CD44 Binds to Macrophage Mannose Receptor on Lymphatic Endothelium and Supports Lymphocyte Migration via Afferent Lymphatics

Marko Salmi, Marika Karikoski, Kati Elima, Pia Rantakari, Sirpa Jalkanen

**Rationale:** Macrophage mannose receptor (MRC) is one of the few molecules known to be involved in lymphocyte trafficking via the lymphatic vessels. In endothelial cells of efferent lymphatics, it binds L-selectin on lymphocytes. In afferent lymphatics, MRC mediates trafficking of both normal and malignant L-selectin–negative cells to the draining lymph nodes.

**Objective:** This work was designed to search for additional lymphocyte ligands of MRC to elucidate how lymphocytes migrate into the draining lymph nodes.

**Methods and Results:** Using immunoprecipitation and binding studies with natural and recombinant proteins, we show that MRC and CD44 can interact with each other. Fine mapping revealed that the cysteine-rich domain of MRC binds to the chondroitin sulfate side chains of CD44. In vivo homing experiments with MRC- and CD44-deficient mice verified that MRC and CD44 function as a receptor-ligand pair in supporting lymphocyte migration via the afferent lymphatics into the draining lymph nodes.

**Conclusions:** These data identify a new counter-receptor for MRC and reveal CD44 as a new molecule involved in the poorly understood process of lymphocyte transit via the lymphatic vasculature. (Circ Res. 2013;112:1577-1582.)

Key Words: endothelium ■ leukocyte ■ lymphatics ■ lymphocytes ■ migration ■ traffic

Leukocyte migration from the periphery into the draining lymph nodes via the afferent lymphatics largely determines the magnitude of immune response within the node. Exit of lymphocytes from the lymph nodes via the lymphoid sinuses into efferent lymphatics also controls the recirculation potential of naive and activated lymphocytes. Furthermore, the lymphatic vasculature also presents an important route for the potential of naive and activated lymphocytes. Furthermore, the lymphatic vasculature also presents an important route for cancer cells to metastasize.

Macrophage mannose receptor (MRC) is a scavenger receptor on macrophages and mediates the uptake of mannosylated glycoconjugates. It is also expressed on lymphatics and is involved in the migration of lymphocytes and melanoma cells into the draining lymph nodes. As a large protein consisting of a cysteine-rich (CR)/ricin R-type lectin domain, a fibronectin type II domain, and 8 C-type lectin domains, it has the potential to interact with diverse ligands. In vitro binding studies indicate that L-selectin functions as a leukocyte ligand for MRC at least in lymphoid sinuses. Because L-selectin–negative cells also migrate in an MRC-dependent manner via the afferent lymphatics, we wanted to identify additional leukocyte counter receptors for MRC.

On the basis of the protein structure and function, we hypothesized that CD44 might interact with MRC. CD44 is a proteoglycan having ≥40 isoforms as a result of the alternative splicing and abundant posttranslational modifications. It is involved in a multitude of functions, including apoptosis, adhesion to extracellular matrix, and lymphocyte homing from the blood into the lymphoid organs. The standard hematopoietic form of CD44 has glycosaminoglycan side chains, which are predominantly chondroitin-4 and -6-sulfates. In addition, the core protein incorporates sulfate directly to its other oligosaccharide components or amino acids. The best-characterized ligand for leukocyte CD44 is hyaluronan, present on the endothelial cell surface of blood vessels, and this interaction is important in the rolling phase of leukocyte extravasation. In addition, CD44 on mature leukocytes binds to endothelial selectins, and it can induce signals activating other adhesion molecules (eg, lymphocyte function-associated antigen-1).© 2013 American Heart Association, Inc.
CR Domain of MRC Binds to Chondroitin Sulfate Side Chains of CD44

To characterize the binding in more detail, we produced recombinant CD44 as an Fc chimera. It showed a smear appearance in the gel, typical for CD44 because of its abundant glycosylation. In addition, a small subpopulation of CD44 molecules were decorated by chondroitin sulfate side chains as demonstrated with 3 sharp bands showing up after chondroitinase ABC treatment (Figure 1B). We then tested the binding of the chimera to transfectants expressing different domains of MRC. The structure of the different domains is depicted in Figure 1C. Flow cytometric analyses showed that CD44 binds to the CR domain of MRC but not to the fibronectin type II domain or to the lectin domain (Figure 1D). Digestion of the chondroitin sulfate side chains of CD44-Fc with chondroitinase ABC abolished the binding to MRC (Figure 1E). Thus, MRC is a novel ligand for CD44, and the interaction takes place via binding of the glycosaminoglycan side chains of CD44 to the distal CR domain of MRC.

Expression Profiles of CD44 and MRC Are Compatible With the Ligand-Receptor Pair Concept

MRC is expressed both on LYVE-1⁺ lymphatic endothelial cells and on a subset of F4/80⁺ macrophages in the skin of WT mice (Figure 2A). In WT mice, CD44 is expressed mainly on hematopoietic cells in normal lymph nodes, whereas lymphatic endothelial cells are negative. MRC, however, is present on both afferent and efferent lymphatics and on many macrophages in the subcapsular sinus of the lymph node (Figure 2B). The MRC⁻/⁻ and CD44⁻/⁻ mice have apparently normal blood and lymphatic vasculature as detected by anti–plasmalemma vesicle-associated protein-1 and anti–LYVE-1, respectively (Figure 2C and Online Table I). Thus, the expression patterns of MRC on lymphatic endothelium and CD44 on lymphocytes are compatible with the possibility that the 2 molecules interact during lymphocyte migration through lymphatics. Furthermore, the expression of the previously reported CD44 counter receptors osteopontin, fibronectin, and E-selectin was not altered in MRC⁻/⁻ mice compared with WT mice (Online Figure I), indicating that the absence of 1 ligand of CD44 (MRC) does not lead to compensatory changes in the expression of other ligands.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CD44⁻/⁻</td>
<td>CD44 knockout</td>
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<tr>
<td>CR</td>
<td>cysteine rich</td>
</tr>
<tr>
<td>MRC</td>
<td>macrophage mannose receptor</td>
</tr>
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<td>MRC⁻/⁻</td>
<td>MRC knockout</td>
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<td>WT</td>
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D. Jackson (University of Oxford) and consisted of a full-length extracellular domain of the standard form of human CD44 fused to human Fc. The CD44 constructs generated are presented in Figure 1.

Coprecipitation and Binding Studies

Binding of CD44 and MRC to affinity-purified human MRC and CD44 was tested by coprecipitation and detected with immunoblotting. The human material was used with the permission of the Ethical Committee of Turku University Hospital.

CD44 Chimera and MRC Constructs

The chimeric CD44 construct was a kind gift from D. Jackson (University of Oxford) and consisted of a full-length extracellular domain of the standard form of human CD44 fused to human Fc. The MRC constructs generated are presented in Figure 1.

Immunohistochemistry

Fiberoptic sections of mouse peripheral lymph nodes and ears were double stained for MRC and lymphatic endothelial hyaluron receptor-1 (LYVE-1), MRC and F4/80, MRC and CD44, and plasmalemma vesicle-associated protein-1 and LYVE-1.

Quantitative Polymerase Chain Reaction

Total RNA was extracted from peripheral lymph nodes, mesenteric lymph nodes, spleen, and liver of WT and MRC⁻/⁻ mice (n=8). TaqMan Gene Expression Assays (Applied Biosystems) for mouse E-selectin, fibronectin-1, and Spp1 (osteopontin) were used in quantitative polymerase chain reaction, and the samples were run as triplicates. The expression values were normalized using mouse β-actin.

In Vivo Studies

Fluorescently labeled lymphocytes isolated from lymph nodes and spleens of CD44⁻/⁻ and WT mice were injected subcutaneously into the footpads of MRC⁻/⁻ and WT mice. After 12 hours, the popliteal lymph nodes were harvested and cell suspensions were analyzed using flow cytometry.

Statistical Analyses

Data were analyzed with the Student t test (2 tailed).

Results

CD44 and MRC Interact With Each Other

The CR domain of MRC recognizes chondroitin sulfate A and B and sulfated oligosaccharides of blood group Lewis a and Lewis x, as well as sulfated N-glycans of lutropin. Because CD44 has chondroitin sulfate side chains and sulfated carbohydrates linked to its core, we tested whether it could serve as a leukocyte counter receptor for MRC. We first affinity-purified CD44 from leukocyte lysates and ran lymphatic endothelial cells containing lymph node lysates through the columns. Immunoblotting analyses showed that MRC had specifically interacted with the immobilized CD44. Reciprocal experiments with immobilized natural MRC molecules confirmed the interaction with CD44. The band of CD44 appeared to be ≈180 kDa, suggesting it to be a certain splice variant or forms containing glycosaminoglycan side chains of CD44 (Figure 1A).

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To characterize the binding in more detail, we produced recombinant CD44 as an Fc chimera. It showed a smear appearance in the gel, typical for CD44 because of its abundant glycosylation. In addition, a small subpopulation of CD44 molecules were decorated by chondroitin sulfate side chains as demonstrated with 3 sharp bands showing up after chondroitinase ABC treatment (Figure 1B). We then tested the binding of the chimera to transfectants expressing different domains of MRC. The structure of the different domains is depicted in Figure 1C. Flow cytometric analyses showed that CD44 binds to the CR domain of MRC but not to the fibronectin type II domain or to the lectin domain (Figure 1D). Digestion of the chondroitin sulfate side chains of CD44-Fc with chondroitinase ABC abolished the binding to MRC (Figure 1E). Thus, MRC is a novel ligand for CD44, and the interaction takes place via binding of the glycosaminoglycan side chains of CD44 to the distal CR domain of MRC.

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Methods

Full methods are described in the online-only Data Supplement.

Animals

The MRC knockout (MRC⁻/⁻) mice were a kind gift from M. Nussenzweig (Rockefeller University). The CD44 knockout (CD44⁻/⁻) mice and their wild-type (WT) controls were from Jackson Laboratories.
Figure 1. CD44 binds to the cysteine-rich (CR) domain of macrophage mannose receptor (MRC) via its chondroitin sulfate side chains. **A**, CD44 and MRC proteins were affinity-purified in immobilized forms from human tissue lysates (precipitation). Thereafter, cell lysates containing CD44 or MRC were run through the column, and the binding of the specific proteins to the immobilized molecules was detected after SDS-PAGE by immunoblotting with the indicated monoclonal antibodies (mAbs; detection). As controls, immunoprecipitations and blottings were performed with a negative control mAb. The arrows point to the specific signals. **B**, Immunoblotting of recombinant CD44-Fc chimera without and with chondroitinase treatment. The bands appearing after chondroitinase treatment are indicated by arrows and presumably reflect the variety of glycosylations typical for CD44. **C**, The domain structure of the MRC constructs used in the study. **D**, Binding of CD44-Fc chimera (CD44lg) to different MRC transfectants. Human embryonic kidney cells were transfected with the indicated myc-tagged plasmids, incubated with CD4lg (neg co) or with CD44lg (x axis), permeabilized, and then stained for the neg co or c-myc (y axis) to visualize the transfection efficacy. **E**, Binding of CD44-Fc chimera without and with chondroitinase pretreatment to MR1 transfectants. Positive populations are indicated by arrows in **D** and **E**. Representative examples from (A, B) 2 to 3 and (D, E) 4 to 8 independent experiments with similar results are shown.
CD44-MRC Mediates Lymphocyte Trafficking via the Afferent Lymphatic Vessels In Vivo

The functional relevance of CD44-MRC interaction in supporting lymphocyte trafficking was then studied in adoptive cell transfer experiments. Fluorescently labeled lymphocytes from spleen and lymph nodes of WT and CD44−/− mice were injected into the footpads of WT and MRC−/− recipient mice, and their homing to the draining lymph nodes was measured. WT lymphocyte migration via the afferent lymphatics was impaired in MRC−/− mice, confirming our previous findings.1 CD44-negative lymphocytes also showed diminished capacity to home from the footpad into the draining lymph nodes in WT recipients (on average, 54% reduction; P<0.01). Furthermore, homing of CD44-negative lymphocytes through MRC−/− lymphatics was reduced to the similar extent (on average, 50%.

Figure 2. Expression analyses of macrophages, lymphatics, and vasculature in wild-type (WT), CD44 knockout (CD44−/−), and macrophage mannose receptor (MRC) knockout (MRC−/−) mice. A, Expression of MRC (green) on lymphatics (LYVE-1, red) and on a subset of macrophages (F4/80, red) in ear skin of WT mice. Examples of double-positive structures are indicated by arrowheads and arrows in the merge. B, Expression of CD44 (green) and MRC (red) in lymph nodes of WT, CD44−/−, and MRC−/− mice. Arrows point to the subcapsular sinus and arrowheads to 2 CD44-positive cells (as examples) within the sinus. C, Morphology of blood vessels (detected by anti–plasmalemma vesicle-associated protein-1 antibody, green) and lymphatic vessels (detected by anti–LYVE-1 antibody, red) in WT, CD44−/−, and MRC−/− mice. Stainings with negative control antibodies are shown in the insets. The figures are representative examples from ≥2 independent experiments, including 2 mice of each genotype in each experiment. LS indicates lymphoid sinus.

Figure 3. Macrophage mannose receptor (MRC)–CD44 interaction mediates lymphocyte migration via the afferent lymphatics in vivo, and the interaction can take place independently of L-selectin. A, Fluorescently labeled lymphocytes from wild-type (WT) mice were injected into the footpads of WT (n=4) and MRC knockout (MRC−/−) mice (n=5), and lymphocytes from CD44 knockout (CD44−/−) mice were injected into WT (n=10) and MRC−/− (n=13) mice. Transferred lymphocytes, which migrated to draining lymph nodes, were detected with flow cytometry. The data are presented as means±SEM percentage of migrated cells from the total number of lymph node lymphocytes. B, Binding of CD44 transfectants and their controls (treated with anti–L-selectin function-blocking antibody) to lymphoid sinuses of human lymph nodes in the presence of anti–MRC or control antibody. The results are shown as means±SEM number of migrated cells from the total number of lymph node lymphocytes. C, A representative micrograph showing binding of CD44-positive transfectants to the lymphoid sinus. Some lymphocytes are pointed out by arrows. mAbs indicates monoclonal antibodies; and Neg co, negative control.
reduction; P<0.005). These functional assays show a new role for CD44 in lymphocyte migration via the lymphatics and indicate that MRC-CD44 is a receptor-ligand pair also in vivo (Figure 3A).

We further tested whether different lymphocyte populations are selectively dependent on CD44-MRC in their homing to draining lymph nodes. In general, T cells homed significantly better than B cells, and more L-selectin-negative cells homed than expected when their relative number in the input population was taken into account. However, their relative percentages were comparable in WT and MRC−/− mice (Online Figure II). Furthermore, because L-selectin can easily be shed from the surface of activated lymphocytes during the homing, we directly tested whether CD44-MRC interaction mediates lymphocyte binding to lymphatic endothelium independently of L-selectin using frozen section ex vivo assays. These experiments clearly showed that CD44 lymphoma transfectants bind better to lymphatic endothelial cells than their controls in the presence of a function-blocking antibody against L-selectin (P=0.03) and that blocking of MRC inhibits this CD44-mediated binding (P=0.03; Figure 3B).

Discussion

In this work, we have identified a new ligand-receptor pair mediating lymphocyte trafficking from the periphery into the draining lymph nodes. Although the role of MRC in guiding lymphocyte migration within lymphatics was known earlier and the contribution of CD44 and hyaluronan interaction to leukocyte trafficking in the blood vessels is well recognized,1,5 CD44-MRC interaction with each other was not known. Furthermore, discovery of CD44 as a lymphocyte surface molecule supporting lymphocyte migration within the lymphatics helps to fill in the so-far incomplete understanding of the molecular mechanisms regulating cell trafficking within lymphatics.

Langerhans cell migration into the draining lymph nodes has been reported to be significantly reduced in CD44−/− mice.11 The reason is thought to be the lack of osteopontin—has been reported to be significantly reduced in CD44 −/− mice (Online Figure II). Furthermore, because L-selectin can easily be shed from the surface of activated lymphocytes during the homing, we directly tested whether CD44-MRC interaction mediates lymphocyte binding to lymphatic endothelium independently of L-selectin using frozen section ex vivo assays. These experiments clearly showed that CD44 lymphoma transfectants bind better to lymphatic endothelial cells than their controls in the presence of a function-blocking antibody against L-selectin (P=0.03) and that blocking of MRC inhibits this CD44-mediated binding (P=0.03; Figure 3B).

In conclusion, leukocyte trafficking within the lymphatics and blood vasculature is equally important for controlling the quality and quantity of the immune response. On the basis of our results, CD44 can be added to those few lymphocyte molecules known to mediate lymphocyte migration within the afferent lymphatics, and its potential as a drug target remains to be tested.

Sources of Funding

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Disclosures

None.

References


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### Novelty and Significance

**What Is Known?**

- Leukocyte trafficking via the lymphatic vessels is fundamental for proper functioning of the immune system.
- Macrophage mannose receptor expressed on the lymphatic endothelial cells can bind to lymphocyte L-selectin to mediate lymphocyte migration into lymphatic vessels.
- CD44 is a multifunctional adhesion molecule that mediates lymphocyte adhesion to extracellular matrix and vascular endothelium.

**What New Information Does This Article Contribute?**

- Lymphocyte CD44 is a novel counter receptor for macrophage mannose receptor.
- CD44–macrophage mannose receptor interaction mediates lymphocyte migration via the afferent lymphatic vessels into the draining lymph nodes under physiological conditions.

Lymphocyte recirculation from the blood to tissues and then via the lymphatic vessels to lymphatic organs and back to blood is crucial for the generation of immune responses. Here, we show that binding of lymphocyte CD44 to macrophage mannose receptor, expressed on lymphatic endothelium, is involved in lymphocyte migration from the periphery to the draining lymph nodes. Identification of this novel receptor-ligand pair reinforces the multifunctionality of these 2 molecules. These findings add to our understanding of the molecular mechanisms guiding lymphocyte entrance into afferent lymphatic vessels and could provide new targets to regulate harmful cell trafficking in inflammatory diseases.
CD44 Binds to Macrophage Mannose Receptor on Lymphatic Endothelium and Supports Lymphocyte Migration via Afferent Lymphatics

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Supplemental Material

CD44 Binds to Macrophage Mannose Receptor on Lymphatic Endothelium and Supports Lymphocyte Migration via Afferent Lymphatics

Salmi: CD44-MRC mediates migration within lymphatics

Supplementary Methods

Animals
The MRC knockout (MRC-/-) mice were a kind gift from M. Nussenzweig (Rockefeller University). The CD44 knockout (CD44-/-) mice (originally made in Hillberg’s laboratory) and their wild type (wt) controls were bought from Jackson laboratories. Age- and sex-matched mice were used in all experiments. The experiments were approved by the ethical committee of Southern Finland.

Immunohistochemistry
Frozen sections of mouse ears were double stained for MRC (FITC-conjugated anti-mouse MRC from BD Pharmingen) and macrophages (PE-TexasRed-conjugated F4/80 from Invitrogen) and MRC and lymphatics (polyclonal anti-LYVE from ReliaTech, followed by Alexa 546 anti-rabbit IgG, Invitrogen). Mouse peripheral lymph nodes were double stained for CD44 (PE-conjugated anti-mouse CD44) and MRC (FITC-conjugated anti-mouse MRC from BD Pharmingen). PV-1 (Meca32, gift from E. Butcher, Stanford University, followed by Alexa488-anti-rat IgG, Invitrogen) and LYVE-1 (polyclonal anti-LYVE from ReliaTech), followed by Alexa 546 anti-rabbit IgG, (Invitrogen) were used to identify vascular and lymphatic endothelial cells, respectively.

CD44-Chimera and MRC Constructs
The chimeric CD44 construct was a kind gift of D. Jackson (University of Oxford) and consisted of a full length extracellular domain of the standard form of human CD44 fused to hinge, CH2 and CH3 of human IgG1Fc in pCDM7Ig vector. It was produced in HEK293 cells and purified by Protein A Sepharose beads. CD4-Ig chimera was used as a control.

The full-length human macrophage mannose receptor cDNA (GeneBank accession number J05550, a kind gift of Dr. M. Taylor, University of Oxford) was first cloned into the expression vector pcDNA3.1 (Invitrogen). It was used as a template to clone distinct protein domains of mannose receptor by amplifying the desired regions by PCR and cloning them into pcDNA3.1. The clones generated encoded the following domains: the cysteine rich/ricin R-type domain (clone MR1; aa 22-142), the fibronectin type II domain (clone MR2+3; aa 161-209), and the C-type lectin domains (MR4; aa 216-1356). In addition, all clones contained the native signal and transmembrane sequences of the human mannose receptor to ensure proper targeting of the expressed proteins (see also Figure 1). The clones were further subcloned into pcDNA3.1/myc-His vector (Invitrogen).

Co-Precipitation Studies
Binding of MRC to purified CD44. Human tonsils were lysed in the lysis buffer (150 mM NaCl, 10 mM Tris-base, pH 7.2, 1.5 mM MgCl2, 1% NP-40, 1% Aprotinin and 1 mM PMSF) and the lysates were clarified by centrifugation. Human CD44 was affinity-purified from the lysate using CnBr-activated Sepharose 4B beads coupled to Hermes-3 monoclonal antibody (5mg/ml beads) against human CD44. Meanwhile, human peripheral lymph node was
minced and depleted of most lymphocytes by squeezing the lymphocytes through a 44 µm mesh. The stromal elements including vessels were then lysed. The lymph node lysate was then run through the CD44-affinity column. The columns were washed and bound proteins were eluted with 50 mM triethylamine, lyophilized, suspended in Laemmlli’s sample buffer and heated 20 min at 37°C before gel loading. 5-12.5% SDS-PAGE was run in non-reducing conditions and blotted to nitrocellulose sheets. MRC was detected with 3-155 antibody followed peroxidase-conjugated rabbit anti-mouse Ig (Dako) using an enhanced chemiluminescence detection method (Hybond-ECL; Amersham Pharmacia Biotech).³

**Binding of CD44 to purified MRC.** Human MRC was affinity-purified from the lymph node lysate using CnBr-activated Sepharose 4B beads coupled to 3-155 monoclonal antibody (5mg/ml beads³). After washings a leukopheresis lysate (peripheral blood leukocytes lysed in a buffer containing 50 mM β-octylglycoside, 1 mM CaCl₂, 1 mM MgCl₂, 1% aprotinin and 1 mM PMSF) was run through the MRC-affinity column. Bound proteins were eluted and separated and detected as above but using Hermes-3, peroxidase-conjugated rabbit anti-mouse Ig (Dako) and enhanced chemiluminescence detection.

**Negative controls.** CnBr-Sepharose 4B beads coupled to the irrelevant negative control antibody (3G6) served as a negative control in affinity purifications. In ECL, 3G6 was similarly used as a negative control mAb. The human material was used with the permission of the Ethical Committee of Turku University Hospital.

**Binding studies with the transfectants**
Different myc-tagged MRC constructs were transfected using Lipofectamine 2000 (Invitrogen) into CHO-cells and to HEK293 cells. After 24 h the cells were stained using 1 microgram of CD44-Fc and control (CD4-Ig) chimera. Binding of the chimeras was detected by sequential incubations with FITC anti-human IgG and Alexa-488 conjugated anti-FITC Ig. In certain experiments, the cells were thereafter permeabilized with acetone, blocked with 5% FCS, and incubated with anti-myc antibody (CRL 1729 from ATCC) or a negative control mAb (both mouse IgGs). Finally the mouse mAbs were detected using PE-conjugated anti-mouse Ig. The cells were analyzed using FACSCalibur and CellQuest software (BD Biosciences). When indicated the chimeras were treated with chondroitinase ABC (0.2U, Sigma).

**Quantitative PCR**
Total RNA was extracted from peripheral lymph nodes, mesenteric lymph nodes, spleen, and liver of wt and MRC-/- mice (n=8) using the Nucleo-Spin RNAII Total RNA Isolation Kit (Macherey-Nagel), and reverse-transcribed with iScript™ cDNA Synthesis kit (BioRad) according to the manufacturer’s instructions. TaqMan Gene Expression Assays (Applied Biosystems) for mouse Sele (E-selectin), FN1 (fibronectin-1), and Spp1 (osteopontin, also known as secreted phosphoprotein 1) were used as primer/probe sets, and the PCR reactions were carried out as suggested by the supplier using the Applied Biosystems 7900HT Fast Real-Time PCR System in the Finnish Microarray and Sequencing Center of Turku Center of Biotechnology. All samples were run as triplicates and the expression values were normalized using mouse β -actin as the endogenous control. The results were analyzed with SDS 2.3 and DataAssist v3.0 softwares. The average mRNA expression of each gene was presented as a percentage of the average β-actin mRNA expression measured from the same samples. Relative expression values were calculated by setting the value of the wild type samples as 1 and comparing the expression values of the knockout samples to that.
Migration into the draining lymph nodes

Peripheral and mesenteric lymph nodes and spleens of CD44-/- and wt mice were passed through a wire mesh to obtain single cell suspensions. After hypotonic lysis of erythrocytes, lymphocytes were labeled for 20 min at 37°C with 0.5 µM CFSE (carboxyfluorescein diacetate succinimidyl ester; Molecular Probes, Oregon, USA). Labeled lymphocytes were injected subcutaneously into the footpads of MRC-/- and wt mice. After 12 hours, the popliteal lymph nodes were harvested and cell suspensions were analyzed using FACS Calibur and CellQuest software (BD Biosciences).

In additional assays, CD44-/- lymphocytes were labelled as ‘CFSE high’ with 0.5 µM CFSE and wt lymphocytes as ‘CFSE low’ with 0.086 µM CFSE for 15 min at 37 °C and mixed at 1:1 ratio for injections into the MRC-/- and wt mice. Both the input cell populations and the lymphocytes isolated from the draining lymph nodes were stained with anti-CD62L / L-selectin Alexa647 (10 µg/ml, Exbio Praha), anti-CD3 PerCP-Cy5.5 (10 µg/ml, BioLegend) and anti-CD45R / B220 Pacific Blue (10 µg/ml, BD Biosciences). Cells were analyzed by LSRII flow cytometry (BD Biosciences) and FACS Diva Software (BD Biosciences) and the data were analyzed using Flowing Software version 2.5.0 (Cell Imaging Core, Turku Centre for Biotechnology). The contralateral popliteal lymph node of each animal was used as control to confirm that the lymphocytes had entered to the draining lymph node via the afferent lymphatics and not via the blood circulation.

Ex vivo frozen section assay

Binding of lymphoma cells (Namalwa) transfected with a construct encoding CD44 and control transfectants (vector only) to lymphatic endothelial cells was studied using frozen sections of human lymph nodes as described earlier. Sections were pre-incubated with an MRC (3-155) and negative control antibodies for 30 min. Thereafter, the anti-L-selectin antibody (Dreg-56, function blocking) treated CD44 and control transfectants were added onto the sections and the sections were incubated in static conditions for 15 min followed by rotation at 60 rpm for 5 min and again without rotation for another 15 min at 7°C. The adherent cells were fixed in 1% glutaraldehyde. The number of cells bound to lymphatic endothelium was counted under dark-field illumination (x200, Leitz Aristoplan, Oberkochem, Germany). Four lymph node sections/assay condition were used, and the experiments were repeated with different lymph node sections from three persons. All lymphoid sinuses in the samples were counted.

Online Table I. LYVE-1 positive lymphatics and PV-1 positive blood vessels in lymph nodes of wt, CD44-/- and MRC-/- mice.

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<th>Blood vessels</th>
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<tr>
<td>MRC-/-</td>
<td>20.0±1.4</td>
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<td>CD44-/-</td>
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*The numbers are given as mean±SD/field analyzed using x200 magnification
Supplementary References


Online Figure I

Online Figure I. There are no compensatory changes in the expression of other known CD44 ligands in MRC-/− mice. The mRNA-levels of fibronectin (FN1), E-selectin (Sele), and osteopontin (Spp1) were measured from wt and MRC-/− tissues using qPCR. The results are shown as relative expression levels (corresponding wt tissue is =1.0, n=8). PLN, peripheral lymph node; MLN, mesenteric lymph node. The data are presented as mean± SD.
Online Figure II

A

Examples of different cell populations in the input and among the homed cells. CD44−/− lymphocytes were labeled CFSE high and wt lymphocytes CFSE low, mixed and transferred to wt and MRC−/− recipients. After 12 h the draining lymph nodes were collected. Cells in the input population and in the lymph nodes were stained for CD3 (T cells), B220 (B cells) and L-selectin.

B

Combined results of all mice (mean ± SEM; wt recipients n=8 and MRC−/− recipients n=9).

Online Figure II. Distribution of different lymphocyte subpopulations in the input and among the homed cells in transfer experiments. CD44−/− lymphocytes were labeled CFSE high and wt lymphocytes CFSE low, mixed and transferred to wt and MRC−/− recipients. After 12 h the draining lymph nodes were collected. Cells in the input population and in the lymph nodes were stained for CD3 (T cells), B220 (B cells) and L-selectin. A, Examples of different cell populations in the input and among the homed cells. B, Combined results of all mice (mean ± SEM; wt recipients n=8 and MRC−/− recipients n=9).