in KPO_4 was added to the remaining pellet. Suspensions were sonicated, freeze-thawed, and incubated for 20 min at 4°C. Supernatants were used to measure MPO. Next, assay buffer that contained 0.2 mg/mL *o*-dianisidine and 158 µM H_2O_2 in 50 mM KPO_4 was added to supernatant. Changes in absorbance were recorded at 460 nm over 3.5 mins. Results were expressed as units of MPO/g of protein of supernatant as determined by bicinchoninic acid assay (Pierce Chemical, Rockford, IL).

Assessment of Neutrophil Trafficking Within the Kidney. To assess renal neutrophil extravasation during ARF, we adapted a recently developed flow-cytometry-based method to determine pulmonary neutrophil extravasation (30) (Fig. 1, *A–C*). Briefly, Alexa 633-labeled antibody against murine neutrophil (GR-1) was injected intravenously 5 mins before anesthetic overdose. This time period is sufficient to label all intravascular but not interstitial neutrophil (30). After euthanizing the animal, we removed nonadherent neutrophils from the renal vasculature by flushing 20 mL of phosphate-buffered saline (perfusion pressure 25 cm H_2O) through the aorta upstream of the renal artery and cutting the inferior caval vein for drainage. Kidneys were removed, minced, and digested with 125 units/mL collagenase type XI, 60 units/mL hyaluronidase type I-s, 60 units/mL DNAse1, and unlabeled anti-GR-1 in order to prevent possible binding of the injected antibody to extravascular neutrophil at 37°C for 60 mins. A cell suspension was made by passing digested kidneys through a 70-µm cell strainer (BD Falcon, Bedford, MA). Erythrocytes were lysed, and remaining leukocytes were resuspended and counted. The fraction of neutrophil in the suspension was determined by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA). Neutrophils were identified by their typical appearance in the forward/sideward scatter (as well as their expression of CD45 [clone 30-F11], 7/4 [clone 7/4], and GR-1 [clone RB6-8C5]. Isotype controls were employed to account for nonspecific antibody binding. All antibodies were purchased from Pharmingen except the anti-mouse GR-1 antibody, which was purified from supernatant of the GR-1 hybridoma (ATCC, Manassas, VA) and fluorescently labeled with an F(ab)-based staining kit following the manufacturer's directions (Alexa Fluor 633, Molecular Probes).

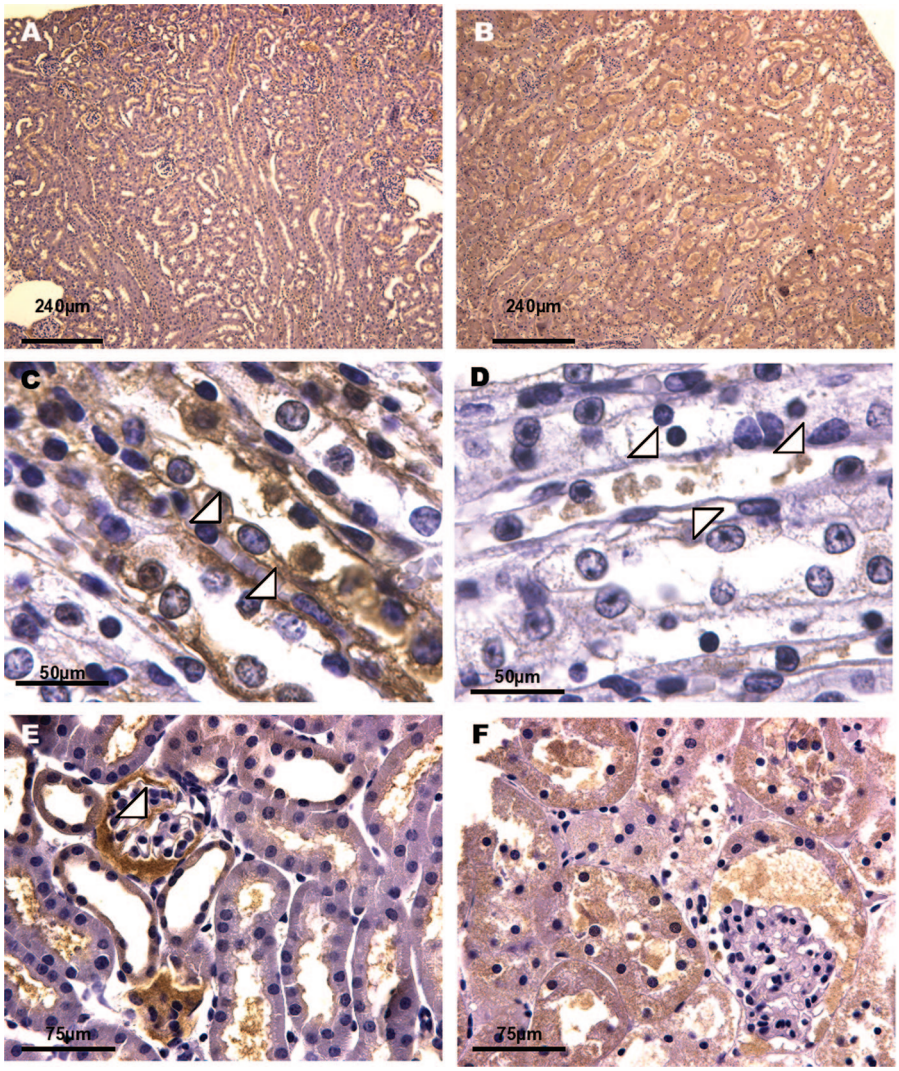

Figure 3. Renal macrophage inflammatory protein (*MIP*)-2 presentation in Duffy antigen receptor for chemokines (*DARC*) controls following renal ischemia-reperfusion. Immunostaining revealed similar overall expression of MIP-2 in kidneys from wild-type (*WT*) mice and DARC$^{-/-}$ mice (*A* and *B*, cortex and outer medulla are shown). At higher magnification, immunostaining showed a striking difference in local MIP-2 presentation between WT mice and DARC$^{-/-}$ mice. In contrast to WT mice (*C* and *E*), DARC$^{-/-}$ mice (*D* and *F*) did not present MIP-2 in renal endothelial cells (*arrowheads*), including glomerular endothelial cells (*E*, WT, *F*, DARC$^{-/-}$). Also, tubular epithelial cells from both WT and DARC$^{-/-}$ mice accounted for a great amount of MIP-2 positive staining (*C–F*).

The expression of GR-1 served to differentiate between intravascular (CD45$^+$7/4$^+$GR-1$^+$) and extravascular/intravascular (CD45$^+$7/4$^+$GR-1$^-$) neutrophils.

MIP-2 Neutralization Experiments. To investigate the role of neutrophil-specific chemokines in our model of postischemic ARF, WT mice received 4 μg of a goat polyclonal anti-mouse MIP-2 antibody intravascularly (R&D Systems, Wiesbaden-Nordenstadt, Germany) 3 hrs before induction of RIR. This dose has been shown to block pulmonary neutrophil-recruitment after hemorrhagic shock completely (31). Control mice received 4 μg of an isotype-matched control antibody intravascularly (R&D Systems, Wiesbaden-Nordenstadt, Germany). After 24 hrs of reperfusion, mice were anesthetized to harvest both kidneys and to collect blood samples.

Immunhistochemistry: MIP-2. Paraffin-embedded kidney sections (5 μm) were incubated with a goat anti-mouse polyclonal antibody against MIP-2 (C-19, Santa Cruz Biotechnology, Santa Cruz, CA) (29, 32). This was followed by a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) and subsequently by avidin-biotin-peroxidase (Vector Laboratories). In preliminary experiments (data not shown), the specificity of the immunostaining was verified by incubating adjacent sections with preabsorbed anti-MIP-2 antibody. Sections stained without primary antibody served as negative controls.

DARC Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from kidneys using trizol/chloroform-protocol (Invitrogen, Karlsruhe, Germany). Total RNA was eluted in diethylpyrocarbonate-treated water. Reverse transcription was accomplished using a DARC-specific primer (5'CCAGTAGCCCAGGTTGCATA'3, Invitrogen SuperScript II Protocol). The detection PCR was carried out with gene-specific primers (upstream primer 5'TGTCTGTATCCGGTGGAAACC'3 and downstream primer 5'CCAGTAGCCCAGGTTGCATA'3) gaining a specific product of 389 base pairs. PCR was performed using the Platinum Taq DNA polymerase kit (Invitrogen, Karlsruhe, Germany). RNA extraction and DARC-RT-PCR were normalized against glyceraldehydes-3-phosphate dehydrogenase specific RT-PCR (upstream primer 5'CGGAGTCAACGGATTTGGTCGTAT'3 and downstream primer 5'AGCCTTCTCCAT GGTGGTGAAGAC'3, gaining a specific product of 307 base pairs).

Kidney, Plasma, and RBC-Bound Chemokine Concentrations. Using commercially available sandwich enzyme-linked immunoabsorbent assays (R&D Systems, Wiesbaden-Nordenstadt, Germany), we measured concentrations of neutrophil-specific chemokines in kidney homogenates (MIP-2) and in plasma (KC) as well as the amount of RBC-bound KC.

Briefly, kidneys were homogenized (1:2 w:v) in extraction buffer, containing Triton X-100, e-aminocapronic acid, heparin, and EDTA. Homogenates were incubated for 18 hrs by 4°C. After incubation samples were centrifuged at 10,000 g for 15 mins, supernatant was collected and stored at −80°C until use.

Whole blood was obtained via cardiac puncture to measure plasma and RBC-bound chemokine concentrations. Plasma was removed and stored at −80° until further use. Then 600 μL of 1% Triton X-100 in phosphate-buffered saline was added to 150 μL of erythrocytes. After incubation for 45 mins at 4°C, samples were centrifuged (8000 g, 4°C, 10 mins) and subsequently stored at −20°C until further use. Enzyme-linked immunoabsorbent assays were carried out according to the manufacturer's instructions.

Statistics. Statistical analysis included one-way analysis of variance with subsequent Student-Newman-Keuls' test, one-way enzyme-linked immunoabsorbent assays on ranks with subsequent Dunn's test, two-sided Fisher's exact test, and Student's *t*-test where appropriate. All data are presented as mean ± SEM.

RESULTS

DARC Deficiency Provides Vital Protection From Postischemic ARF. Whereas only 44% of WT mice survived RIR for 48 hrs, DARC$^{-/-}$ mice demonstrated 100% survival following RIR ($p < .05$, Fig. 1*A*).

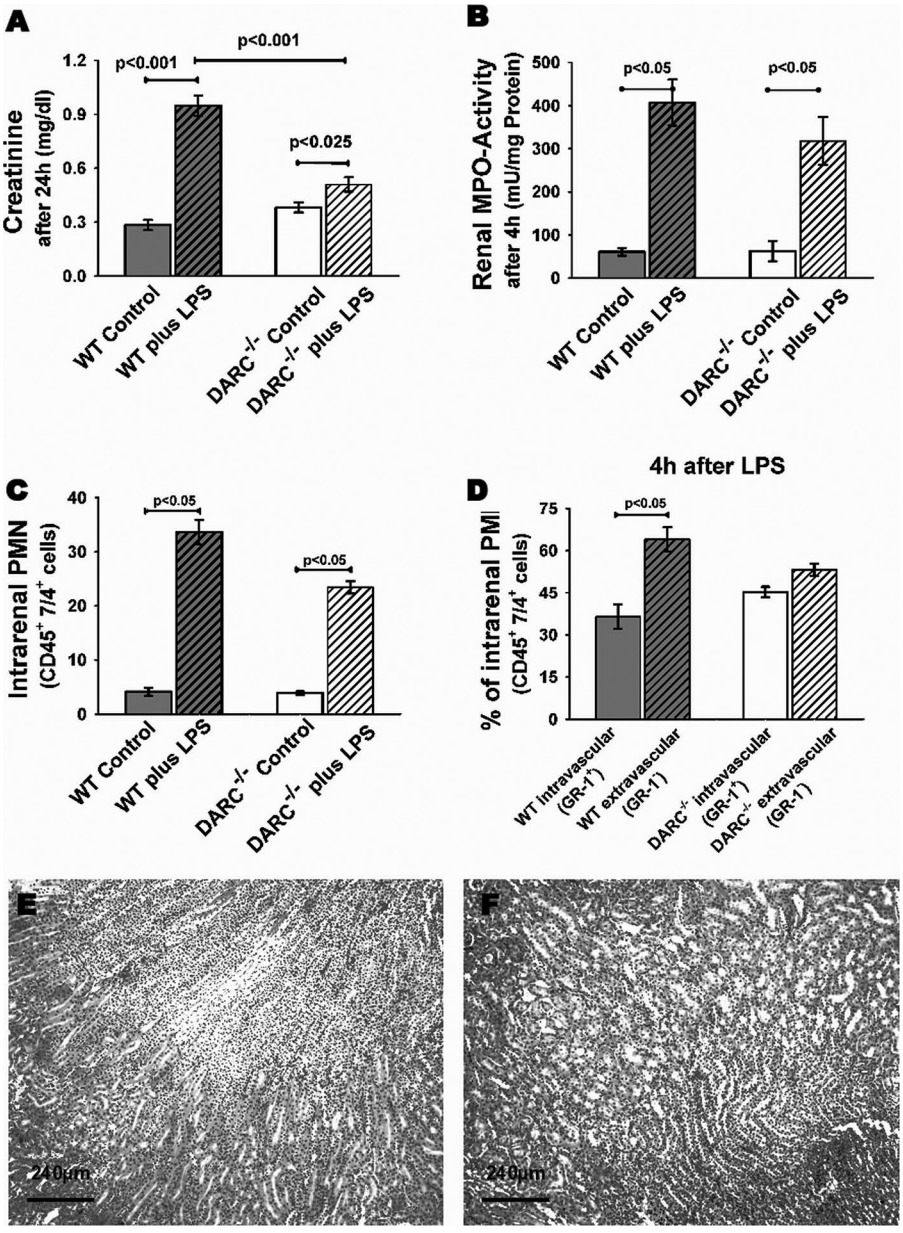

Figure 4. Systemic LPS administration causes acute renal failure in wild-type (*WT*) mice but not in Duffy antigen receptor for chemokines (*DARC*) DARC$^{-/-}$ mice. WT mice showed a significant rise in plasma creatinine concentrations over 24 hrs, whereas DARC$^{-/-}$ mice displayed only a small, clinically irrelevant increase (*A*). As shown by myeloperoxidase (*MPO*) (*B*) and flow cytometry (*C*), WT mice as well as DARC$^{-/-}$ mice exhibited a comparable, statistically significant influx of neutrophils into the kidney 4 hrs after LPS. Note the excellent agreement between MPO and flow cytometry data. WT mice also exhibited a significantly greater accumulation of neutrophils in the extravascular than in the intravascular compartment of the kidney (*D*). DARC$^{-/-}$ mice, by contrast, demonstrated a rather even distribution of neutrophils between the extravascular and intravascular compartment, suggesting an extravasation defect in these mice (n = 5–8). Histologic examination failed to demonstrate signs of overt renal cell death in both WT (*E*) and DARC$^{-/-}$ mice (*F*); only hyperemia and tubular edema were present. *LPS*, lipopolysaccharide; *PMN*, neutrophils.

WT mice exhibited a strong, significant rise in plasma creatinine and BUN concentrations at 48 hrs after RIR (Fig. 1, *B* and *C*). By contrast, DARC$^{-/-}$ mice showed no increase in creatinine or BUN at all. Renal MPO in WT mice was significantly elevated at 24 hrs after RIR, indicating massive influx of neutrophil into the kidneys. DARC$^{-/-}$ mice, on the other hand, displayed no rise in MPO after RIR (Fig. 1*D*). RIR produced great morphologic damage in kidneys from WT mice, including massive tubular edema and loss of tubular epithelial cells. DARC$^{-/-}$ mice only revealed medulla hyperemia but no signs of cellular destruction (Fig. 1, *F* and *G*).

DARC is Expressed in Murine Kidney and Controls Expression as Well as Presentation of MIP-2 After Renal Ischemia-Reperfusion Injury. Using RT-PCR, we could detect DARC RNA in kidneys from WT mice but not in those from DARC$^{-/-}$ mice. Both RIR and LPS administration gave rise to a strong up-regulation of renal DARC RNA expression in WT mice (Fig. 1*E*).

As MIP-2 is a neutrophil-specific chemokine and high-affinity ligand for DARC, we measured renal MIP-2 concentration following RIR or sham surgery. Only WT mice, but not DARC$^{-/-}$ mice, showed a significant increase in renal MIP-2 concentrations at 24 hrs after RIR (Fig. 2*A*). Mice that had received MIP-2 antibody before RIR exhibited significantly lower renal MPO, plasma creatinine, and BUN concentrations than mice injected with a control antibody (Fig. 2, *B–D*). Mice pretreated with an anti-MIP-2 antibody also lacked signs of severe morphologic damage (Fig. 2*E*). Immunostaining revealed similar overall expression of MIP-2 in kidneys from WT mice and DARC$^{-/-}$ mice (Fig. 3, *A* and *B*). Immunostaining showed MIP-2 expression in renal endothelial cells of WT mice, including glomerular endothelial cells (Fig. 3, *C* and *E*). However, immunostaining failed to detect MIP-2 expression in endothelial cells of DARC$^{-/-}$ mice (Fig. 3, *D* and *F*). There were no differences between WT and DARC$^{-/-}$ mice regarding tubular MIP-2 staining (Fig. 3, *A–F*). MIP-2 plasma concentrations were identical in all groups (data not shown).

DARC Modulates Endotoxemia-Induced Renal neutrophil Recruitment and Subsequent ARF. In agreement with our previous findings (29), systemic LPS application led to intrarenal, neutrophil-dependent ARF in WT mice. DARC$^{-/-}$ mice, on the other hand, experienced a significant protection from ARF and exhibited only a small, clinically irrelevant increase in plasma creatinine concentrations after 24 hrs (Fig. 4*A*). MPO data indicated a large influx of neutrophil into the WT mice kidneys, peaking 4 hrs after LPS injection (data not shown). MPO in DARC$^{-/-}$ mice was statistically not different from that in WT mice (Fig. 4*B*). Whole-kidney flow cytometry revealed similar results for total intrarenal neutrophil content (Fig. 4*C*) and thereby validated MPO as a marker for global renal

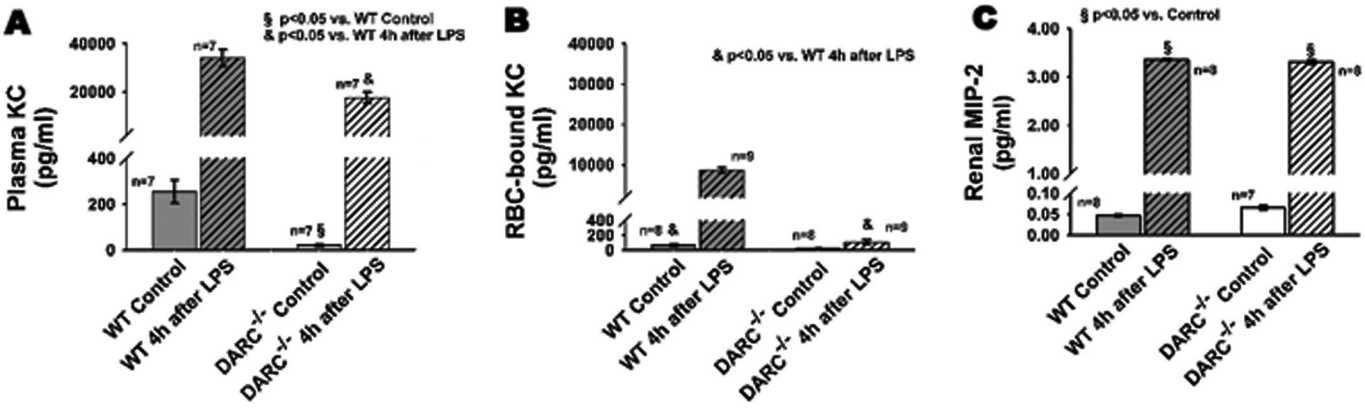

Figure 5. Duffy antigen receptor for chemokines (*DARC*) modulates systemic chemokine homeostasis. Both wild-type (*WT*) mice and DARC$^{-/-}$ mice exhibited strong elevations in plasma concentrations of keratinocyte-derived chemokine (*KC*) following lipopolysaccharide (*LPS*) administration, which were greater in WT mice than in DARC$^{-/-}$ mice (*A*). In WT mice, the amount of red blood cell (*RBC*)-bound KC was significantly larger at 4 hrs after LPS administration, when compared with control. This amount, however, equaled at most 30% of the total plasma KC concentration at all times. As to be expected, in DARC$^{-/-}$ mice, RBC-bound concentrations of KC were extremely low before as well as 4 hrs after LPS injections (*B*). WT mice and DARC$^{-/-}$ mice showed comparable renal macrophage inflammatory protein (*MIP*)-2 concentrations before and 4 hrs after LPS administration (*C*).

neutrophil recruitment. However, flow cytometry also showed that WT mice, but not DARC$^{-/-}$ mice, exhibited a significantly greater accumulation of neutrophils in the extravascular than in the intravascular compartment. This suggests a renal extravasation defect in DARC$^{-/-}$ mice during endotoxemia (Fig. 4*D*; intravascular, CD45^{+}7/4^{+}GR-1^{+}; extravascular, CD45^{+}7/4^{+}GR-1^{-}). Systemic LPS injection led to renal hyperemia and tubular edema but no overt signs of cell death in WT mice (Fig. 4*E*); DARC$^{-/-}$ mice showed similar changes (Fig. 4*F*).

Systemic and Renal Chemokine Homeostasis During Endotoxemia. To evaluate the impact of DARC on local and systemic chemokine homeostasis during endotoxemia, we measured plasma, RBC-bound, and renal chemokine concentrations. WT mice showed greater plasma KC concentrations than DARC$^{-/-}$ mice before and after LPS injection (Fig. 5*A*). LPS administration led to a large increase of RBC-bound KC in WT mice. However, RBC-bound concentrations of KC always equaled only 30% of the plasma KC concentrations. RBC from DARC$^{-/-}$ mice did not bind any relevant amounts of KC (Fig. 5*B*).

Renal MIP-2 concentrations in WT mice and DARC$^{-/-}$ mice were identical before and after LPS injection (Fig. 5*C*). Immunostaining demonstrated similar overall expression of MIP-2 in kidneys from WT mice and DARC$^{-/-}$ mice after LPS injection (Fig. 6, *C* and *D*). Immunostaining could not detect any MIP-2 in renal endothelial cells of DARC$^{-/-}$ mice, including glomerular endothelial cells (Fig. 6, *F* and *H*) during endotoxemia. By contrast, renal endothelial cells from WT mice stained positive for MIP-2 (Fig. 6, *E* and *G*). Renal tubular epithelial cells stained strongly positive for MIP-2 in both WT and DARC$^{-/-}$ mice (Fig. 6, *G* and *H*).

DISCUSSION

In this study, we demonstrate that DARC gene deficiency provides protection from ARF after local (renal ischemia-reperfusion) and systemic (endotoxemia) renal injury. DARC gene deficiency greatly impairs renal neutrophil recruitment during ARF as well as quantitative and spatial expression of neutrophil-specific chemokines. In WT mice, renal DARC messenger RNA expression is strongly up-regulated during ARF. As both models of ARF are largely neutrophil-dependent, DARC-dependent neutrophil recruitment emerges as an attractive hypothesis to explain our findings.

Although serum creatinine and BUN concentrations are not ideal markers of renal function, their use in mice is valid during early phases of renal injury (11) and resembles clinical practice. Due to differences in relative muscle mass, adult mice usually display lower serum creatinine concentrations than adult humans (33). Nonetheless, tripling in serum creatinine concentrations in mice equals a 75% reduction in glomerular filtration rate (11). Thus, the creatinine concentrations and their relative changes demonstrated here are clinically relevant. Both our models of renal injury represent very severe forms of ARF when assessed by validated clinical classifications, such as the RIFLE classification (34). The high mortality rate in WT mice after RIR further supports this conclusion. Hypovolemia and subsequent prerenal azotemia are other frequent concerns in animal models of ARF. However, as BUN/creatinine ratios remain unchanged in our models of ARF (26, 27), it is unlikely that prerenal azotemia is a confounding factor in our study.

Several factors, such as microcirculatory disturbances, reactive oxygen species, recruitment of inflammatory cells, and cytokines, have been implicated in the development of postischemic as well as endotoxemic ARF (35, 36). We have recently shown that both our models of ARF are largely neutrophil-dependent (27, 29). Any impairment in neutrophil recruitment, activation, or function is therefore expected to attenuate renal dysfunction in these settings. Previously published studies have produced conflicting results regarding the role of DARC in neutrophil recruitment (24, 25). Here, MPO studies indicate that DARC gene deficiency almost completely blocks postischemic recruitment of neutrophils into the kidney. MPO data also suggest that global neutrophil recruitment into the kidney is not altered in DARC$^{-/-}$ mice during endotoxemia. However, MPO assays, which measure global neutrophil recruitment, do not differentiate between intravascular and extravascular neutrophils. We have therefore adapted a recently developed flow-cytometry-based method to determine pulmonary neutrophil extravasation (30). This approach validated the results obtained with MPO assays and also revealed impaired renal

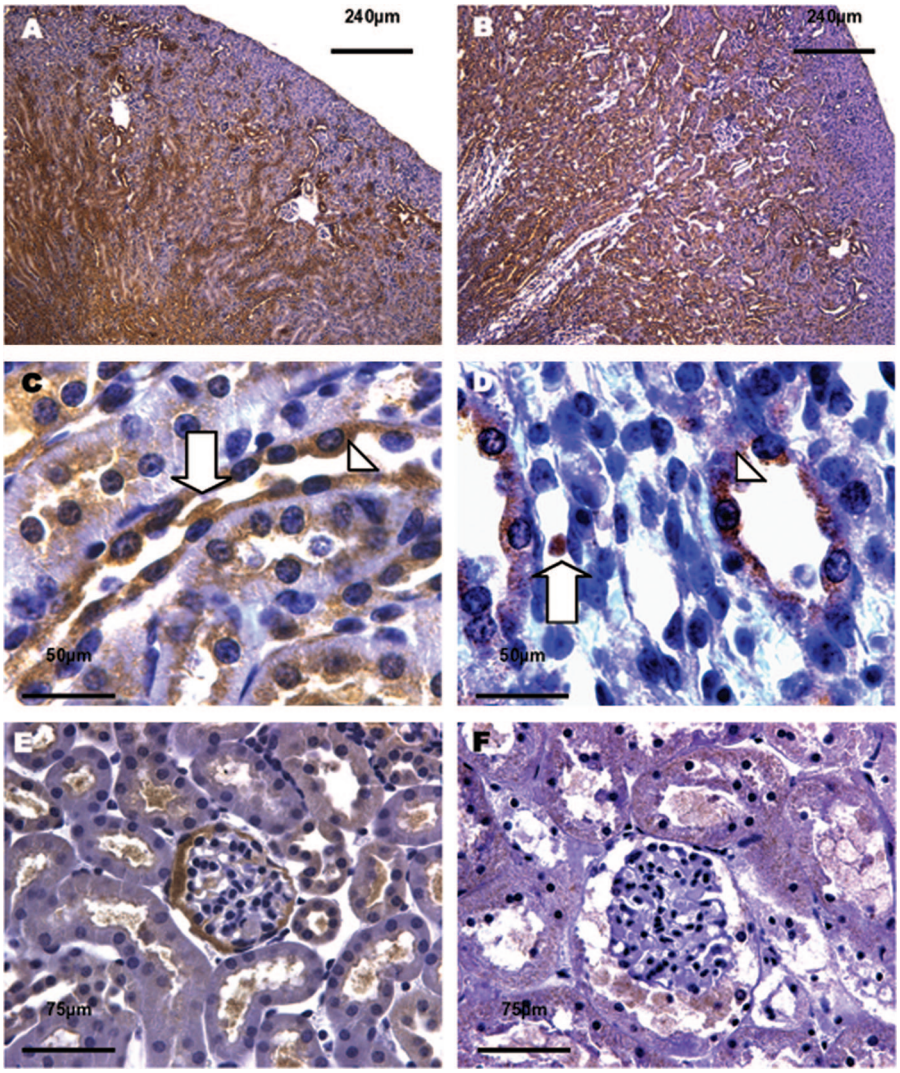

Figure 6. Duffy antigen receptor for chemokines (DARC) also alters renal chemokine presentation during endotoxemic acute renal failure. Whereas immunostaining revealed similar overall expression of macrophage inflammatory protein (MIP)-2 in kidneys from wild-type (WT) mice and DARC$^{-/-}$ mice (*A* and *B*), it showed a striking difference in local MIP-2 presentation between WT mice and DARC$^{-/-}$ mice. There was strong MIP-2 expression in renal endothelial cells (*arrowheads*) of WT mice at 4 hrs after lipopolysaccharide injection (*C*), including glomerular endothelial cells (*E*). By contrast, immunostaining did not detect any MIP-2 presentation in renal endothelial cells (*arrowheads*) of DARC$^{-/-}$ mice (*D* and *F*). A large portion of all MIP-2 staining was localized to tubular epithelial cells (*C–F*). Arrows denote MIP-2^{+} cells close to either WT mice endothelial cells (MIP-2^{+}, *C*) or DARC$^{-/-}$ mice endothelial cells (MIP-2^{-}, *D*).

neutrophil extravasation in DARC$^{-/-}$ mice during endotoxemia.

DARC binds neutrophil-specific chemokines, such as MIP-2 and KC, with high affinity. Two recent studies identified key roles for both MIP-2 and KC in experimental models of ARF. Blockade of either KC or MIP-2 provided equal protection from neutrophil recruitment and subsequent ARF in animal models of postischemic ARF (12) and LPS-induced ARF (37). In our study, preliminary data suggested similar expression kinetics for MIP-2 and KC (data not shown). For an exemplary proof of concept, we therefore analyzed the role of MIP-2 in our model of postischemic ARF. Injection of anti-MIP-2 antibody before RIR led to a significant reduction in postischemic renal MPO and kidney dysfunction. Subsequent measurements of renal MIP-2 concentrations revealed that only WT mice, but not DARC$^{-/-}$ mice, exhibited moderate increases in tissue MIP-2 following RIR. However, it is questionable whether a 30% elevation in tissue MIP-2 in WT mice after RIR can solely account for the drastic increase in postischemic renal neutrophil content. Importantly, WT mice and DARC$^{-/-}$ mice also displayed strikingly different patterns of spatial MIP-2 expression. Whereas immunostaining failed to localize MIP-2 to endothelial cells in DARC$^{-/-}$ mice after RIR, it demonstrated abundant expression of MIP-2 in renal endothelial cells from WT mice. Comparing WT mice and DARC$^{-/-}$ mice during endotoxemia resulted in similar findings. WT mice and DARC$^{-/-}$ mice yielded almost identical renal MIP-2 concentrations before and after LPS administration, despite a significant deficit in neutrophil extravasation in DARC$^{-/-}$ mice. Spatial distribution of MIP-2, however, was very different between WT mice and DARC$^{-/-}$ mice. Renal endothelial cells from WT mice, but not from DARC$^{-/-}$ mice, stained positive for MIP-2 during endotoxemia. We therefore hypothesize that DARC gene deficiency prevents MIP-2 from being displayed to neutrophils rolling on renal endothelial cells, which in turn impedes leukocyte activation and adhesion. Recent *in vivo* and *in vitro* data (23) substantially support this proposal. This concept of DARC-dependent chemokine presentation in endothelial cells can explain why rather drastic differences in renal neutrophil recruitment occur without substantial differences in total renal MIP-2 content. Our data suggest that spatial (here, endothelial) rather than global chemokine expression ultimately determines neutrophil recruitment and subsequent organ damage.

Whereas our findings are in agreement with the proposal of DARC as a facilitator of transendothelial chemokine transport, our results contradict the concept of DARC as a chemokine sink during systemic inflammation (24). Although the binding of the neutrophil-specific chemokine KC to RBC was almost exclusively dependent on DARC expressed on RBC, the total amount of RBC-bound KC was <30% of that circulating in the plasma before or after LPS injections.

Together with other data, our results suggest a critical and functionally relevant role for DARC as a conductor of quantitative and spatial chemokine homeostasis. However, the current lack of a blocking antibody or other blocking molecule hampers the final proof of this concept. Until such agents are available, one has to interpret the potential impact of our findings on ARF under clinical conditions carefully.

CONCLUSIONS

We show that DARC gene deficiency provides excellent protection from ARF induced by local or systemic renal injury. We hypothesize that the protection seen in DARC$^{-/-}$ mice is largely due to a lack of endothelial chemokine presentation and a subsequent defect in neutrophil recruitment. Additionally, our findings argue against previous hypotheses that DARC might act as a chemokine sink during systemic inflammation.

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