Exaggerated response to endotoxin in mice lacking the Duffy antigen/receptor for chemokines (DARC)

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Introduction

The Duffy blood group was first described as an alloantigen in a multiply transfused hemophiliac. The finding that expression of this blood group protein was significantly under-represented in individuals of African ancestry, particularly in regions where the population was resistant to infection by Plasmodium vivax, led to the recognition that the Duffy-negative erythroid phenotype was protective for infection by P. vivax.

A second major advancement of insight into the physiologic role of the Duffy blood group antigen was the discovery that it was identical to a promiscuous receptor for chemokines expressed on the surface of erythrocytes. Chemokines promote the directed migration of specific leukocyte subsets. The chemokine family can be separated into branches based on the configuration of the 2 amino-proximal, of 4 positionally conserved, cysteine residues. In the CXC branch they are separated by a single residue, whereas they are juxtaposed in the CC branch. In general, CXC and CC chemokines promote the formation of acute and chronic inflammatory infiltrates, respectively. The receptors that transduce the signals of chemokines are members of the serpentine receptor family, and separate subsets of receptors mediate signaling by CXC and CC chemokines. The Duffy antigen was shown to be identical to the erythroid chemokine receptor, which has the unique capacity to bind members of both CXC and CC branches. In contrast to other receptors, the binding of ligands to Duffy antigen/receptor for chemokines (DARC) does not induce signal transduction, complicating the interpretation of its function.

The genetic mechanism for the erythroid Duffy-negative phenotype preserved the expression of this receptor in endothelium, inviting speculation that it plays an important role in the pathophysiology of inflammation. In the current studies, the physiological role of DARC was studied in mice rendered deficient of this receptor by gene targeting. These mice showed an exaggerated inflammatory response to intraperitoneal administration of bacterial lipopolysaccharide (LPS), indicating that DARC may be a regulatory sink for chemokines.

Materials and methods

Targeted disruption of the mouse DARC gene

A 9-kilobase (kb) EcoRI fragment containing the DARC locus was cloned from an isogenic mouse genomic library. This fragment was used to generate the targeting construct shown in Figure 1A, which contained 4.3-kb and 1.2-kb arms of homology in the 5′ and 3′ regions, and a neo gene in opposite transcriptional orientation that replaced 90 base pairs (bp) of the DARC open reading frame. Correctly targeted embryonic stem (ES) cells were injected into C57BL/6 blastocysts and subsequently implanted into CD1 host pseudopregnant females to obtain 10 chimeric pups. Three of these chimeras were able to readily transmit the DARC mutation through the germline when mated with C57BL/6 mice, resulting in F1 heterozygotes. F1 heterozygotes were intercrossed to obtain F2 homozygous DARC-deficient mice, which served as the experimental animals (along with the wild-type littermate controls) in these studies. All animal experiments were approved by institutional committees and were in compliance with National Institutes of Health guidelines.

Genotype analysis

The genotypes of all ES cells and mice were determined by Southern blot or polymerase chain reaction (PCR) analysis. Insertion of neo introduces a unique EcoRI site, reducing the 9-kb EcoRI fragment to 4.5 kb in the correctly targeted allele by Southern blotting with the use of a 3′ DARC

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probe. PCR was initially used to screen ES cells and to genotype F2 mice. The wild-type DARC allele was detected by amplification of a 400-bp PCR product, using D1 and D2 primers that flank the DARC coding exon. Primer D1 (5′-GCTAGA TGTCCTGACTGTCC) is a sequence that is deleted in the correctly targeted allele. Primer D2 (5′-CCAGTAGCCCAGGTTGCA TA) is complementary to sequences in exon 2. Primer D neo (5′-TA TGGCGCGC-CA TCGA TCTC-3′) is complementary to a sequence within the inserted neo gene. D2 and D neo were used to amplify a 300-bp fragment from the targeted DARC gene.

RT-PCR analysis

Total RNA for RT-PCR analysis was obtained from spleens of F2 mice by using TRIzol Reagent (Gibco-BRL). Complementary DNA (cDNA) was synthesized from RNA templates with a poly-T primer and M-MLV RT (Gibco-BRL). DARC cDNA was amplified by PCR, using primers D2 and D3 (5′-GCCCTGAGCCTGCAGTGCCA T-3′) in exon 1, resulting in a 400-bp product.

Red blood cell chemokine binding assay

Red blood cells (RBCs), obtained from F2 mice, were incubated with 0.5 nmol/L [125I]-labeled interleukin-8 (IL-8) and MCP-1 (DuPont NEN) and saturating amounts of unlabeled ligands. The incubation was terminated by centrifugation through an oil mixture. Erythrocyte pellets were counted in a gamma counter.

LPS challenge

LPS (Escherichia coli, 0111:B4; Sigma Chemical, St Louis, MO) was administered to wild-type and nullizygous mice by intraperitoneal injection.
(30 mg/kg). Control mice (+/+) received phosphate-buffered saline (PBS). Mice were killed 2 hours after injection, and tissues were sampled for myeloperoxidase (MPO) determination and histopathology.

**Results**

Chimeras generated from correctly targeted ES cells transmitted the mutation through the germline when mated with C57BL/6J mice. ES cell lines and F1 heterozygotes were genotyped by Southern blot analysis (Figure 1B). F2 mice were genotyped by PCR analysis (Figure 1C).

The nullizygous mice exhibited normal growth, development, fertility, and were healthy at 1 year. No significant differences between DARC+/+ and DARC−/− mice were detected in their standard hematological parameters (data not shown).

Normal DARC messenger RNA (mRNA) expression was completely abolished in the −/− mice as measured by RT-PCR analysis from splenic total RNA (Figure 1D), indicating the absence of DARC expression by endothelial cells. The effect of the DARC gene disruption on expression of the erythrocyte chemokine receptor was examined, using [125I]-labeled IL-8 and MCP-1. The RBCs from the DARC−/− mice showed a marked reduction in specific binding relative to the cells from control mice (Figure 1E,F), indicating that they lack expression of DARC on erythroid cells.

Histologic examination of tissues from animals treated with LPS revealed a mild granulocytic infiltrate in lung and liver of +/+ mice (Figure 2A,D). In contrast, nullizygous mice had intense granulocytic infiltrates in lung (Figure 2B) and scattered granulocytes in hepatic sinusoids, with foci of microabscess formation (Figure 2E). Organ neutrophil accumulation was assessed indirectly by measuring tissue MPO content. Lung and liver from −/− mice had significantly higher MPO levels than +/+ mice (Figure 2C,F). Mice receiving PBS injections lacked evidence of a significant infiltrate. There was no difference between +/+ and −/− mice in LPS-induced leukocyte recruitment into the peritoneal cavity (data not shown).

**Discussion**

The exaggerated inflammatory response observed in nullizygous mice suggests that DARC may serve as a chemokine sink and that normal expression of DARC regulates leukocyte trafficking during inflammation.

*Note added in proof.* Following the resubmission of our manuscript, Luo et al.13 reported that DARC knockout mice have lower MPD values than wild-type controls in lung and intestine 24 hours after intraperitoneal injection of 10 mg/kg LPS. This represents a lower dose and a longer time interval than employed in the current study.

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**References**


**Erratum**

In the article by El-Daher et al entitled “Distinct localization and function of $^{1,4,5}$IP$_3$ receptor subtypes and the $^{1,3,4,5}$IP$_4$ receptor GAP1/IP4BP in highly purified human platelet membranes,” which appeared in the June 1, 2000, issue of Blood (Volume 95:3412-3422), there were 2 incorrect numbers in Table 2. The $^{45}$Ca$^{2+}$ uptake for row 4 should be 35 000 ± 1400* and for row 5 should be 52 300 ± 700. [*P < .05 compared with respective controls using Mann-Whitney U Confidence Interval and Test using the Minitab Data Analysis Software (Coventry, UK).]