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SIGN-R1, a novel C-type lectin expressed by marginal zone macrophages in spleen, mediates uptake of the polysaccharide dextran

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Abstract

The marginal zone macrophages of the spleen are implicated in the clearance of polysaccharides, but underlying mechanisms need to be pinpointed. SIGN-R1 is one of five recently identified mouse genes that are homologous to human DC-SIGN and encode a single, external, C-terminal C-type lectin domain. We find that a polyclonal antibody to a specific SIGN-R1 peptide reacts primarily and strongly with a subset of macrophages in the marginal zone of spleen and lymph node medulla. In both sites, SIGN-R1 exists primarily in an aggregated form, resistant to dissociation into monomers upon boiling in SDS under reducing conditions. Upon transfection into three different cell lines, high-mol.-wt forms bearing SIGN-R1 are expressed, as well as reactivity with ER-TR9, a mAb previously described to react selectively with marginal zone macrophages. SIGN-R1-expressing macrophages preferentially sequester dextrans following i.v. injection. Likewise, when phagocytic cells are enriched from spleen and tested in culture, dextran is selectively endocytosed by a subset of very large SIGN-R1$^+$ cells representing ~5% of total released macrophages. Uptake of FITC–dextran by these macrophages *in vivo* and *in vitro* is blocked by ER-TR9 and polyclonal anti-SIGN-R1 antibodies. Following transfection with SIGN-R1, cell lines become competent to endocytose dextrans. The dextran localizes primarily to compartments lacking transferrin receptor and the LAMP-1 CD107a panlysosomal antigen. Therefore, SIGN-R1 mediates the uptake of dextran polysaccharides, and it is predominantly expressed in the macrophages of the splenic marginal zone and lymph node medulla.

Introduction

The marginal zone of the spleen [reviewed in (1)] is an anatomical region that surrounds each white pulp nodule, separating the lymphocyte-rich white pulp from the vascular and macrophage-rich red pulp. It is comprised of distinctive B cells (2,3) and macrophages (1). The IgM$^+$ marginal zone B cells are distinguished from the more abundant B cells in the follicles of splenic white pulp and other peripheral lymphoid organs by a lack of Igδ and CD23 (a C-type lectin that can act as a low-affinity Fcε receptor), but high levels of CD21 (a receptor for the C3d complement fragment) and CD1d (which presents glycolipids to NKT cells). Marginal zone B cells can produce antibody to polysaccharides (2,4), a function that has been solidified by new experiments with mice selectively depleted of this B cell subset (5). The marginal zone macrophages also have distinctive properties. They react selectively with a mAb called ER-TR9 (6,7), which does not

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react with macrophages in other regions of the spleen, such as CD169+ marginal metallophils and F4/80+ red pulp macrophages. Marginal zone macrophages avidly internalize polysaccharides like dextran, Ficoll and hydroxyethylated starch (7–10), although the consequences of this uptake remain to be pinpointed.

In a separate line of research on the mechanism whereby dendritic cells (DC) initiate immune responses from resting T lymphocytes, Geijtenbeek *et al*. identified a new DC-restricted, C-type lectin that binds to an Ig superfamily adhesion molecule, ICAM-3/CD50, expressed on resting T cells (11). The lectin was named DC-SIGN, for DC-Specific ICAM-3 Grabbing Non-Integrin, to emphasize the fact that this new lectin was distinct from the integrins that had been pinpointed previously as ligands for the ICAM family. Upon cloning, DC-SIGN proved to be identical to a previously defined gene expressed in placenta and capable of binding the HIV-1 envelope glycoprotein (12). Geijtenbeek *et al*. (13) then demonstrated that DC-SIGN was responsible in large part for the capacity of DC to bind HIV-1 and transmit the virus to T cells, where it then replicates vigorously (14,15). The function of DC-SIGN in HIV-1 sequestration and transmission requires its cytosolic domain (16), which contains three different motifs for endocytic uptake and intracellular traffic (16,17). In a series of recent developments, it has been shown the DC-SIGN is also involved in the binding of several other infectious agents including at this time the Ebola virus (18), human cytomegalovirus (19), *Leishmania major* (20) and *Mycobacterium tuberculosis* (21,22).

To better understand DC-SIGN function *in vivo*, we decided to identify the mouse homologue. To our surprise, the experiments revealed an additional family of four closely linked homologous genes, termed SIGN-R1, 2, 3 and 4, yet none of these were detectably expressed in several populations of mouse DC (23). We therefore wanted to identify the cellular sites of expression and function of the new SIGN-R, beginning here with SIGN-R1. We will show that SIGN-R1 is expressed at high levels in the spleen and lymph node, but this expression is confined to a subset of macrophages in the splenic marginal zone and lymph node medulla. The lectin proves to be responsible for the uptake of polysaccharides *in vivo* in the marginal zone and *in vitro* in selected populations of splenic macrophages. Another laboratory, working independently, has also reported similar findings (24).

Methods

Mice and cell culture

C57BL/6 mice were purchased from Taconic (Germantown, NY) and Japan SLC (Hamamatsu, Shizuoka, Japan). Mice of both sexes were kept under specific pathogen-free conditions until use at 6–10 weeks of age. All experiments were conducted according to institutional guidelines. 293 human renal epithelial cells, CHO cells, and RAW264.7 and P388D1 mouse macrophage cell lines (ATCC, Manassas, VA) were cultured in DMEM with 10% FCS, 100 U/ml penicillin G and 100 μg/ml streptomycin. Two other mouse macrophage cell lines, J774.1 and IC-21 cells, were cultured in RPMI 1640 with 10% FCS and antibiotics.

Antibodies and microscopy

Rabbit polyclonal antibodies against the C-terminal 13-amino-acid peptide of SIGN-R1 (amino acid sequence: N-WICKKSATPCTEG-C) were raised and purified with a peptide-affinity column by ResGen/Invitrogen (Huntsville, AL). Anti-CD169 (SER-4) and F4/80 antibodies to antigens abundant on macrophages were purified from supernatants of the SER-4 and F4/80 hybridomas, kind gifts of Dr Siamon Gordon (Oxford University, Oxford, UK). Anti-MHC class II β chain antibody was purified from supernatants of the KL295 hybridoma (ATCC). Anti-human DC-SIGN antibody was provided by Dr Thomas Moran (Mount Sinai School of Medicine, New York, NY). Antibodies to the following targets were purchased: green fluorescent protein (GFP; BD Biosciences Clontech, Palo Alto, CA), actin (Research Diagnostics, Flanders, NJ), marginal zone macrophages (ER-TR9; BMA Biomedicals, Augst, Switzerland), macrophages (MARCO; Serotec, Raleigh, NC), DC (CD11c; BD Biosciences PharMingen, Palo Alto, CA) and B cells (B220, BD Biosciences PharMingen). A deconvolution microscope (Olympus America, Melville, NY), and one-, two- or three-color fluorescence labeling was used to visualize subsets of leukocytes as well as endocytosed polysaccharides in 1-μm sections through lymphoid tissues, primary macrophages, and various cell lines and their transfectants. The identity and colors of the fluorescent reagents are listed in the micrographs.

Vector construction and transient expression

A eukaryotic expression vector encoding the open reading frame (ORF) of GFP, pEGFP-N1 (BD Biosciences), was modified to make fusion proteins between GFP and the C-type lectin domains of mouse DC-SIGN, human DC-SIGN and mouse SIGN-R1. In brief, the termination codon in the ORF of GFP was removed and then the ORF of GFP cDNA was ligated in-frame with that of each C-type lectin domain. To express SIGN-R1 without a cytosolic domain, a fragment of SIGN-R1 cDNA encoding the first 47 amino acids was deleted. Then an initiation ATG codon was inserted before the codon corresponding to amino acid 48 of the SIGN-R1 protein and the mutant named SIGN-R1#48. Expression vectors containing the ORF of various GFP–C-type lectin fusion proteins, as well as a full-length and a deletion mutant of SIGN-R1 cDNA were transfected into 293, CHO and RAW 264.7 cells with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Two days after transfection, cells were harvested and lysed for analysis.

SDS–PAGE and Western blot analysis

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with protease inhibitor cocktails (Sigma-Aldrich, St Louis, MO) and stored at –80°C. Each lysed sample, equivalent to 1–2 × 10^5 cells, was mixed with an equal volume of 2 × SDS sample buffer with/without β-mercaptoethanol (β-ME) and boiled at 95°C for 5 min. Then, the samples of lysate were separated in 8, 10, 12 or 4–15% gradient SDS–PAGE and transferred onto PVDF membranes, followed by incubation with antibodies. Antibody-reactive bands on the blots were visualized by incubation with

peroxidase-labeled secondary antibodies followed by treatment with ECL Plus chemiluminescent substrate (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure in Kodak BioMax Light film (Eastman Kodak, Rochester, NY).

Polysaccharides

FITC–anionic dextran and Texas Red–dextran (mol. wt 70 kDa; Molecular Probes, Eugene, OR) and FITC–dextran (mol. wt 2000 kDa; Sigma-Aldrich) were purchased. To study endocytosis of these polysaccharides *in vivo*, 100 μg/mouse was administered by the i.v. route and uptake studied from 1 to 48 h later; alternatively, the polysaccharides were added *in vitro* at 10 μg/ml for 2–12 h on ice or at 37°C to cultured macrophages from spleen and to cell lines transfected with SIGN-R1 or empty vector as negative control. To test inhibition of uptake with anti-SIGN-R1 antibodies, we used doses of 25 μg antibody per animal given i.v. prior to 100 μg of dextran, or 10 μg/ml antibody *in vitro* during the administration of 2 μg/ml dextran, with non-reactive rat and rabbit Ig serving as negative controls.

Isolation and enrichment of marginal zone macrophages from mouse spleen

Paramagnetic particles with or without a fluorescent label (1–2 μm, 2.73% solution, cat. no. 19133; Polysciences, Warrington, PA) were washed 5 times with PBS and 0.2 ml of the 0.273% solution was injected i.v. After 4 h, spleens were digested with collagenase as described previously (25) and the single suspension was depleted of erythrocytes by hemolysis. The cells were suspended in RPMI 1640 supplemented with 5% FCS and antibiotics (R5 medium) at 3×10^7/ml, and passed through nylon mesh. The cells phagocytosing parmagnetic particles were collected on a magnetic apparatus (Dynal, Oslo, Norway), suspended in 5 ml of R5 medium and further enriched by a second round of magnetic beading. The isolated cells were suspended in R5 and seeded onto poly-L-lysine-coated coverslips placed in 24-well plates at 4×10^4 cells/600 μl. One hour after incubation in a CO_2 incubator, the cells were fixed with 3% paraformaldehyde in PBS for 20 min at room temperature, washed 3 times with PBS, and then permeabilized for 15 min at room temperature with 0.05% saponin in RPMI 1640 supplemented with 10% FCS and 10 mM HEPES at pH 7.4. To identify SIGN-R1$^+$ macrophages, the cells were first incubated with 2.4G2 mAb (anti-CD16/32) supernatant at 1/5 dilution for 2 h and then stained with 7.5 μg/ml SIGN-R1 or control rabbit IgG followed by biotinylated F(ab′)$_2$ donkey anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA) and avidin–Texas Red (Perkin Elmer Life Sciences, Boston, MA). After washing 3 times with PBS and 2 times with double-distilled water, the specimens were mounted in glycerol solution (1:1 mixture with PBS) containing 1% propylgallate. To assess uptake of FITC–dextran *in vitro*, the polysaccharide was applied at 2–10 μg/ml for 12–16 h in culture.

Results

SIGN-R1 is expressed mainly in lymph nodes and spleen

In our previous report (23), the expression of SIGN-R1 was detected by Northern blot analysis, which included RNA samples from testis, kidney, skeletal muscle, liver, lung, spleen, brain, heart, thymus, lymph nodes and intestine. The Northern blot analysis showed highest expression of SIGN-R1 in lymph nodes, some expression in spleen, but little or no expression in other tissues. In both lymph nodes and spleen, the Northern blot revealed two major bands at 1.3 and 2.1 kb, which would be expected to be generated from alternative use of poly(A)$^+$ signals in the 3′-untranslated region of SIGN-R1 mRNA (26).

Rabbit polyclonal antibody against the C-terminal 13 amino acid peptide of SIGN-R1 (23) was induced and purified by affinity to the same SIGN-R1 peptide. The specificity of this

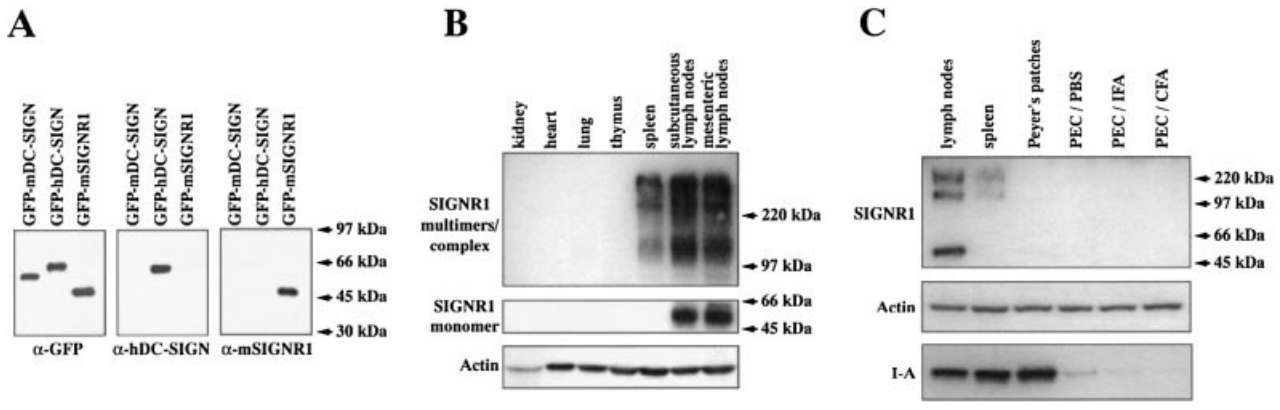


Fig. 1. SIGN-R1 is expressed mainly in lymph nodes and spleen. (A) Specificity of rabbit anti-mouse SIGN-R1 polyclonal antibody, confirmed by Western blot analyses on a series of closely related C-type lectin proteins. Fusion proteins of GFP and each C-type lectin domain of mouse DC-SIGN, human DC-SIGN (the fusion protein contained both C-type lectin and neck domains, hence the higher mol. wt) and mouse SIGN-R1 were transiently expressed in 293T cells. Equivalent amounts of each cell lysate protein were analyzed by the Western blots detected with anti-GFP antibody, anti-human DC-SIGN antibody and anti-mouse SIGN-R1 antibody respectively. (B) Western blot analyses of SIGN-R1 on cell lysates from mouse lymph nodes, spleen, thymus, lung, heart and kidney. Signals corresponding to the monomer and complex forms of SIGN-R1 are illustrated in two separate blots from SDS-PAGE gels of different densities, in order to provide better resolution. (C) Western blot analyses of SIGN-R1 on cell lysates from lymph nodes, spleen, Peyer's patches and peritoneal exudate cells (PEC). Peritoneal exudate cells were harvested 7 days after peritoneal injection of PBS, incomplete Freund's adjuvant (IFA) or complete Freund's adjuvant (CFA).

antibody was assessed by Western blot analysis on the closely related C-type lectins, human DC-SIGN and mouse DC-SIGN, each expressed as a soluble GFP fusion protein (Fig. 1A). Rabbit anti-SIGN-R1 antibody only bound to the C-type lectin domain of mouse SIGN-R1, but not the homologous lectin domains of human and mouse DC-SIGN. Although the sequences of SIGN-R1 and DC-SIGN have considerable similarity, even in the C-terminal 13 amino acids, the rabbit anti-SIGNR-1 polyclonal appeared highly specific. The expressed SIGN-R1 GFP fusion proteins had a mol. wt appropriate for monomeric forms of the lectin (Fig. 1A).

Using this antibody, we examined the expression of SIGN-R1 protein in various mouse tissues and cells by Western blot analysis. SIGN-R1 protein was only detectable in lymph nodes and spleen (Fig. 1B and 1C), as was the case for SIGN-R1 mRNA in these organs by Northern blot analysis. However, SIGN-R1 protein was detected at very low levels or not at all in B cells, T cells or CD11c$^+$ DC isolated from lymph nodes and spleen (data not shown), as expected from similar results of previous RT-PCR experiments (23). Furthermore, SIGN-R1 protein was not detected in lysates from Peyer's patches or peritoneal cells (comprised primarily of macrophages), the latter with and without activation to form inflammatory exudates (Fig. 1C). Similarly, SIGN-R1 protein was not found in four macrophage cell lines: RAW264.7, IC-21, J774.1 and P388D1 (data not shown). These Western blotting results indicate strong reactivity in spleen and lymph node, but did not reveal the reactive subset of macrophages that will become apparent below.

SIGN-R1 forms monomer and multimer/complexes

We first pursued the fact that SIGN-R1 protein by Western blotting was heterogeneous relative to that expected for a 50-kDa monomer. In lysates of lymph nodes, multiple forms were noted, including a low-mol.-wt 50 kDa monomer and others of higher mol. wt, 100 kDa or larger, presumptive multimers (Figs 1B and C, and 2B). In spleen, the 50-kDa monomeric form of SIGN-R1 was absent but higher-mol.-wt species were evident (Figs 1B and C, and 2B).

To further characterize these multiple forms of SIGN-R1 proteins, the full-length cDNA of SIGN-R1 was transiently transfected into 293 cells, followed by lysis and Western blotting on gradient gels (Fig. 2A). Without β-ME treatment, most SIGN-R1 protein was in high-mol.-wt multimer/complex forms, with less intense monomer and dimer bands. After the β-ME treatment, the majority of SIGN-R1 protein migrated as a monomer, but significant amounts of presumed dimer, trimer and tetramer forms remained. The formation of complex and β-ME-resistant multimers in 293 cells was also observed in CHO and RAW264.7 cells, transfected with SIGN-R1 and SIGN-R1#48 cDNA (data not shown).

Unlike SIGN-R1 expressed in transfected cell-lines, most of the multimer/complex forms of SIGN-R1 in spleen and lymph nodes were resistant to β-ME treatment (Fig. 2B). The

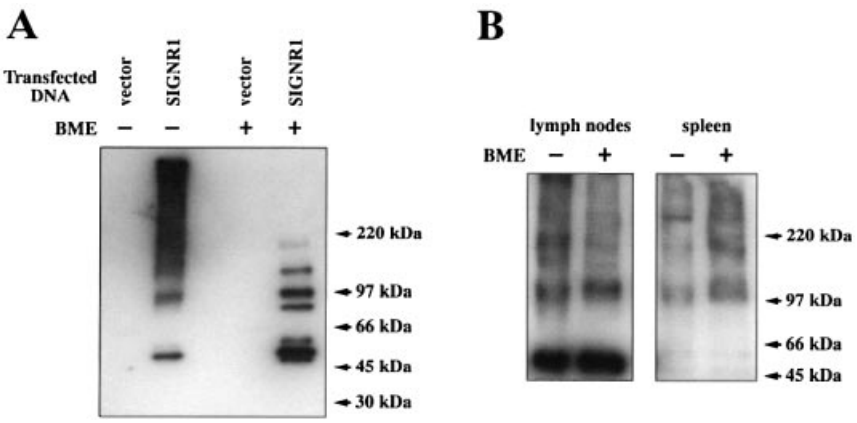
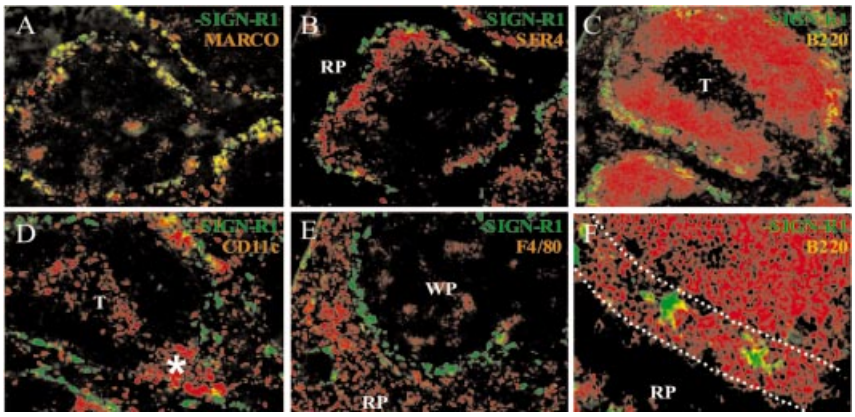


Fig. 2. SIGN-R1 proteins can form multimers resistant to β-ME treatment. (A) Eukaryotic expression vectors containing no insert or SIGN-R1 cDNA were transfected into 293 cells and cell lysates were prepared 2 days later. Equivalent amounts of protein were boiled in sample buffers without (–) or with (+) β-ME prior to Western blot analysis with rabbit anti-SIGN-R1 antibody. (B) Cell lysates were prepared from lymph nodes and spleen. Equivalent amounts of lysate protein were boiled in sample buffer, – or + β-ME prior to Western blot analysis with rabbit anti-SIGN-R1 antibody. For improved resolutions, 4–15% gradient SDS–PAGE gels were used for the Western blots in (A) and (B).

Fig. 3. Antibodies to SIGN-R1 selectively label macrophages in splenic marginal zone, but not the red pulp (RP). SIGN-R1 is in green and other cell-specific antibodies are in red, including: (A) MARCO scavenger receptor, (B) CD169 sialoadhesin [SER-4 antibody (28)], (C) B220 B cells, (D) CD11c DC particularly in the T cell areas (T) and in T cell transit regions (*) that interrupt the marginal zone (29), and (E) F4/80 red pulp macrophages. (F) At a higher magnification, the marginal zone is comprised of SIGN-R1 macrophages and B cells.

Fig. 4. SIGN-R1$^+$ macrophages (green) are restricted to the medulla of the lymph node. The second antibodies are listed on each panel and identify: (A) B cell follicles overlying the T cell areas, (B and C) medullary macrophages labeling with the MARCO and F4/80 antibodies, and (D) CD169$^+$ [SER-4 antibody (28)] macrophages in the subcapsular sinus and medulla. (E) CD11c$^+$ DC in the T cell regions (T) beneath the B cell areas (B).

Fig. 5. Only a subset of released splenic phagocytes are SIGN-R1$^+$. (A) FITC-labeled, iron-laden latex particles were given to mice i.v. 4 h prior to isolating the phagocytes with a magnet and then labeled *ex vivo* with control rabbit antibody (red). (B) Same as (A), but labeled *ex vivo* with rabbit anti-SIGN-R1. A subset of very large phagocytes labels for SIGN-R1, as shown as 3 examples.



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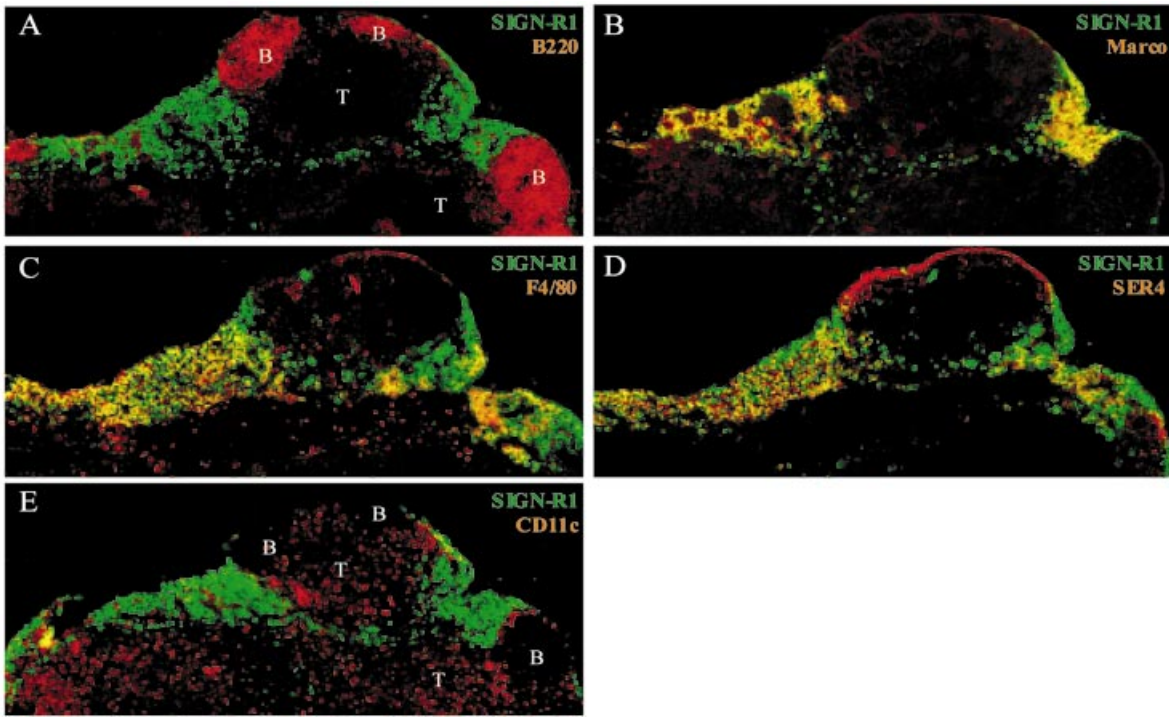

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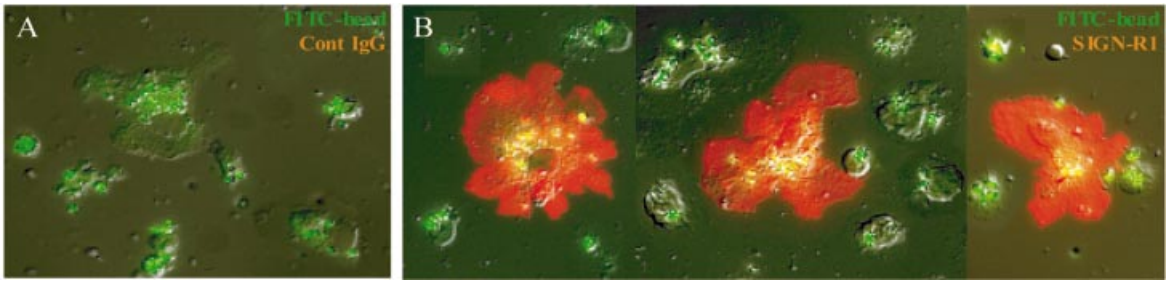

Fig. 5. (legend on facing page).

monmeric form of SIGN-R1 protein only increased by a small amount in lymph nodes after β-ME treatment. Interestingly, there was no monomeric form of SIGN-R1 protein detectable in spleen before and after β-ME treatment. Moreover, after β-ME treatment, the Western blot signals for multimer/complex forms of SIGN-R1 protein in spleen increased, possibly because the β-ME treatment made epitopes for the SIGN-R1 antibody more accessible. As illustrated in the gradient gels in Fig. 2, a large proportion of the complex forms of SIGN-R1 showed a mol. wt much larger than the expected 200-kDa for tetramers. We interpret these results to mean that much of the SIGN-R1 protein in spleen and lymph node is aggregated in a manner that is largely resistant to dissociation with SDS and β-ME.

Immunolabeling of SIGN-R1⁺ macrophages in tissue sections of spleen and lymph node

To determine the cellular sites for SIGN-R1 expression, we carried out immunolabeling studies on sections from spleen and lymph node, the two organs where mRNA and protein were detected. In spleen, rabbit anti-SIGN-R1 antibody strongly labeled the large phagocytic cells in the marginal zone that occupy much of the perimeter of each white pulp nodule (Fig. 3, green color). In two-color studies, most of the marginal zone macrophages also expressed MARCO, a previously identified (27) scavenger-type receptor (Fig. 3A). In contrast, there was no staining for SIGN-R1 on macrophages termed marginal metallophils, labeled with SER-4 anti-sialoadhesin (CD169) antibodies on the white pulp side of the marginal zone (28) (Fig 3B). Likewise anti-SIGN-R1 did not label B220⁺ B cells (Fig. 3C), CD11c⁺ DC found on the external aspect of the marginal zone and in the T cell areas (29) (Fig. 3D), and F4/80⁺ red pulp macrophages (30) (Fig. 3E). Thus SIGN-R1 labels a subset of macrophages embedded within the marginal zone of B cells (Fig. 3F), but none of the other cellular elements of the spleen.

In lymph node, SIGN-R1 labeling was intense on macrophages found in the medullary region (Fig. 4A). These cells also labeled for the MARCO and F4/80 macrophage antigens (Fig. 4B and C). In contrast, the lymph node cortex did not express SIGN-R1, including the rim of subcapsular sinus, CD169⁺ macrophages (Fig. 4D). No labeling for SIGN-R1 was found in the B cell areas or on CD11c⁺ DC in the T cell areas (Fig. 4E). The large numbers of SIGN-R1⁺ medullary macrophages probably accounts for the fact that the most intense signals in any organ in Northern and Western blots are found in lymph node.

Isolation of SIGN-R1⁺ macrophages from spleen

We developed methods to release and enrich macrophages to test if only a subset were SIGN-R1⁺ and eventually study their properties further. Mice were injected i.v. with iron-containing latex particles (either labeled with FITC or unlabeled). The spleens were then subject to collagenase digestion and the cell suspensions selected with a magnetic field to provide a population enriched in phagocytes corresponding to 0.2% of the total cell suspension. The isolated cells were highly enriched in latex-laden phagocytes and ~5% of the phagocytes were distinct, large, 'flower or petal-shaped' cells labeling strongly for SIGN-R1 (Fig. 5). These cells occasionally contained two or three nuclei (data not shown).

Selective uptake of dextrans by SIGN-R1⁺ macrophages in vivo and in vitro

To verify that the marginal zone macrophages marked by SIGN-R1 would take up polysaccharides, we administered polysaccharides at a dose of 100 μg/mouse i.v. The SIGN-R1⁺ cells were the major site for binding of 2000- and 70-kDa forms of anionic FITC–dextran (Fig. 6A). These polysaccharides were present in the marginal zone for 1–20 h and then disappeared over the subsequent day (Fig. 6A). In addition to the marginal zone, FITC–dextran was found in a web in the B cell area (white arrows, Fig. 6A), up to the 48 h that we followed their persistence. Dextran presumably activated the alternative complement pathway in the mice and were then recognized by CD21 complement receptors on follicular DC in the B cell areas. Small amounts of FITC–dextran also were noted in presumptive DC in the T cell areas (not shown). When we compared the splenic localization of FITC–dextran with FITC–latex following i.v. injection, the FITC-latex was found in both marginal zone and red pulp, and did not localize to the B cell follicles (not shown).

To phenotype the cells internalizing FITC–dextran, two-color immunofluorescence studies were performed (Fig. 6B). The FITC–dextran-bearing cells expressed the marginal zone macrophage antigens, ERTR9 and SIGN-R1 (Fig. 6B, left panels), but little or no CD11c (DC), B220 (B cells), SER4/CD169 (marginal zone metallophils) or F4/80 (red pulp macrophages) (Fig 6B).

In vitro experiments were carried out to ensure that FITC–dextran would have access to SIGN-R1⁺ and SIGN-R1⁻ phagocytes, isolated as in Fig. 5. When the phagocytes were exposed to 10 μg/ml FITC–dextran, only the SIGN-R1⁺ phagocytes took up the polysaccharide (Fig. 6C). Therefore SIGN-R1⁺ macrophages are the principal phagocytes involved in the splenic uptake of dextrans.

SIGN-R1 mediates endocytic uptake of polysaccharide in culture and in vivo

To determine if SIGN-R1 could itself mediate dextran uptake, the cDNA was transfected into three different cell lines (293 human renal epithelium, CHO and the RAW267.4 macrophage cell line). The transfected cells became reactive with SIGN-R1 antibody as well as the previously described ER-TR9 antibody, a reagent that selectively stains marginal zone macrophages (6) and blocks uptake of dextran *in vivo* (7). Using the FACS to monitor the uptake of FITC–dextran at the single-cell level, we found that SIGN-R1 transfectants acquired the capacity to take up FITC–dextran at low doses (10 μg/ml, Fig. 7A). By deconvolution microscopy, SIGN-R1 transfected cells [identified in blue with either monoclonal ER-TR9 (not shown) or polyclonal SIGN-R1 (Fig. 7B)] internalized dextran into numerous endocytic vesicles. At 37°C, the FITC–dextran containing intracellular compartments showed little or no double labeling with antibodies to either LAMP-1/CD107a, which labeled abundant lysosomes in these cells, or with antibody to the transferrin receptor, which labeled abundant endosomes (Fig. 7B). Using either microscopic or FACS assays, cells transfected with the vector control did not internalize above

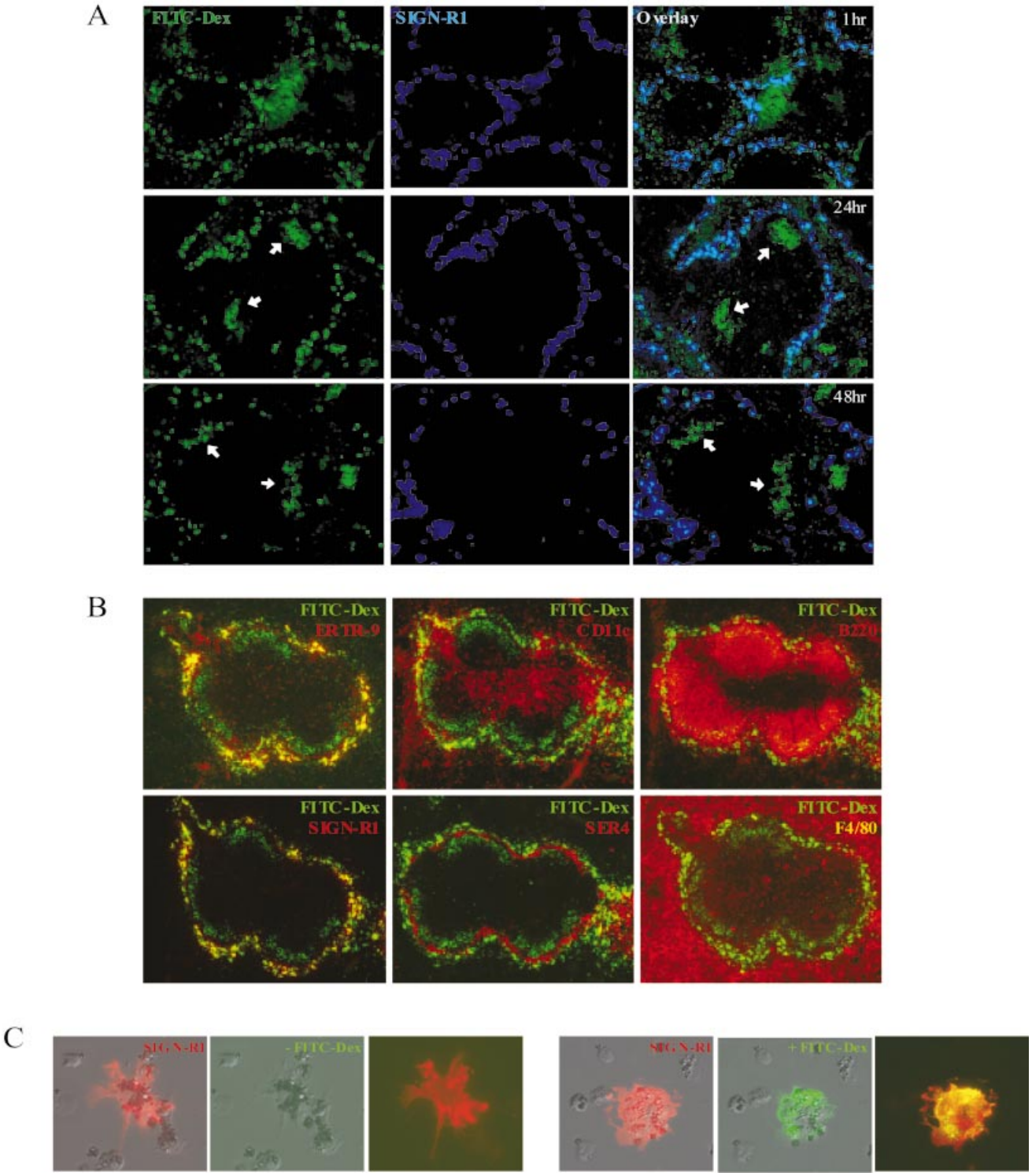

Fig. 6. Uptake of FITC–dextran into SIGN-R1$^+$ macrophages. (A) Uptake *in vivo* monitored 1, 24 and 48 h after injection of 100 μg/mouse of anionic 2000-kDa FITC–dextran. The FITC (left) and SIGN-R1 (lavender, middle) labels are in similar cells in the single-color micrographs, resulting in a light blue color in the overlay micrograph (right panels). Retention of dextran in the B cell follicles (white arrows) was apparent for longer periods than retention within marginal zone macrophages. (B) Only macrophages in the marginal zone take up FITC–dextran (green). These phagocytes express high levels of SIGN-R1, but little or no F4/80 and CD169 antigen, found on red pulp and marginal metallophil macrophages respectively. CD11c$^+$ DC and B220$^+$ B cells also show little or no uptake of FITC–dextran. (C) Selective uptake of FITC–dextran by isolated marginal zone. SIGN-R1$^+$ macrophages (red) cultured overnight in 10 μg/ml FITC–dextran.

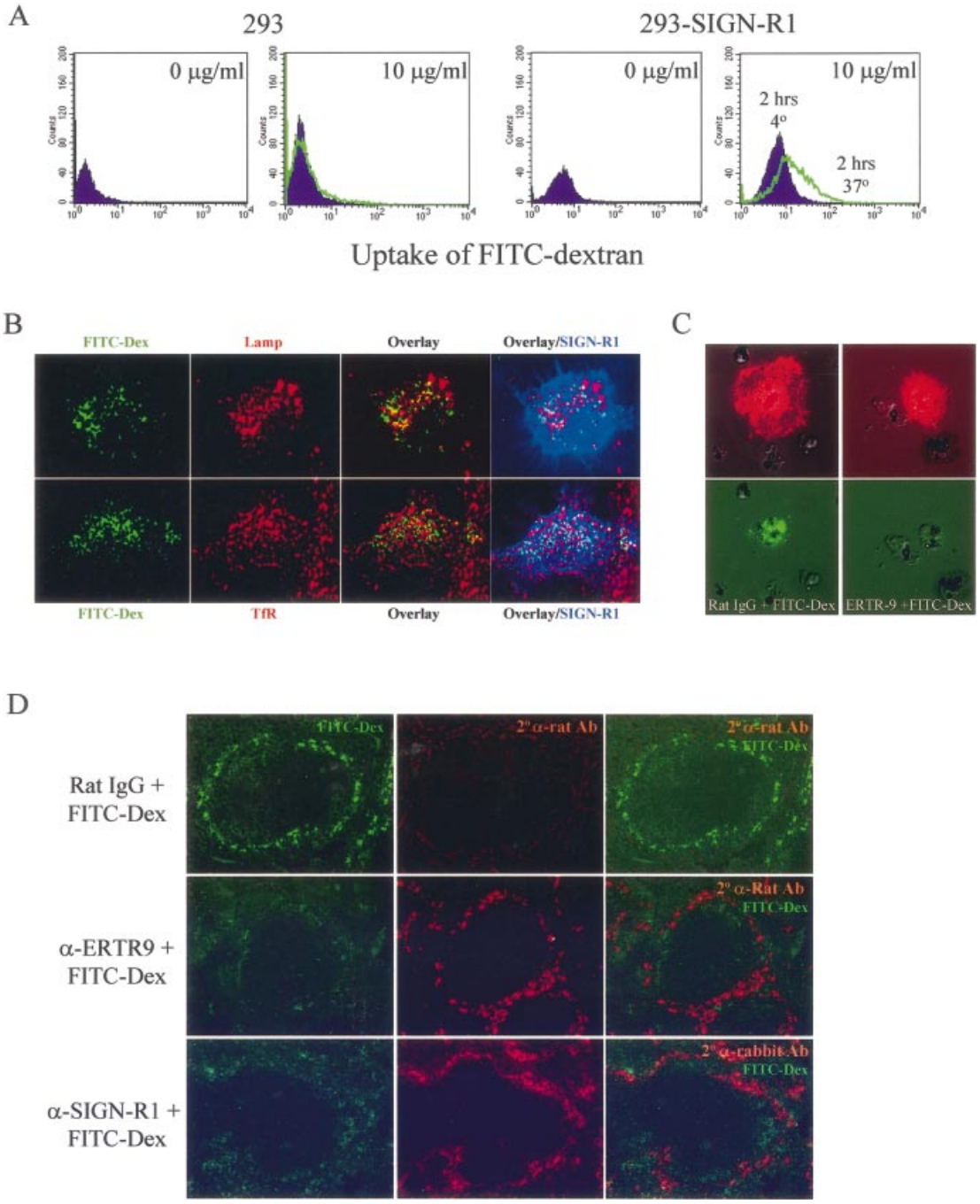

Fig. 7. SIGN-R1 mediates the uptake of FITC–dextran. (A) FACS staining of 293 cells without or with transfection of SIGN-R1, and exposed to 0 or 10 μg/ml FITC–dextran for 2 h at 4 and 37°C. In data that are not shown, the SIGN-R1 transfectants acquired reactivity to ER-TR9 mAb. (B) Stably transfected RAW264.7 SIGN-R1 transfectants were mixed with an excess of non-transfected RAW264.7 cells and cultured in 10 μg/ml FITC–dextran (green) for 2 h followed by double labeling with rabbit anti-SIGN-R1 antibody (lavender) and either rat anti-LAMP-1 (top) or anti-transferrin receptor (bottom) in red. (C) Single examples of splenic macrophages cultured for 12 h in 2 μg/ml FITC–dextran and either 10 μg/ml control rat Ig (left) or 10 μg/ml of blocking ER-TR9 antibody (right). (D) Blocking of uptake of 100 μg of 2000-kDa FITC–dextran into splenic marginal zone macrophages *in vivo*. FITC–dextran was administered i.v. for 1 h in mice pretreated 30 min earlier with 25 μg of control Ig (top row), ER-TR9 rat anti-SIGN-R1 mAb (middle row) or rabbit anti-SIGN-R1 polyclonal antibody (lower row).

the background seen with SIGN-R1$^-$ cells and no uptake was seen if the SIGN-R1 transfectants were exposed to polysaccharide at 4°C (not shown).

To evaluate the function of SIGN-R1 on primary populations of macrophages, we performed blocking studies with anti-SIGN-R1 antibodies. Rat ER-TR9 mAb at 10 μg/ml blocked FITC–dextran uptake by isolated splenic macrophages (Fig. 7C). Both rat and rabbit anti-SIGN-R1 antibodies, given at a dose of 25 μg per mouse, blocked the uptake of i.v. FITC–dextran by marginal zone macrophages in spleen sections (Fig. 7D). No blocking was observed with non-reactive control antibody (Fig. 7D top row) or if anti-SIGN-R1 was used to try to block FITC–latex uptake in culture (not shown). Therefore SIGN-R1 is able to mediate the uptake of polysaccharides, as is characteristic of marginal zone macrophages *in vivo* and *in vitro*.

Discussion

The new family of C-type lectins, termed SIGN-R1, 2, 3 and 4 (23), is so named because of homology to a lectin termed DC-SIGN that is expressed by DC. However, the SIGN-R themselves are not clearly expressed by DC. Instead, we now find that SIGN-R1 is primarily found on subsets of macrophages in splenic marginal zone and lymph node medulla. In spleen cell suspensions, these macrophages represent ~0.01% of the cells that can be released by current methods. SIGN-R1 is the target for the previously described antibody ER-TR9 (6,7) and, importantly, this lectin on a minor fraction of spleen cells serves as the major endocytic receptor for dextrans. Our findings have one difference from a conclusion in the prior literature, i.e. that neutral polysaccharides are selectively captured by marginal zone macrophages (7–10). With the reagents that we studied, we observed clearance by SIGN-R1$^+$ macrophages of both anionic and neutral forms of dextran, and with a wide variety of mol. wt, 70–2000 kDa. The efficient uptake of these dextrans could be replicated when cell lines from three different species and tissues (293 human renal epithelium, CHO and RAW267.4 mouse macrophages) were transfected with SIGN-R1.

Dextrans are often used to study endocytosis by DC, which express little or no SIGN-R1. However, it is possible that DC use other receptors like the macrophage mannose receptor and/or that much higher concentrations of dextrans (~1 mg/ml) are needed to observe strong dextran uptake in DC (31).

While SIGN-R1 protein is readily identified in lysates of spleen and lymph node, little of it exists as a simple monomer. Instead, the lectin migrates as a complex in SDS–PAGE, most likely multimers or larger complexes. Such complexes could originate following binding of SIGN-R1 to large complex carbohydrates in tissues, which are somehow stable in SDS and β-ME treatment. The molecular basis and significance of the dominant high-mol.-wt SIGN-R1 forms are currently unclear. One possibility is that the aggregation of SIGN-R1 enhances the binding and/or uptake of polysaccharides.

In the case of HIV-1 uptake via the homologous DC-SIGN lectin, the virus can be sequestered for days in non-lysosomal compartments (16,32) and then the HIV-1 is transferred in an infectious state to other T cells. Perhaps this sort of traffic into and out of the cell occurs with SIGN-R1 ligands in macrophages. At this point, it is evident that uptake via SIGN-R1 primarily delivers dextrans to intracellular compartments lacking in the LAMP-1 lysosomal and transferrin receptor endosomal markers. These findings parallel the observations on HIV-1 uptake via DC-SIGN, but the fate of polysaccharides internalized via SIGN-R1 needs to be pursued.

Although macrophages are a major cellular component of the spleen, it is difficult to liberate these cells into suspension, and then separate them from most of the lymphocytes and DC. To be able to study splenic macrophages in isolation, we have developed a method that enriches the cells on the basis of substantial particle uptake *in vivo*. DC primarily take up only 1–2 latex particles per cell (33), but when we administer iron-laden latex particles to mice i.v. and then dissociate the spleens in collagenase 4 h later, we are able to use magnets to isolate phagocytes with >3 particles/cell and to >90% purity. These phagocyte populations lack CD11c$^+$ DC, but do contain a subset (~5%) of very large 'petal'-shaped cells that stain strongly with monoclonal and polyclonal anti-SIGN-R1 antibodies. These macrophages selectively internalize FITC–dextran at low doses and the uptake is blocked by ER-TR9 mAb to SIGN-R1. Such results are valuable since events other than receptor expression may influence the *in vivo* distribution and uptake of macromolecules. Our results with macrophages *in vivo* and *in vitro* indicate that phagocytes ignore low concentrations of dextrans unless they express the newly defined SIGN-R1 receptor for these polysaccharides. SIGN-R1 may be an important mediator for the uptake of microbes in both spleen and lymph node, particularly through the recognition of microbial polysaccharides.

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Abbreviations

β-ME	β-mercaptoethanol
DC	dendritic cells
GFP	green fluorescent protein
ORF	open reading frame

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