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A high endothelial venule-expressing promiscuous chemokine receptor DARC can bind inflammatory, but not lymphoid, chemokines and is dispensable for lymphocyte homing under physiological conditions

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Abstract

Chemokines displayed on the luminal surface of blood vessels play pivotal roles in inflammatory and homeostatic leukocyte trafficking *in vivo*. However, the mechanisms underlying the functional regulation of chemokines on the endothelial cell surface remain ill-defined. A promiscuous chemokine receptor, the Duffy antigen receptor for chemokines (DARC), has been implicated in the regulation of chemokine functions. Here we show that DARC is selectively expressed at the mRNA and protein levels in the high endothelial venules (HEV) of unstimulated lymph nodes (LN). To examine the biological significance of DARC expression in HEV, we performed competitive binding experiments with 20 different chemokines. The results showed that DARC selectively bound distinct members of the pro-inflammatory chemokines such as CXCL1, CXCL5, CCL2, CCL5 and CCL7, but not lymphoid chemokines such as CCL21, CCL19, CXCL12 and CXCL13 that are normally expressed in HEV. CCL2 bound to DARC failed to induce a significant cytosolic [Ca^{2+}] elevation in CCR2B-expressing cells, whereas the free form of CCL2 induced a distinct [Ca^{2+}] elevation, suggesting that DARC down-regulates activities of pro-inflammatory chemokines upon binding. Targeted disruption of the gene encoding DARC did not induce any obvious changes in the cell number or leukocyte subsets in the peripheral and mesenteric LN. Neither did DARC deficiency significantly affect lymphocyte migration into LN. These results suggest that DARC may be a scavenger for pro-inflammatory chemokines, but not a presenting molecule for lymphoid chemokines at HEV and that it is probably functionally dispensable for lymphocyte trafficking to HEV-bearing lymphoid tissues under physiological conditions.

Introduction

Lymphocyte recirculation plays a vital role in propagating immunological memory and also ensures the immune surveillance of the body (1). During this process, lymphocytes selectively migrate into lymph nodes (LN) and Peyer's patches

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by interacting with the specialized high endothelial venules (HEV) (2,3). A multistep model has been proposed for the selective interactions between lymphocytes and HEV cells that involves lymphocyte rolling followed by firm adhesion and diapedesis, which require rapid activation of lymphocyte integrins (4). Recent studies established that lymphocyte integrins are activated by chemokines that trigger specific G-protein-coupled receptors expressed on the lymphocyte cell surface (5). CCL21 (SLC), one of the chemokines expressed in HEV cells, can activate LFA-1 on lymphocytes (5–7) and a mutation in the gene encoding CCL21 (8) results in severely impaired migration of T cells across HEV (6,7) [chemokine nomenclature used in this study follows guidelines reviewed in (9)]. A few other chemokines, including CXCL12 (SDF-1) (10), CXCL13 (BLC) (10), CCL19 (ELC) (11), CCL2 (MCP-1) (12) and CXCL9 (MIG) (13), have also been implicated in leukocyte trafficking across HEV by acting differentially on various leukocyte subsets, thus enabling them to adhere to and migrate across HEV under physiological or pathological conditions. Chemokine expression is tightly controlled temporally and spatially, an indication of the biological importance of these molecules (14,15), but the exact mechanism of this regulation is not well understood.

To identify novel molecules expressed specifically in HEV, we previously performed gene expression analyses in mouse HEV cells expressing peripheral node addressin (PNAd) (16) or mucosal addressin cell adhesion molecule (MAdCAM)-1 (17). We found that the PNAd$^+$ HEV cells and MAdCAM-1$^+$ HEV cells formed overlapping, but partly different, gene expression patterns (16,17). One of the genes commonly found in both types of HEV in mice codes for a promiscuous chemokine receptor, the Duffy antigen receptor for chemokines (DARC) (16,17). DARC has also been identified in the lumen of HEV of human tonsils (18).

DARC carries an antigenic determinant of the Duffy blood group system and is a type I glycoprotein. It spans the plasma membrane 7 times like other chemokine receptors and is the receptor on red blood cells (RBC) for the human malarial parasite *Plasmodium vivax* (19,20). Unlike other heptahelical chemokine receptors, DARC is probably not functionally linked with G-proteins, because it lacks in its cytoplasmic portion the highly conserved DRY motif that is required for G-protein coupling and, hence, it may not transmit chemokine signals intracellularly (21). DARC can bind a variety of the CXC and CC chemokines, but not C chemokine (21–26). Two main functions have been postulated for DARC. One is that DARC functions as a sink or scavenger receptor for chemokines (19,27). The other is that DARC is involved in the transcytosis of chemokines across endothelial cells and presents them to leukocytes (28–31). Recently, DARC-deficient mice have been established, but, to our knowledge, the function of DARC in chemokine transport to and presentation at HEV has not been addressed (32,33).

In the present study, having confirmed the expression of DARC in LN HEV, we have examined in detail DARC's repertoire of chemokine binding using both inflammatory and lymphoid chemokines, some of which have been implicated in lymphocyte trafficking across HEV. We have also examined the functional consequence of chemokine binding to DARC in terms of the chemokine's ability to induce intracellular Ca^{2+} influx. In addition, we have examined the lymphoid tissues of DARC-deficient mice to elucidate the physiological significance of DARC in lymphocyte trafficking.

Methods

Animals and antibodies

All animal experiments were performed under an experimental protocol approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine. Specific pathogen-free male C57BL/6 mice were from Japan SLC (Hamamatsu, Japan). DARC-deficient mice were established and used as described (34). The MECA-367 mAb (35) was kindly provided by Dr E. C. Butcher (Stanford University, CA). Biotin-conjugated MECA-367 mAb was prepared in our laboratory. All of the following biotin- or FITC-conjugated mAb were obtained from BD PharMingen (San Diego, CA): anti-CD3ε (145-2C11), anti-B220 (RA3-6B2), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-Gr-1 (RB6-8C5) and anti-CD11b (M1/70) mAb.

Chemokines

CXCL1 (MGSA), CXCL4 (PF4), CXCL5 (ENA-78), CXCL8 (IL-8), CXCL10 (IP-10), XCL1 (lymphotactin), CCL2, CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL7 (MCP-3) and CCL8 (MCP-2) of human origin were purchased from PeproTech (Rocky Hill, NJ). Recombinant human CXCL12 was kindly provided by Dr K. Tashiro (Kyoto University, Kyoto, Japan). Recombinant human CCL17 (TARC), CCL18 (PARC), CCL20 (LARC) and CX3CL1 (fractalkine) fused with the secreted form of the placental alkaline phosphatase were kind gifts of Dr O. Yoshie (Kinki University, Osaka, Japan). CCL21 was purchased from Dako (Kyoto, Japan). CXCL13 and CCL19 were from R & D Systems (Minneapolis, MN). [^{125}I]Human CXCL1 and [^{125}I]human CCL2 were purchased from NEN Life Science Products (Boston, MA).

Cell culture

A mouse endothelial cell line, F-2 (36), was maintained in DMEM (Sigma, St Louis, MO) supplemented with 10% FCS (Gibco/BRL, Grand Island, NY), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% (v/v) 100 × non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol. A murine pre-B cell line, L1.2, that expresses human CCR2B (L1.2/CCR2B) (37), was kindly provided by Dr O. Yoshie. These cells were cultured in RPMI 1640 (Sigma) containing the same supplements as above and 0.8 mg/ml Geneticin (G418; Sigma).

Establishment of stable transfectants expressing murine DARC

The expression plasmid pcDNA3.1 (Invitrogen, Groningen, The Netherlands) containing a full-length cDNA insert encoding the murine DARC (pcDNA3.1/DARC) was a gift of Dr A. S. Pogo (New York Blood Center) (25). Transfection of F-2 cells with the cDNA was performed using Lipofectamine (Gibco/BRL) according to the manufacturer's instructions. The transfected cells were selected with G418 (1.0 mg/ml). A

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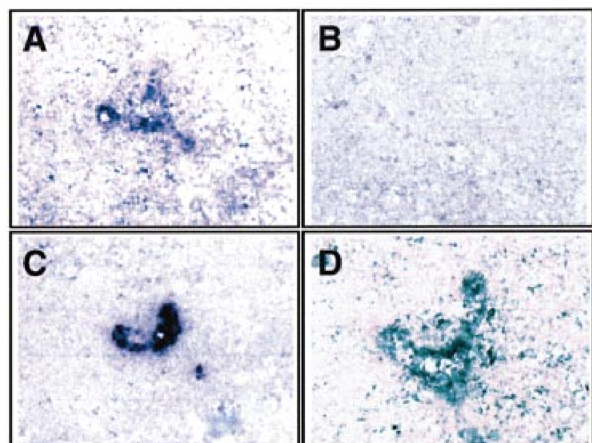

Fig. 1. *In situ* hybridization analysis of DARC in LN. Cryostat sections of LN were hybridized with digoxigenin-labeled DARC antisense riboprobe (A and D), DARC sense riboprobe (B) or GlyCAM-1 antisense riboprobe (C). The sections were then incubated with alkaline phosphatase-conjugated anti-digoxigenin. (A–C) Mesenteric LN, (D) inguinal LN.

murine DARC+ transformant from a single colony was obtained and designated as F-2/DARC.

In situ hybridization

A digoxigenin-labeled single-strand RNA probe for DARC was prepared as follows. The expression plasmid pcDNA3.1/DARC was digested with *Kpn*I and *Hind*III, and the resultant cDNA fragment, containing the coding region of DARC (0.7 kb), was inserted into the *Kpn*I–*Hind*III-digested pBluescript SK vector (Stratagene, La Jolla, CA). Digoxigenin-labeled antisense and sense RNA probes were prepared with T3 polymerase (Stratagene) and T7 polymerase (Stratagene) respectively using the DIG RNA Labeling Mix (Roche, Mannheim, Germany). *In situ* hybridization with mouse LN sections was performed as described (16).

Generation of rabbit polyclonal antibody (pAb) against murine DARC

A pAb was raised against the C-terminal intracellular region of mouse DARC by s.c. immunization of rabbits with a keyhole limpet hemocyanin-conjugated synthetic peptide, LPTRQASQMDALAGK, which corresponds to amino acids 319–333 of the mouse DARC. The pAb was purified from immunized rabbit serum using a synthetic peptide-conjugated Sepharose column.

Immunohistochemistry

Frozen sections (8 μm) of LN were fixed in acetone and then in 4% paraformaldehyde in a 1% calcium chloride solution. After blocking, sections were incubated with biotinylated MECA-367 mAb or biotinylated anti-DARC pAb. The sections were washed in PBS and then incubated with the alkaline phosphatase-conjugated ABC reagent (Vector, Burlingame, CA). After gentle fixation in 1% glutaraldehyde in PBS, the sections were stained using Vector Red (Vector). For the analysis of DARC-deficient mice, fixed cryosections were incubated with biotin-conjugated mAb against cell-surface markers as indicated. After washing, the sections were stained with the horseradish peroxidase-conjugated ABC reagent (Vector) and Metal Enhanced DAB (Pierce, Rockford, IL), followed by counter-staining with hematoxylin.

Chemokine-binding analysis

For the competitive chemokine-binding analysis, the F-2 and F-2/DARC cells (4×10^5 cells/100 μl) were incubated at 4°C for 1 h with 0.2 nM ^{125}I-labeled CXCL1 in the presence or absence of excess amounts of unlabeled chemokines as competitors (20 nM). The cell suspension was then layered over 200 μl of a mixture of 80% dibutyl phthalate/20% olive oil (Sigma). The cell-associated radiolabeled CXCL1 was separated from unbound radioactivity by centrifugation and quantified in a γ-counter. All reactions were performed in triplicate and all experiments were performed at least twice.

Calcium mobilization assay

Calcium mobilization was measured by loading L1.2/CCR2B cells (1×10^6/ml) with 2 μM Indo-1 acetoxymethylester (Sigma) at 37°C for 30 min in RPMI medium containing 1% FCS. The cells were analyzed by an Epics Elite flow cytometer (Beckman Coulter, Miami, FL) using UV excitation with fluorescence emission collected as a ratio of 406:525 nm, as described previously (38). The mean ratio plotted against time was acquired using Multitime software for analysis of kinetic flow cytometry data (Phoenix Flow Systems, San Diego, CA). The RBC-associated CCL2 was prepared as follows: mouse RBC (2×10^9 cells) in 0.5 ml of PBS containing 1% FCS (PBS/FCS) were incubated with 10 nM CCL2 at 37°C for 20 min. Unbound CCL2 was removed by two 250-μl washes with PBS/FCS. The RBC were then mixed with Indo-1-loaded L1.2/CCR2B cells (at an indicator cell:RBC ratio of 1:200) in a total volume of 0.5 ml and the mixture was immediately injected into the flow cytometer. The RBC were electronically gated using a combination of the light scatter and Indo-1 fluorescence. To determine the amount of CCL2 bound to the RBC, the binding of ^{125}I-labeled CCL2 to mouse RBC was measured as follows. Freshly isolated mouse RBC (2×10^9 cells) (27) in 0.5 ml of PBS/FCS were incubated with 10 nM ^{125}I-labeled CCL2 at 37°C for 20 min. Unbound CCL2 was removed by two 250-μl washes with PBS/FCS and the radioactivity in the cell pellet was quantified in a γ-counter. To monitor release of bound CCL2 from RBC, the RBC were resuspended in 0.5 ml of PBS/FCS and incubated at room temperature for up to 60 min. Measurement of RBC-associated radioactivity showed no significant release of bound CCL2 from RBC (data not shown).

Cell counts and flow cytometric analysis

Single-cell suspensions from the spleen and LN from wild-type and DARC-deficient mice were prepared by gentle mincing. Cell numbers were counted manually using a hemocytometer. For flow cytometry, cells (1×10^6) were incubated with FITC-conjugated mAb (5 μg/ml) against cell-surface markers as indicated at 4°C for 30 min in 50 μl of PBS containing 1% BSA and 0.05% NaN_3. The cells were washed twice and analyzed on an Epics XL flow cytometer using System II software (Beckman Coulter).

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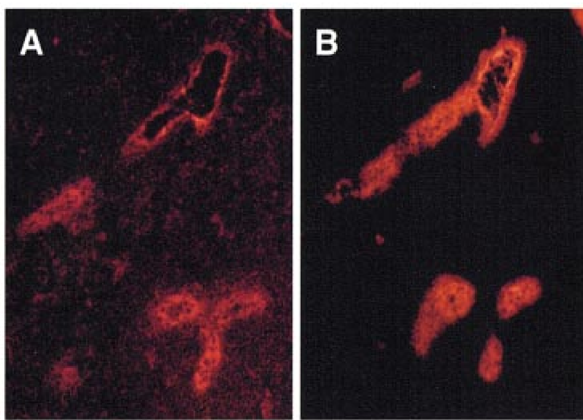

Fig. 2. Immunohistochemical analysis of DARC expression in LN. Sections were incubated with biotin-conjugated anti-DARC pAb (A) or biotin-conjugated MECA-367 mAb (B), followed by alkaline phosphatase-conjugated ABC reagent. The sections were stained with Vector Red as the substrate. Original magnification ×100.

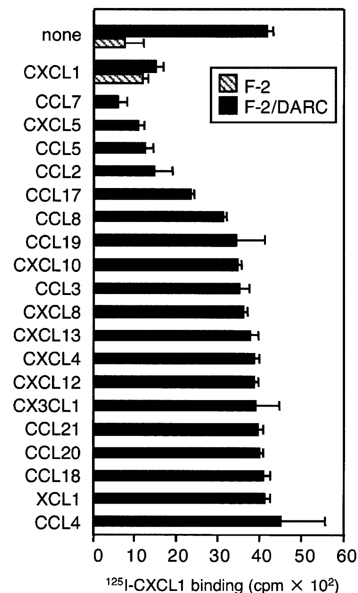

Fig. 3. Competitive inhibition of chemokine binding to DARC. F-2 and F-2/DARC cells (4×10^5 cells) were incubated at 4°C for 1 h with ^{125}I-labeled CXCL1 (0.2 nM) as a ligand probe for DARC in the absence or presence of 20 nM unlabeled chemokine, as indicated. After the incubation, cell-bound ^{125}I-labeled CXCL1 was separated and measured on a γ-counter. The assay was performed in triplicate. The data are shown as the mean ± SD.

In vivo migration assay

Total spleen cells were prepared from green fluorescent protein (GFP)-transgenic mice of the C57BL/6 background (39). The GFP$^+$ spleen cells (1×10^7 cells) were injected i.v. into age- and sex-matched wild-type mice or DARC-deficient mice. The mice were sacrificed 6 h after injection, and the spleen, mesenteric LN, peripheral LN and Peyer's patches were harvested. The numbers of T cells, B cells and Mac-1$^+$ cells that had migrated into these tissues were determined by flow cytometry using an Epics-XL and FACScan (BD Biosciences, San Jose, CA).

Results

DARC is expressed in the HEV of unchallenged, quiescent LN

It has been reported that DARC is expressed in the post-capillary venules of various tissues (40,41), including HEV of the palatine tonsils in humans (18). However, because the post-capillary venules are the sites where inflammatory cells often extravasate under pathological conditions and because tonsils are exposed to various types of immunological assaults due to their strategic location, it remained unclear whether the DARC expressed at these locations is induced by exogenous stimulation or is constitutively expressed.

An mRNA hybridization analysis *in situ* showed that an antisense probe to DARC hybridized preferentially to HEV cells of mesenteric LN from unchallenged mice (Fig. 1A), as did the antisense probe to an HEV marker, GlyCAM-1 (Fig. 1C). In contrast, no significant signal was observed with the DARC sense probe (Fig. 1B). Similar results were observed in inguinal LN of unchallenged mice (Fig. 1D). Immunofluorescence staining with a pAb to DARC confirmed the expression of the DARC protein in MAdCAM-1$^+$ HEV cells, which appeared to be preferentially distributed to the basolateral and apical surface of HEV cells (Fig. 2). A much weaker immunoreactive signal was also observed in a small proportion of flat non-HEV-type endothelial cells. The anti-DARC pAb failed to react with any cell components in LN sections of DARC-deficient mice, verifying the specificity of this antibody (data not shown). These results demonstrate that DARC is preferentially and constitutively expressed at the mRNA and protein levels in HEV cells of unchallenged, quiescent LN of specific pathogen-free mice, and not induced by antigenic stimulus.

DARC binds pro-inflammatory, but not lymphoid, chemokines.

Although previous studies showed that DARC binds a variety of inflammatory chemokines belonging to the CXC and CC classes (21–26), whether DARC can bind members of the lymphoid chemokines has not been determined to our knowledge. To examine the binding specificity of DARC to various chemokines by a competitive binding inhibition analysis, we established an F-2 endothelial cell line that stably expressed DARC (F-2/DARC). To assess the binding specificity of DARC, 20 different chemokines were used as cold competitors (at a final concentration of 20 nM) against 0.2 nM of ^{125}I-labeled CXCL1. As shown in Fig. 3, the binding of ^{125}I-labeled CXCL1 to F-2/DARC was strongly inhibited by the addition of excess amounts of inflammatory chemokines, including CXCL1, CCL7, CXCL5, CCL5 and CCL2, and moderately inhibited by CCL17 and CCL8. No significant displacement of ^{125}I-labeled CXCL1 binding was observed with chemokines, including CXCL10, CCL3, CCL4 and CXCL8. These results are consistent with the previous observations showing that DARC binds multiple, but restricted, members of the inflammatory chemokines (21–26). In contrast, the binding of ^{125}I-

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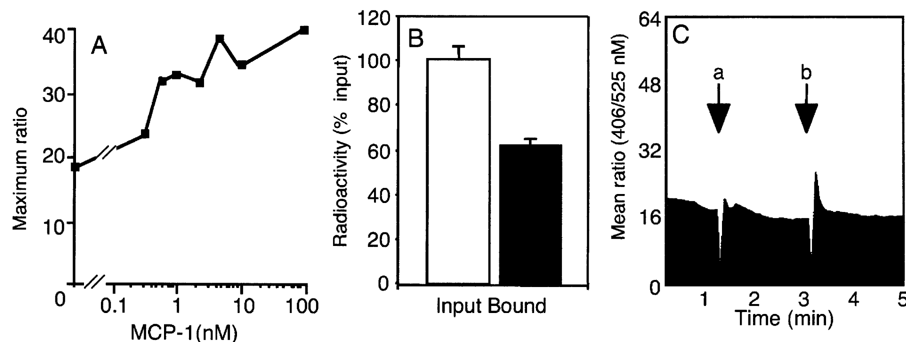

Fig. 4. Accessibility of DARC-bound CCL2 to CCR2B-expressing cells. (A) The dose–response curve of the Ca^{2+} influx induced by free CCL2 in L1.2/CCR2B cells. Indo-1-labeled L1.2/CCR2B cells were stimulated with increasing concentrations of CCL2, and Ca^{2+} mobilization was measured by flow cytometry. (B) CCL2 binding to RBC. Mouse RBC (2×10^9 cells) were incubated with ^{125}I-labeled CCL2 (10 nM) for 20 min at 37°C. After extensive washing, [^{125}I]CCL2 binding was measured in a γ-counter. The binding assay was performed in triplicate. (C) Ca^{2+} signals in L1.2/CCR2B cells stimulated with RBC-associated or free CCL2. Mouse RBC (2×10^9 cells) were pre-loaded with 10 nM CCL2 as above. Indo-1-labeled L1.2/CCR2B cells were stimulated with the RBC pre-loaded with CCL2 (arrow a) and then free CCL2 (100 nM) (arrow b). Ca^{2+} influx was measured by flow cytometry.

labeled CXCL1 was uniformly unaffected by the addition of any of the lymphoid chemokines examined, including CCL21, CCL19, CXCL12 and CXCL13, indicating that DARC does not bind these chemokines. Furthermore, the CXCL1 binding was not affected by CX3CL1, CCL20, CCL18 or XCL1. Lack of CCL21 binding to F-2/DARC was also confirmed by flow cytometric analysis using pAb specific to CCL21 (data not shown). These results clearly indicate that, although DARC binds the ELR-CXC and CC classes of chemokines in a seemingly promiscuous manner, it actually binds only a certain member of the pro-inflammatory chemokines and fails to bind the lymphoid chemokines.

CCL2 bound to DARC fails to induce Ca^{2+} signals in CCR2B-expressing cells

Because of its capacity to bind multiple chemokines, DARC has been implicated in the modulation of chemokines' biological activities. To address this issue, we examined the ability of CCL2 bound to DARC to induce a Ca^{2+} influx in the L1.2/CCR2B cells. Free CCL2 was used as a control and found to induce a prominent Ca^{2+} response in the L1.2/CCR2B cells in a dose-dependent manner with a threshold concentration of ~0.6 nM (Fig. 4A). Next, CCL2 (10 nM) was added to RBC that express DARC abundantly (19,20) to prepare cells with CCL2 bound to the cell-surface DARC (CCL2-loaded RBC). Determination of the amount of CCL2 bound to RBC using ^{125}I-labeled CCL2 indicated that ~60% of the CCL2 added bound to the RBC (Fig. 4B). No significant release of bound CCL2 from RBC was observed under the conditions used in this study (data not shown). In a separate series of experiments using RBC from DARC-deficient mice, we confirmed that essentially all the CCL2 binding to RBC was bound to DARC (data not shown). The CCL2-loaded RBC were then mixed with the L1.2/CCR2B cells at an indicator cell:RBC ratio of 1:200, which would provide a CCL2 concentration of ~6 nM (~10-fold higher than the threshold concentration) in the test sample. The measurement of Ca^{2+} influx by flow cytometry showed that the CCL2 bound to DARC did not induce any significant Ca^{2+} influx in the L1.2/CCR2B cells (a in Fig. 4C). Enhancing the physical contact between the DARC-bound RBC and the L1.2/CCR2B cells with strong centrifugal force prior to the measurement of Ca^{2+} influx did not result in the induction of a significant Ca^{2+} influx in the receptor-bearing cells (data not shown). In contrast, free CCL2 (6 nM) added to the same cells elicited a rapid Ca^{2+} influx (b in Fig. 4C), showing that DARC did not present CCL2 effectively to the L1.2/CCR2B cells, at least under the experimental conditions we used. Admittedly, these experiments were performed under rather artificial conditions and also did not directly address the function of the DARC expressed on HEV cells. The results are, however, compatible with the hypothesis that the membrane-bound DARC does not present chemokines effectively, but rather scavenges them (19,27).

DARC-deficient mice show no apparent abnormalities in their LN

To further investigate the physiological significance of DARC in lymphocyte trafficking, we established DARC-deficient mice by targeted gene disruption. The secondary lymphoid tissues of DARC-deficient mice were then examined according to cell number, composition of leukocyte subsets and tissue architecture. In the mesenteric and peripheral LN of DARC-deficient mice, there were no significant differences in the overall cell number and composition of leukocyte subsets including T cells, B cells, macrophages and granulocytes, as compared with the age- and sex-matched wild-type mice (Fig. 5A, upper and lower panels). The compartmentalization of T and B cells was also apparently normal in the DARC-deficient mice (Fig. 5B). In addition, Gr-1$^+$ cells, CD11b$^+$ cells and F4/80$^+$ cells were also distributed normally in the DARC-deficient mice (data not shown). The DARC deficiency also did not significantly affect migration of T cells, B cells and Mac-1$^+$ cells into the spleen, mesenteric and peripheral LN, and Peyer's patches as evaluated in the short-term *in vivo* migration assay (data not shown). The only noticeable change was observed in the spleen of the DARC-deficient mice. In 6- to 9-week-old DARC-deficient mice, there was ~1.7-fold increase in the total cell number (Fig. 6A), but there was no significant

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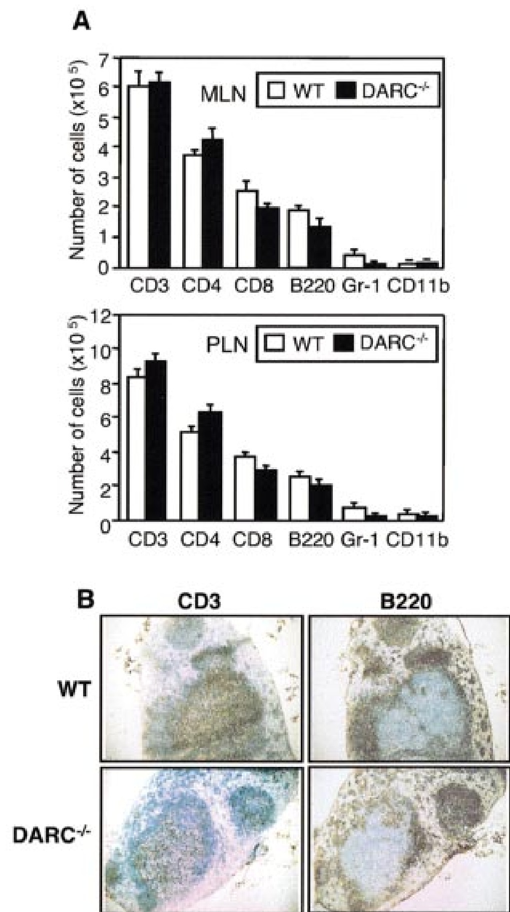

Fig. 5. Leukocyte subsets in LN of DARC-deficient mice. (A) A comparison of absolute cell numbers among leukocyte subsets in mesenteric and peripheral LN from DARC-deficient (DARC$^{-/-}$) and wild-type (WT) mice. Total cell numbers were counted by hemocytometer. The percentage of each subset was determined by flow cytometry. (B) Distribution of T and B cells in mesenteric LN from DARC-deficient (DARC$^{-/-}$) and wild-type (WT) mice. The sections of mesenteric LN were fixed and incubated with biotin-conjugated anti-CD3 or -B220 mAb, and stained with horseradish peroxidase-conjugated ABC reagent and Metal Enhanced DAB, followed by counter staining with hematoxylin. Original magnification ×100.

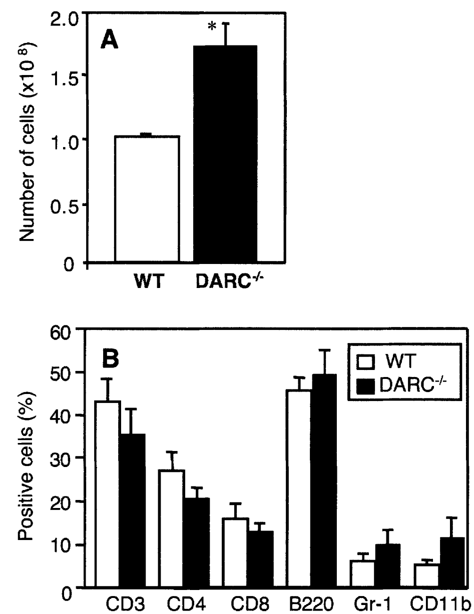

Fig. 6. Cell number and leukocyte subsets in the spleen of DARC-deficient mice. (A) The numbers of spleen cells in DARC-deficient (DARC$^{-/-}$) and wild-type (WT) mice. Single-cell suspension from spleen was prepared and counted using a hemocytometer. Data are shown as the mean ± SD from 6-week-old mice (N = 3). *P < 0.05. (B) Leukocyte subsets in the spleen of DARC-deficient (DARC$^{-/-}$) and wild-type (WT) mice. Spleen cells were stained with the indicated mAb and analyzed by flow cytometry. Data are shown as the mean ± SD from 6- to 9-week-old mice (N = 6).

change in the leukocyte subset composition (Fig. 6B). In older mice, there was no overt splenomegaly. Collectively, the DARC-deficient mice showed no apparent abnormalities in their LN, with only a moderate and transient splenomegaly of unknown etiology in young mice, indicating that DARC is dispensable, at least for lymphocyte entry and egress from LN under physiological conditions.

Discussion

Here we confirm and extend our previous observation by showing that DARC is constitutively expressed in the HEV of unchallenged LN (16,17) and that it binds selected inflammatory, but not lymphoid, chemokines. In agreement with the binding studies using mouse RBC (24–26), the competitive inhibition of chemokine binding to mouse DARC expressed on endothelial cells indicated that DARC binds only restricted members of the inflammatory chemokines including CXCL1, CCL7, CXCL5, CCL5 and CCL2. Interestingly, DARC bound none of the lymphoid chemokines tested, including CCL21, CCL19, CXCL13, CXCL12, CX3CL1, CCL20 and CCL18. Lack of CCL21 binding to DARC expressed on endothelial cells was also confirmed by flow cytometric analysis by the use of specific pAb to CCL21 (data not shown). DARC's uniform inability to bind lymphoid chemokines, which was also confirmed using mouse RBC (data not shown), is interesting, since one of the postulated functions of DARC is the docking of chemokines to concentrate them on the surface of endothelial cells for presentation to specific receptors on immune cells (28–31). However, most of the chemokines detectable in HEV, such as CCL21 (6,7), CCL19 (11), CXCL12 (10) and CXCL13 (10), failed to bind DARC, clearly indicating that these chemokines are localized to the HEV independent of DARC. Although CXCL9 has recently been shown to be expressed in HEV in inflamed LN (13), we have not examined its ability to bind DARC.

The CCL2 bound to RBC failed to induce Ca^{2+} influx in the CCR2B-expressing cells (Fig. 4). The failure to respond to the RBC-associated CCL2 was not due to damage to the CCR2B-expressing cells or a loss of sensitivity of these cells to CCL2, since the same cells showed a distinct increase in intracellular free calcium when free CCL2 was added to the mixture. Virtually all CCL2 binding to RBC was mediated by DARC, because no significant binding of CCL2 was observed in

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DARC-deficient RBC (data not shown). Therefore, these results indicate that CCL2 became inaccessible to the receptor-bearing cells upon binding to DARC, which is in accordance with the report by Darbonne *et al*. (27) in which CXCL8 absorbed to RBC was also shown to be incapable of stimulating neutrophils. While Darbonne *et al*. (27) used an indicator cell (neutrophil):RBC ratio of 1:100, we used a higher indicator cell (CCR2B transfectant):RBC ratio of 1:200, so that a higher chemokine concentration would be obtained in the assay. With this ratio, ~6 nM of CCL2 was present as a DARC-associated form (Fig. 4B); 1 nM CCL2 was sufficient to induce an increase in Ca^{2+} influx in the L1.2/CCR2B cells in this assay (Fig. 4A). However, the CCL2 associated with DARC failed to induce any Ca^{2+} influx in the L1.2/CCR2B cells (Fig. 4C). When CXCL1 was used, the RBC-associated CXCL1 also failed to induce a significant Ca^{2+} influx in its cognate receptor-bearing cells (data not shown).

At present, several possibilities could account for the inability of DARC-bound chemokines to stimulate the receptor-bearing cells. First, DARC may physically mask the chemokines' binding site for their cognate receptors. Supporting this hypothesis, DARC has been shown to occupy CXCL1's binding sites for its cognate receptor (CXCR2) at least partially (42). Second, a conformational change may be induced in the chemokines upon binding to DARC that prevents them from binding their cognate receptors. This hypothesis is, however, currently difficult to verify in the absence of structural data from chemokines complexed with DARC. Finally, while the DARC-bound chemokines may still have the capacity to bind to the receptors, they could be physically inaccessible to the receptor-bearing cells under the experimental conditions used. Although we cannot as yet formally exclude this possibility, we do not think it very likely, since basically the same result was obtained when the chemokine-associated RBC were forced to make contact with the receptor-bearing cells by strong centrifugal force (data not shown). In addition, it may be argued that these results were obtained with cultured endothelial cells and not with HEV. However, the observed ability of DARC to bind restricted members of the inflammatory chemokines is in accordance with previous observations by others (21–26). We therefore speculate that DARC expressed in HEV also binds selected members of inflammatory chemokines and probably limits their access to immune cells, helping to ensure the highly selective transendothelial migration of lymphocytes through HEV.

To address the role of DARC in physiological lymphocyte trafficking across HEV, we established DARC-null mice by targeted disruption of the DARC gene and examined their lymphoid tissues. Consistent with previous reports (32,33), we observed no significant alterations in the composition of leukocyte subsets and the histological architecture of LN in the DARC-deficient mice (Fig. 5). In addition, we found that lymphocyte migration into LN was not compromised in DARC-deficient mice significantly (data not shown). These findings suggest that lymphocyte trafficking through HEV is unaffected by the deletion of DARC in HEV and that DARC is functionally dispensable in this cellular event. In this regard, it is of note that HEV in unchallenged LN express a pro-inflammatory chemokine, CXCL1 (KC), at the mRNA and protein levels (Y. Ebisuno and T. Tanaka, unpublished observation). Given that HEV do not allow trafficking of neutrophils and monocytes into the LN under physiological conditions, the biological activity of CXCL1 might be masked by chemokine-binding molecules such as DARC *in situ*, while molecules other than DARC such as proteoglycans (43,44) and/or various sialomucins (45) could also be involved in the sequestration of inflammatory chemokines at the HEV cell surface.

The function of DARC in the spleen is unclear. In 6- to 9-week-old DARC-deficient mice, the spleen showed a significant increase in wet weight and cell number (Fig. 6A), although there was no remarkable alteration in the composition of leukocyte subsets or the histological architecture (Fig. 6B). In older mice, no splenomegaly was observed. DARC is expressed in the endothelial cells lining the sinusoids in the splenic red pulp (19). Although the study of DARC-null mice by others indicates that DARC regulates leukocyte trafficking during inflammation (32,33), we have found no evidence that suggests the involvement of DARC in cellular trafficking in the unstimulated spleen in the present study.

Previous studies by the use of DARC-deficient mice yielded conflicting results regarding the DARC's role in inflammatory responses *in vivo*; Dawson *et al*. (33) reported significantly increased leukocyte infiltration into the lung and liver after lipopolysaccharide challenge in DARC-deficient mice, which is in favor of the hypothesis that the DARC functions as a chemokine scavenger. In contrast, Luo *et al*. (32) reported apparently contradictory results showing that leukocyte recruitment into the lung was decreased in mice lacking DARC. Although our *in vitro* and *in vivo* results appear concordant with the notion of DARC being a chemokine scavenger, DARC's role under pathological conditions remains to be fully investigated. Clearly further studies are needed to define the role of DARC in the regulation of chemokine activities and leukocyte trafficking *in vivo* more precisely.

Collectively, the results from our study demonstrate that DARC binds only a subset of the inflammatory chemokines and fails to bind the lymphoid chemokines that have been implicated in the regulation of lymphocyte trafficking into secondary lymphoid tissues. Upon binding certain inflammatory chemokines, DARC may limit their access to the cells in the vicinity, thus negatively regulating the chemokines' activities on the endothelial cells. However, under physiological conditions, the DARC expressed in HEV is apparently redundant in terms of lymphocyte trafficking into the LN.

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Abbreviations

DARC	Duffy antigen receptor for chemokines
HEV	high endothelial venule
GFP	green fluorescent protein
LN	lymph node
MAdCAM	mucosal addressin cell adhesion molecule
pAb	polyclonal antibody
PNAd	peripheral node addressin
RBC	red blood cell

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