Expression of mannose receptor and ligands for its cysteine-rich domain in venous sinuses of human spleen

Luisa Martinez-Pomares[1,*], Leif G Hanitsch[1,†], Richard Stillion[1], Satish Keshav[2] and Siamon Gordon[1]

[1]*Sir William Dunn School of Pathology, University of Oxford, Oxford, UK and* [2]*Department of Medicine, Royal Free and University College Medical School, London, UK*

The mannose receptor (MR) is a type I membrane molecule with two lectin activities. Mannose recognition takes place through the C-type lectin-like carbohydrate recognition domains, while recognition of sulphated glycans is mediated by the cysteine-rich domain (CR). In murine spleen CR ligands are present in a subpopulation of macrophages (Mϕ) placed in the marginal zone whereas MR-expressing cells consisting of Mϕ and nonvascular endothelia are located in the red pulp. No colocalisation of MR with CR ligands has been observed in murine tissues. In this manuscript we describe the distribution of MR and CR ligands in human spleen. In this organ we have detected a perfect colocalisation of MR with CR ligands in Lyve-1$^+$ cells lining venous sinuses. These cells form a physical barrier for blood cells as they need to migrate through the sinuses in order to exit the splenic parenchyma and, in this way, contribute to the unique filtration function of this organ. Furthermore, unlike murine spleen, CD68$^+$ red pulp Mϕ lack MR expression. Our results suggest an unexpected contribution of MR to splenic function through the recognition of sulphated ligands that could influence the filtering capability of this organ.

The mannose receptor (MR) is a type I membrane protein with three types of domain in the extracellular region;[1–3] a cysteine-rich domain (CR), that is a member of the β-trefoil protein family,[4] a domain containing fibronectin type II repeats and 8 C-type-lectin-like domains (CTLD). The cytopasmic tail of the MR contains signals that mediate receptor internalisation and recycling.[5,6] Two well-characterised lectin activities are mediated by the extracellular region of MR: CR binds sulphated sugars terminated in SO_4-3-galactose (Gal), SO_4-4-N-acetyl-galactosamine (GalNAc) and SO_4-3-GalNAc; these modifications are present in glycoprotein hormones produced by the anterior pituitary, in chondroitin sulphate A and B and in sulphated Lewis^a and Lewis^x.[7–9] The second lectin activity is mediated by

several of its CTLD.[10] CTLD4-7 recognise complex sugars terminated in mannose, fucose and N-acetyl-glucosamine.[5]

Although MR is considered a macrophage (Mϕ) marker its expression by other cell types has been well documented.[11] In mice in addition to most tissue Mϕ, MR is present in hepatic and lymphatic endothelia and mesangial cells. In humans MR expression has been demonstrated in monocyte-derived Mϕ and dendritic cells *in vitro* and in myeloid cells in dermis and tonsils and lymphatic endothelium in lymph nodes *in situ*.[12–14]

The spleen is a major filtration organ for microbes and blood cells and it is required to elicit responses against TI-2 antigens. It is divided into a lympho-cyte-rich area (white pulp) and an erythrocyte-rich area (red pulp) where filtration and clearance take place, likely mediated by specialised Mϕ populations. Splenic architecture varies among species. In rodents (mice and rats) a well-developed structure, the marginal zone,[15] has been described surrounding a clearly defined marginal sinus and containing specialised Mϕ and B-cell populations. A more confusing picture emerges in the case of human

Correspondence: Dr L Martinez-Pomares, PhD, Sir William Dunn School of Pathology, South Parks Road, Oxford OX1 3RE, UK.
E-mail: luisa.martinez-pomares@path.ox.ac.uk

*Current address: School of Molecular Medical Sciences, Faculty of Medicine and Health Sciences, University of Nottingham Queen's Medical Centre, Nottingham NG7 2UH, UK

†Current address: Engeldamm 28, Berlin D-10179, Germany

spleen where the presence of a marginal sinus has been questioned; a marginal zone is solely defined on the basis of an accumulation of mature B cells and a perifollicular area has been described with increased numbers of red blood cells.[16] Regarding the splenic red pulp, cords and sinuses have been clearly demonstrated in rats and humans. Splenic cords act as filtration beds receiving blood from terminal arterial vessels and conveying it to venous sinuses[17] that contain, among others cells, mature Mϕ involved in phagocytosis. The walls of the splenic sinuses consist of specialised endothelial cells with intercellular slits, that express the alpha chain of CD8 and thrombomodulin and are weakly positive for CD31.[16] These cells are fixed to ring fibres of basement membrane-like material transversing the endothelial cells on their basal surface. Blood cells circulating through the red pulp flow across the wall of splenic sinuses between endothelial cells.

Analysis of MR and of CR ligands in mouse spleen showed a clear compartmentalisation with MR present in Mϕ and nonvascular endothelia in the red pulp and CR ligands expressed by specialised Mϕ surrounding B-cell follicles.[11] MR in red pulp is likely to be involved in the clearance of endogenous and microbe-associated mannosylated ligands. Cells bearing CR ligands migrate within the B-cell areas upon stimulation and then express CD11c, suggesting a role in antigen transport.[18–20] CR ligands in spleen are exposed at the cell surface and mediate specific binding and internalisation of CR-bearing recombinant proteins introduced into the circulation.[21] No association of MR$^+$ cells and cells expressing ligands for CR has been observed in this animal model[11] and a soluble form of MR, identified in the human and mouse, has been proposed as the counter receptor for these CR ligands.[22–25]

In order to assess how the MR/CR ligand system may function across species, we have generated a chimaeric protein containing the human CR fused to mouse IgG2a Fc to investigate the presence of CR ligands in human spleen and to compare their expression with that of the MR.

In this manuscript, we demonstrate that CR ligands (able to bind mouse and human CR) are present in equivalent areas in mouse and rat spleens suggesting that the role of CR ligands is likely to be conserved between these species. However, in human spleen CR ligands are present in venous sinuses in the red pulp. Remarkably and in contrast to the results obtained with rodent spleen, we observed a perfect colocalisation between MR and CR ligands and the absence of MR on CD68$^+$ red pulp Mϕ. The presence of MR and CR ligands in venous sinuses suggests a specific role for the MR/CR ligand interaction in human spleen in mediating adhesion of the venous sinuses cells and/or in controlling cell trafficking through the sinus.

Materials and methods

Source of Tissues

Rat tissues were obtained from animals bred at the Sir William Dunn School of Pathology.

Spleen tissue for immunohistochemistry was obtained from samples that were surplus to clinical requirements following tissue typing for solid organ transplantation. Unfixed 5 mm square blocks were frozen in OCT-embedding medium (Bayer Diagnostics, Berkshire, UK) and frozen slowly in a bath of dry ice and ethanol.

Reagents

Emr2–muFc was kindly provided by Dr Martin Stacey (University of Oxford) and muCR-huFc was prepared as described.[20]

Generation of huCR-muFc

The CR domain of the human MR was amplified using the primer 5′ Hu CR: GGC ATC GGA TCC TGT CCA TCA GGA GAA GG and 3′ Hu CR: GCC ATC GCG GCC GCG CCT AGT AGC GTA TAC ATG GCT T using as template the plasmid pMMR265, kindly provided by Dr Maureen Taylor, University of Oxford. The PCR product contained a *BamH*I site at the 5′ end and a *Not*I site at the 3′ end. The hinge-CH2 and CH3 regions of a non-Fc receptor-binding form of murine IgG2b was amplified using as template plasmid DNA generously provided by Lisa Gilliland (SWDSOP) using the primers 5′ muFc: GGC ATG GCG GCC GCA GAG CCC AGC GGG CCC ATT TCA AC and 3′muFc: GGC ATG TCT AGA TCA TTT ACC CGG AGA CCG GGA GAT. This PCR product had a *Not*I site at the 5′ end and an *Xba*I site at the 3′ end. The DNA fragments encoding huCR and muFc were cloned into the expression vector pcDNA.3 digested with *BamH*I and *Xba*I. This cloning strategy introduced three alanine residues between the huCR and mFc. The resulting plasmid was transfected into 293T cells using genejuice (Novagen, Merk Biosciences Ltd, Nottingham, UK) and the recombinant huCR-muFc protein was purified from cell supernatants using protein A sepharose (Amersham Biosciences, UK).

Sugar Binding Assay

All washings and incubations were performed in 10 mM Tris-HCl, pH 7.5, 10 mM Ca^{2+}, 0.154 M NaCl and 0.05% (w/v) Tween 20.

Sugar polyacrylamide (PAA) substrates (Lectinity, Moscow) were coated onto the wells of ELISA plates (Nunc; Maxisorb) by incubation in 154 mM NaCl overnight at 37°C (50 μl/well, sealed in a damp box) at 5 μg/ml. After coating, plates were washed five times. HuCR-muFc was incubated in the wells of

coated plates (50 µl/well) for 2 h at room temperature. Binding was detected by incubation with anti-mouse IgG Fc-specific alkaline phosphatase-conjugated (Chemicon, Hampshire, UK). Plates were washed five times and developed with *p*-nitrophenyl phosphate substrate (Sigma-Aldrich, UK) in 100 mM Tris-HCl, pH9.5, 100 mM NaCl, 5 mM MgCl$_2$. Absorbance was measured at 405 nm. All assays were carried out in duplicate.

Immunohistochemistry

Bright field

Detection of sialoadhesin (Sn) and CR ligands in rat spleen: Fresh rat tissues were embedded in Tissue-Tek OCT compound (Bayer Diagnostics) and were frozen on dry ice-cooled isopentane. Sections (5 µm) were cut, air dried and stored at −20°C. Sections were thawed at room temperature, fixed in 2% paraformaldehyde in Hepes-buffered saline, permeabilized in 0.1% Triton X-100 in PBS, quenched using glucose oxidase, and blocked in 5% normal sheep sera in PBS. Primary antibody (ED3 or isotype-matched control at 2 µg/ml from Serotec Ltd, Kidlington, UK) or murine Fc chimaeric proteins (huCR-muFc or Emr2-muFc) diluted in blocking buffer (5 µg/ml) were added and incubated for 1 h at room temperature. Binding was detected using peroxidase-conjugated sheep anti-mouse immunoglobulin Ig (Vector, Peterborough, UK). When the human Fc-derived protein muCR-huFc was used, blocking was performed using normal goat serum and binding was detected using peroxidase-conjugated goat anti-human IgG (Vector).

Detection of MR, CD68, Lyve-1 and CR ligands in human spleen: Fresh human spleens were embedded in Tissue-Tek OCT compound (Bayer Diagnostics) and were frozen as described above. Sections (5 µm) were cut, air dried and frozen. Sections were thawed at room temperature, fixed in 2% paraformaldehyde in Hepes-buffered saline, permeabilized in 1% Triton X-100 in PBS, quenched using glucose oxidase, and blocked with 5% normal sera in PBS. Sections were then incubated with primary mouse antibodies (15.2.2 (anti-MR) (HyCult biotechnololgy,) or anti-CD68 (Clone EBM11, Dakocytomation, Denmark), muFc-derived chimaeric proteins (huCR-muFc or Emr2-muFc) or polyclonal rabbit serum against human Lyve-1 (kindly provided by Dr David Jackson, University of Oxford) diluted in blocking buffer. In the case of mouse IgG and rabbit antiserum, incubation was performed at room temperature for 1 h. Incubation with huCR-muFc and Emr2-muFc was performed at room temperature overnight. In all cases binding was detected using biotinylated horse anti-mouse IgG (Vector, Peterborough, UK) and the peroxidase-conjugated-ABC detection system (Vector). In some instances, huCR-muFc was used in the presence of 10 mM SO$_4$-4-GalNAc (Dextra Laboratories, Reading, UK).

Fluorescence

Detection of CR ligands in human spleen was performed using a three-step amplification system by which huCR-muFc binding was detected using digoxigenin-conjugated anti-mouse (Chemicon) and FITC-conjugated anti-digoxigenin Ab (Roche Switzerland). For detection of MR, biotinylated 15.2.2 mAb and Cy5-conjugated streptavidin was used; for detection of CD68, nonconjugated antibody and Alexa 488-conjugated anti-mouse IgG was employed. To detect Lyve-1, polyclonal rabbit serum against human Lyve-1 and FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch Europe Ltd, Cambridgeshire, UK) were used. Sections were counterstained using DAPI and mounted using fluorescence-mounting media (Dakocytomation). Sections were analysed using a Zeiss Axioplan 2e microscope fitted with a 16 bit, 1024 × 1024 pixels CCD camera. Images were processed using Metamorph software. Adjustments were the same for all the images acquired for each experiment.

Preparation of Tissue and Cell Lysates

Human and rat spleen samples were cut using a sterile scalpel and placed in ice cold lysis buffer (2% Triton X-100, 10 mM Tris-HCl pH8, 150 mM NaCl, 2 mM EDTA, 10 mM NaN$_3$) containing protease inhibitors (Roche, 1 tablet/10 ml lysis buffer). Tissue pieces were processed using a Dounce homogeniser and protein solubilisation was aided by further incubation at 4°C for 45 min. Nonsolubilised material was removed by centrifugation at 4°C in a microfuge (2000 r.p.m. for 5 min followed by 13 000 r.p.m. for 15 min). Protein concentration was measured using the BCA method (Pierce, Perbio Science, Cheshire, UK). Lysates were aliquoted and kept frozen at −80°C. Cell lysates from human blood monocyte-derived Mφ cultured in six-well plates, were prepared by washing cells in ice cold PBS, addition of lysis buffer containing protease inhibitors and incubation at 4°C for 45 min. Lysates were collected and cleared as described above. Culture supernatants were collected, centrifuged at 3000 r.p.m. 4°C for 10 min and stored at −80°C.

Detection of MR in Human Spleen Lysates and in Cell Lysates and Supernatants from Human Monocyte-Derived Mφ by Western Blot

Protein lysates were electrophoresed in a 6 or 10% SDS-PAGE under nonreducing conditions and transferred to nitrocellulose filters (Amersham). Filters were blocked using 5% (w/v) nonfat milk in PBS-T (PBS containing 0.01% (v/v) Tween 20). Presence of MR was assessed by incubation with the anti-human MR mAb clone 15.2.2 (5 µg/ml in blocking buffer) and species-absorbed horseradish peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch). Binding was detected using the ECL reagent (Amersham Biosciences, UK).

Detection of CR Ligands in Human and Rat Spleen Lysates

Protein lysates were electrophoresed and transferred to filters as before. CR ligands were detected using huCR-muFc or muCR-huFc (10 μg/ml in blocking buffer) and species-absorbed horseradish peroxidase-conjugated anti-mouse IgG or anti-human IgG (Jackson ImmunoResearch) and visualised using the ECL system.

Detection of Sn in Rat Spleen Lysates and in Preparations of CR Ligands

Total protein lysates were electrophoresed and transferred to filters as before. Sn was detected using ED3 mAb (10 μg/ml in blocking buffer) and species-absorbed horseradish peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch) and visualised using the ECL system. To select for CR ligands, huCR-muFc and muCR-huFc were crosslinked to protein A sepharose using dimethyl pimelimidate (Sigma) as coupling reagent as described.[26] Rat spleen lysates (1.5 ml) were precleared using protein A sepharose (300 μl) for 2 h at 4°C. 300 μl of precleared lysate was incubated with 30 μl of huCR-muFc-sepharose or muCR-huFc-sepharose in the presence or absence of 10 mM SO$_4$-4-GalNAc, or with 30 μl of protein A-sepharose for 4 h at 4°C. After incubation beads were collected and washed three times in lysis buffer containing protease inhibitors. Bound material was released by boiling in SDS-PAGE-loading buffer, electrophoresed and transferred to nitrocellulose as before. Sn was detected as described above but using ED3 at 5 μg/ml.

Detection of CR Ligands in Human Spleen Lysates Through Enrichment and Ligand Blot Analysis

Human spleen lysate (1 ml) was precleared using protein A sepharose (300 μl) for 2 h at 4°C. In all, 500 μl of precleared lysate was incubated with 50 μl of muCR-huFc-sepharose in the presence or absence of 10 mM SO$_4$-4-GalNAc over night at 4°C. After incubation beads were collected and washed three times in lysis buffer containing protease inhibitors. Bound material was released by boiling in SDS-PAGE-loading buffer, electrophoresed and transferred to nitrocellulose as before. CR ligands were detected as described above but using huCR-muFc at 10 μg/ml.

Results

HuCR Binds SO$_4$-3-Galactose, SO$_4$-3-*N*-Acetyl-Galactosamine and GalNAc *In Vitro*

To confirm that the CR from human MR has binding properties similar to those of its murine counterpart,

binding of the chimaeric protein huCR-muFc (Figure 1a) to PAA-derived glycoconjugates containing SO$_4$-3-Gal, SO$_4$-3-GalNAc and SO$_4$-4-GalNAc or α-1-3-GalNAc was assayed as described in Materials and methods. As shown in Figure 1b human CR displayed binding properties similar to those described for its murine counterpart and preferentially recognised galactose and *N*-acetyl-galactosamine sulphated in position 3 in a dose-dependent manner. No binding to nonsulphated ligands was observed.

Detection of CR Ligands in Rat Spleen: huCR-muFc Has the Same Binding Properties as muCR-huFc *In Situ*

As a means to assess the conservation among rodents of the selective expression of CR ligands in the marginal zone, and to compare the ability of huCR and muCR to recognise sulphated sugars in tissue sections, rat spleens were incubated with huCR-muFc and muCR-huFc as described in
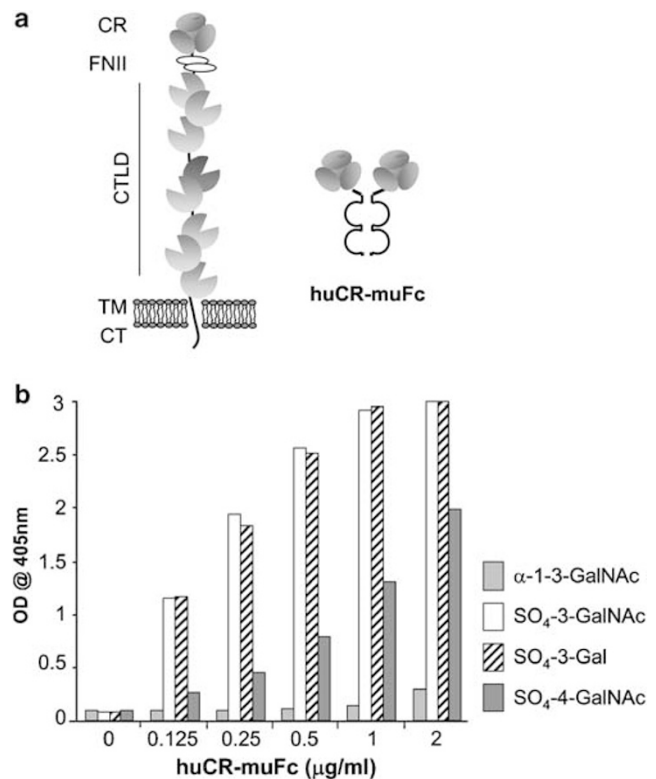

Figure 1 huCR-muFc recognises SO$_4$-3-Gal, SO$_4$-3-GalNAc and SO$_4$-4-GalNAc *in vitro*. (**a**) Schematic representation of human MR and the Fc chimaeric protein huCR-muFc. CR: cysteine-rich domain, FNII: domain-containing fibronectin type II repeats, CTLD: C-type carbohydrate recognition domain-like domain, TM: transmembrane domain, CT: cytoplasmic tail. (**b**) HuCR-muFc specifically binds the sulphated glycoconjugates SO$_4$-3-Gal-PAA, SO$_4$-3-GalNAc-PAA and SO$_4$-4-GalNAc-PAA in a dose-dependent manner. Enhanced recognition of glycoconjugates bearing SO$_4$ at position 3 was observed as described previously for muCR-huFc and sMR.

Materials and methods. As shown in Figure 2, both proteins labelled populations of cells placed in the marginal zone. These cells correspond to marginal zone Mϕ and marginal zone metallophilic Mϕ, respectively, as they express Sn shown by the labelling of serial sections with the anti-Sn mAb ED3.[16] Unlike in mice, CR ligands in rat spleen extend to the outer and inner marginal zone and do not show the restricted expression to areas of the marginal zone associated with B-cell follicles. Restricted expression of CR ligands in cells observed in the white pulp could correspond to migrated myeloid cells. No specific labelling was observed with the control proteins Emr2-muFc and human IgG1, or with the secondary detection reagent alone. Specific recognition through the lectin region of huCR was confirmed by the complete inhibition observed in the presence of soluble SO$_4$-4-GalNAc.

Detection of huCR and muCR Ligands in Rat Spleen by Ligand Blot

In mice several CR ligands can be detected in protein lysates after electrophoresis and transfer to filters. These ligands include species with rMW ranging from 240 to 100 kDa two of which have been characterised as Sn and low MW isoforms of CD45.[26] To investigate the nature of the ligands recognised by huCR and muCR in rat spleen, splenic protein lysates were electrophoresed, transferred to nitro-cellulose and incubated with huCR-muFc and muCR-huFc as described in Materials and methods. In agreement with the identical distribution of huCR and muCR ligands observed in spleen sections, huCR-muFc and muCR-huFc recognised the same ligands in rat spleen lysates by ligand blot analysis. A major band that comigrated with rat Sn (detected using mAb ED3) was observed (Figure 3a). To
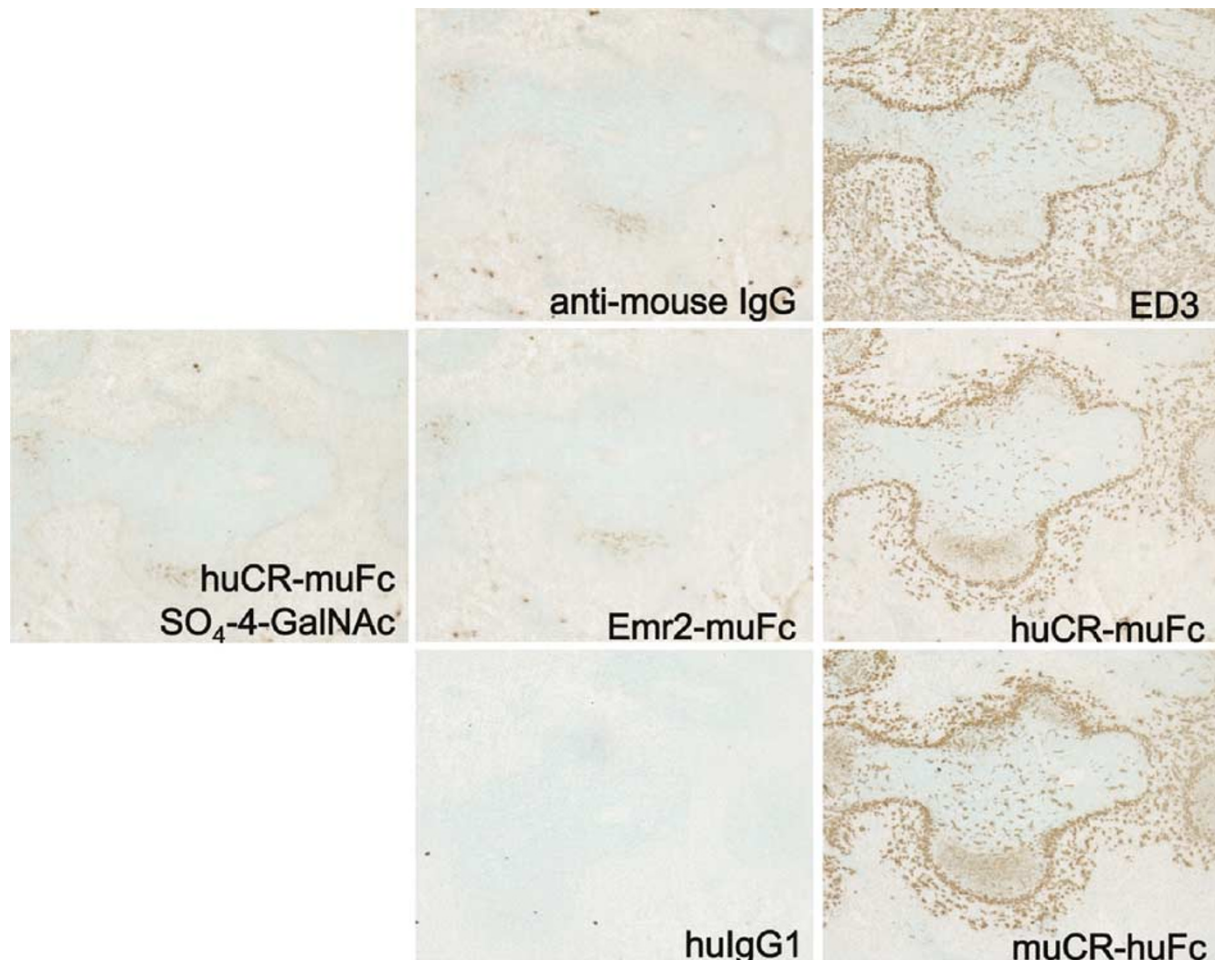

Figure 2 CR ligands can be detected using muCR-huFc and huCR-muFc in the marginal zone of rat spleen. Rat spleen sections were incubated with the chimaeric proteins huCR-muFc, muCR-huFc and the anti-rat Sn-specific mAb ED3 as described in Materials and methods. Human IgG1, Emr2-muFc and mouse IgG1 (isotype control for ED3, data not shown) were used as negative controls. HuCR-muFc binding was inhibited in the presence of SO$_4$-4-GalNAc. Identical specific labelling of cells located in the outer and inner marginal zone can be observed with huCR-muFc and muCR-huFc. These cells correspond to marginal zone and marginal zone metallophilic Mϕ based on the expression of sialoadhesin. Additional cells bearing CR ligands can also be observed in the white pulp. These could correspond to migrating cells as described in the mouse. Magnification $\times 10$.

confirm that rat splenic Sn was a counter-receptor for huCR and muCR, huCR and muCR ligands in rat spleen lysates were selected as described in Materials and methods and the presence of Sn in these preparations was tested by Western blot. Dependence for sulphated sugar recognition was determined by performing the selection in the presence or absence of soluble SO_4-4-GalNAc. As shown in Figure 3b two bands immunoreactive with the anti-Sn mAb could be detected in the absence of inhibitor. The lower band (labelled with an arrow) colocalised with the band recognised by this mAb in total protein lysates. The upper band (labelled with an arrowhead) could correspond to a protein complex containing Sn. This was also observed in

the case of mouse Sn selected based on its ability to bind muCR.[26] A band with a similar rMW was observed in the ligand blots presented in Figure 3a (labelled with a star). These results reinforce the similarity between the mouse and rat system and demonstrate that the detection of CR ligands by ligand blot using huCR-muFc is feasible.

HuCR-Ligands can be Detected in the Red Pulp of Human Spleen

The marginal zone of human spleen lacks the classical marginal zone-associated Mϕ characterised in rodents and it was of interest to investigate which cell population in this organ, if any, would display the sulphated ligands recognised by MR. Once we had determined that the huCR-muFc protein provided a suitable probe for *in situ* studies, we investigated its binding to normal human spleen. As shown in Figure 4a, a distinct pattern could be observed in which sinuses occupying the entire red pulp were recognised by huCR-muFc. Specificity was confirmed by the lack of labelling observed with Emr2-muFc (data not shown) and the inhibition of huCR-muFc binding in the presence of SO_4-4-GalNAc. Though CR ligands could be consistently found in three independent spleen samples, their detection was favoured by tissue permeabilisation and overnight incubation with huCR-muFc. We were unable to detect CR ligands in human spleen lysates by ligand blot analysis under conditions in which the ligands present in rat spleen were readily detectable (Figure 5a). To increase the level of detection we selected CR ligands from human spleen lysates using muCR-huFc coupled to protein A and probed them with huCR-muFc (Figure 5b). This analysis revealed the presence of three putative CR ligands in human spleen lysates that were absent when the selection was performed in the presence of
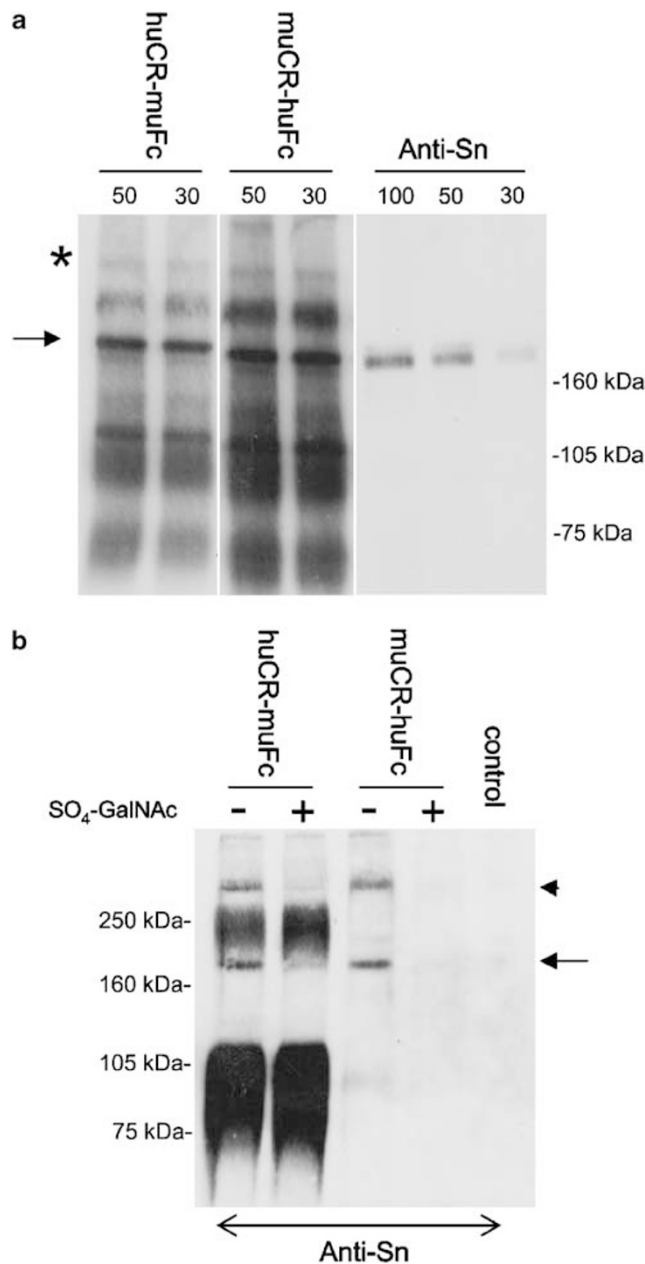

Figure 3 Analysis of CR ligands in rat spleen lysates. (a) HuCR and muCR recognise the same ligands in rat spleen lysates. Different amounts of protein lysates from rat spleen were transferred to nitrocellulose and incubated with huCR-muFc or muCR-huFc (50 and 30 μg) or with anti-Sn mAb (100, 50 and 30 μg) as described in Materials and methods. HuCR-muFc and muCR-huFc recognised a major band situated between the 160 and 250 kDa MW markers (see arrow) that could correspond to Sn as it colocalises with the band recognised by the anti-Sn mAb. Other minor species with rMW above 50 kDa were also detected. No binding of the secondary reagent used for detection was observed (data not shown). (b) Sn is present in preparations of huCR and muCR ligands. HuCR and muCR ligands were enriched in the presence (+) or absence (−) of SO_4-4-GalNAc as described in Materials and methods. Selected proteins were analysed by Western blot using anti-Sn mAb. Two bands immunoreactive with this mAb (marked with arrow and arrowhead) were detected in the absence of inhibitor. Additional bands present in the preparation of huCR ligands in the presence and absence of inhibitor could correspond to huCR-muFc eluted from the sepharose as they were recognised by the anti-mouse IgG reagent used for detection (data not shown). Control line contained proteins selected using protein A sepharose.
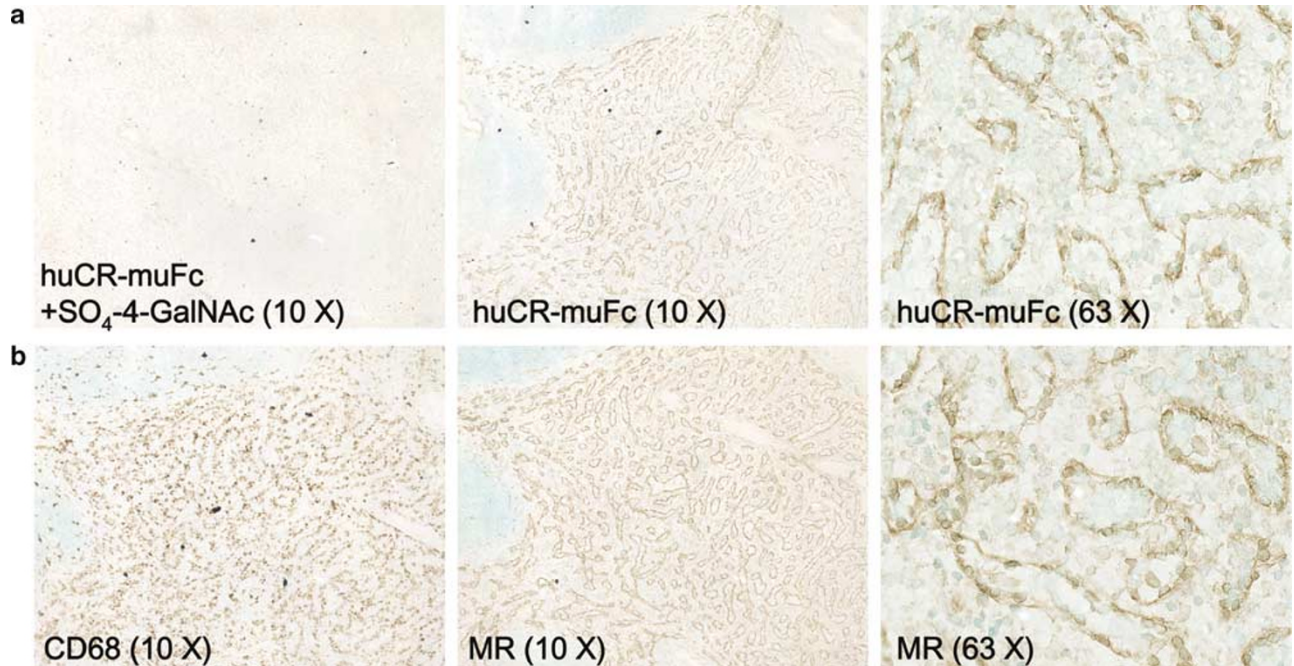
Figure 4 Analysis of CR ligands and MR distribution in human spleen by immunohistochemistry. (**a**) Detection of CR ligands in normal human spleen. Human spleens were incubated with 5 μg/ml of huCR-muFc and Emr2-muFc (data not shown) in the presence or absence of SO_4-4-GalNAc and binding was detected as described above. Specific labelling was observed in the red pulp of the spleen of structures resembling venous sinuses. Similar results were obtained using 2.5 and 10 μg/ml (data not shown). (**b**) Analysis of MR distribution in normal human spleen. Distribution of MR in human spleen was analysed using the anti-MR mAb 15.2.2. MR expression was largely confined to the red pulp to structures similar to those containing CR ligands. No similarities with the distribution of the Mϕ-restricted marker CD68 were observed.

SO_4-4-GalNAc. Two of these displayed a very high rMW which would suggest that the major counter-receptors for CR in human spleen are large molecules or form SDS-resistant complexes. The third CR ligand migrated just below the 75 kDa MW marker.

MR is Absent in CD68^{+} Mϕ in Human Spleen, but is Highly Expressed by Lyve-1^{+} Cells Lining Venous Sinuses where it Colocalises with CR Ligands

In order to investigate the functional relationship between MR and CR ligands in human spleen, we investigated MR distribution in this organ and compared it to that of CD68 (macrosialin), a lysosomal-associated glycoprotein abundant in Mϕ. When the expression of both markers was compared in serial sections it was clear that both receptors were present in completely different cell populations. Furthermore, MR distribution closely resembled the pattern observed for CR ligands as it was found in cells lining venous sinuses in the red pulp (Figure 4b). These results were confirmed by double immunofluorescence as no colocalisation was observed between MR and CD68 in the splenic red pulp parenchyma. Detailed analysis of the sections showed the presence of very few MR^{+}CD68^{+} cells with typical Mϕ morphology. In

contrast to the string-like labelling observed in the sinus-lining cells, MR in these Mϕ had a distinct endosomal distribution in agreement with its role as an endocytic receptor. In contrast to CD68, a perfect colocalisation was observed between the lymphatic endothelial marker Lyve-1[27] and MR supporting the characterisation of these MR^{+} cells as sinus-lining cells (Figure 6). Fluorescence labelling was also employed to determine if the resemblance between MR and CR ligand-distribution observed in bright field was due to colocalisation. Results shown in Figure 7 demonstrate that CR ligands and MR colocalise in red pulp venous sinuses, in the area of the cell facing the splenic parenchyma suggesting a role for this interaction in cell adhesion. It is noteworthy that the MR^{+}CD68^{+} cells observed within the sinuses do not contain CR ligands (Figure 6). No expression of collagen I or III was found associated with MR (data not shown).

MR in Human Resembles the Cell-Associated form of MR Expressed by Human Monocyte-Derived Mϕ

In the mouse, a soluble form of MR (sMR) has been suggested as the counter-ligand for the CR ligands observed in the marginal zone as it is thought that under some circumstances, sMR present in serum could be targeted to these cells. To investigate if the
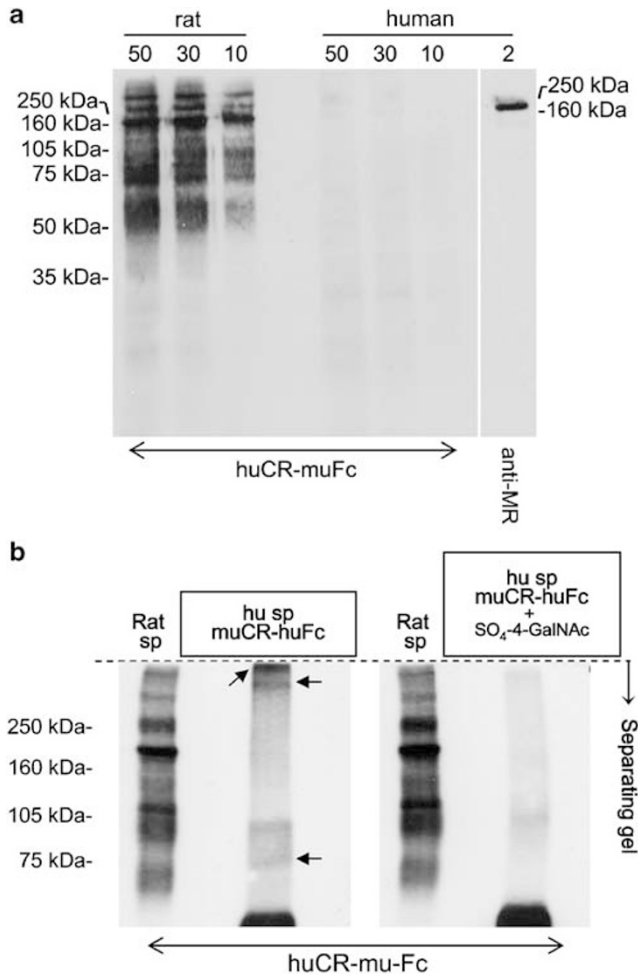
Figure 5 Characterisation of CR ligands in human spleen. (**a**) HuCR ligands in human spleen were not detected by ligand blot analysis. Different amounts (10, 20 and 50 μg) of protein lysates from human and rat spleen were transferred to nitrocellulose and incubated with huCR-muFc as described. Specific binding of huCR-muFc to rat, but not human spleen lysates was observed. Integrity of the protein preparation was demonstrated by the detection of full-size MR in 2 μg of lysate (anti-MR). No additional ligands were found at the lower MW range in the rat lysate. (**b**) Detection of huCR ligands in preparation of human spleen enriched for CR ligands. MuCR ligands in human spleen lysate were selected in the presence or absence of SO$_4$-4-GalNAc as described in Materials and methods. MuCR-huFc was used because of the high background obtained when using huCR-muFc-sepharose, probably due to inefficient crosslinking of the mouse Fc to protein A. Enriched products were analysed by ligand blot using huCR-muFc. Three putative CR ligands (labelled with arrows) were observed in the absence of competitor.

colocalisation of MR with CR ligands could be due to the trapping of sMR in the sinuses, comparison of the rMW of human splenic MR with that of cMR and sMR produced in human Mϕ cultures was performed by Western blot analysis. As shown in Figure 8, no sMR could be detected in splenic lysates even at high protein concentrations suggesting that the bulk of MR observed in human spleen corresponds to cell-associated receptor.

Discussion

In this manuscript, we describe the distribution of the MR and its CR ligands in human spleen. The results we have obtained can be summarised as follows: (i) MR is widely expressed in the red pulp of human spleen but is not present in red pulp CD68$^+$ Mϕ. It is located in cells lining venous sinuses that express the nonvascular endothelial marker Lyve-1; (ii) MR is absent from the human splenic white pulp suggesting lack of expression by splenic dendritic cells at least under normal conditions; (iii) the CR of human MR recognises SO$_4$-3-Gal, SO$_4$-3-GalNAc and SO$_4$-4-GalNAc *in vitro* and marginal zone-associated ligands in rat spleen *in situ*; (iv) CR ligands colocalise with MR in the venous sinuses.

We consider it highly likely that the ligands recognised by the CR domain in rat and human spleen correspond to sulphated carbohydrates based on the following observations: (a) Under no circumstances has a nonsulphated ligand been described for CR.[7] (b) Crystallographic studies have determined the residues in muCR required for sulphated carbohydrate binding.[4] Sequence analysis demonstrated that all these residues are conserved in huCR. (c) The interaction between muCR and sulphated sugars takes place through a neutral binding pocket where soluble monomeric SO$_4$-4-GalNAc would bind. Therefore, it is unlikely that this reagent would block hypothetical additional binding sites through steric hindrance. (d) CR does not undergo conformational changes upon interaction with sulphated carbohydrates.[4] Therefore, it is unlikely that a second binding site could have been eliminated through conformational changes induced by our inhibitor. (e) Our results indicate that muCR and huCR have the same binding specificity: (e.1) the sugar-binding assay shown in Figure 1, is consistent with data published for muCR, (e.2) the distribution of muCR-huFc and huCR-muFc ligands in rat spleen sections is identical (Figure 2), (e.3) muCR-huFc and huCR-muFc recognised the same bands in rat spleen protein lysates by ligand blot analysis (Figure 3a) and (e.4) muCR-huFC and huCR-muFc shared the ability to interact with rat Sn in a sulphate-dependent manner (Figure 3b).

The Marginal Zone in Rodents and Humans

Using rat spleen as a model to validate the probe used to search for huCR ligands we have further confirmed the differences observed between mice and rats regarding the phenotype of marginal zone-associated Mϕ.[28,29] In mice only marginal zone metallophilic Mϕ express high levels of Sn and CR ligands[11,20] while in rats the expression of both markers extends to the Mϕ placed at the outer marginal zone. Further work is required to test if CR ligands are also exposed to the extracellular milieu
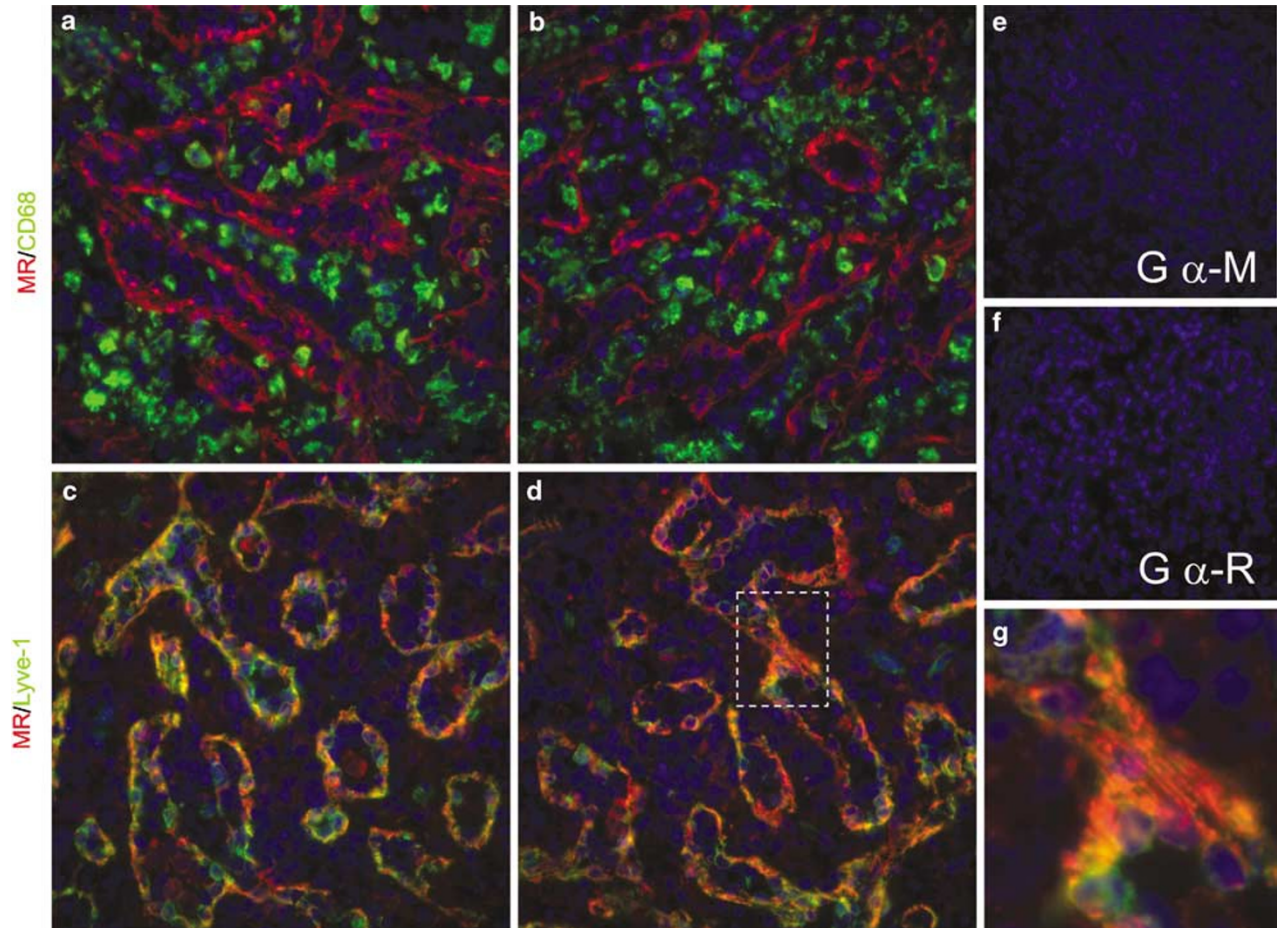
Figure 6 MR is present in Lyve-1^{+} cells lining venous sinuses in human spleen. MR-specific fluorescent labelling in human spleen (in red) does not colocalise with CD68-specific labelling (in green, (**a**) and (**b**)) but colocalises with Lyve-1-specific labelling (in green, (**c**) and (**d**)). (**e**) and (**f**) show labelling obtained with anti-mouse IgG (G α-M) and anti-rabbit IgG (G α-R) secondary antibodies. Inset (**g**) shows detail of (**d**) in which putative ring fibres double labelled with MR and Lyve-1 can be observed.

in rats[21] and how their expression relates to MR distribution. We have been unable to detect CR ligands in any area surrounding the white pulp in human spleen. Even though Sn expression has been described in Mφ located in the perifollicular zone,[16] our results suggest that in these cells it lacks the post-translational modification recognised by human CR.

The Role of MR and CR Ligands in Venous Sinuses

MR is present in most Mφ populations and in liver sinusoidal cells of the mouse where it is predicted to mediate clearance of endogenous and pathogen-associated molecules. MR expression has also been observed in nonvascular endothelia in mouse lymph nodes and spleen.[11] Little information is available regarding the role of MR in these endothelial-like cells. In human lymph nodes a role has been proposed for MR expressed by lymphatic endothelia in cell adhesion through an interaction with L-selectin on T cells.[30] Further work is required to understand how this receptor, mostly located in the endosomal compartment in Mφ and cultured cells (NIH3T3 and CHO cells), could mediate selective cell adhesion. These studies should include the analysis of the cellular biology of MR in endothelial-like cells. A role for MR in cell adhesion is further supported by its newly recognised ability to bind collagens through its FNII domain (Martinez-Pomares *et al*, manuscript in preparation). In this regard, it is noteworthy that no colocalisation of MR and collagens I and III has been observed in human spleen (data not shown).

Preliminary analysis of the cellular distribution of MR within venous sinus-lining cells indicated a fibrillar distribution that in some cases was associated with structures that resemble ring fibres where Lyve-1 was also present (see panel g in Figure 6). We consider two possible explanations for the presence of CR ligands colocalising with MR in venous sinuses. First, that the sulphated carbohydrates recognised by CR, such as chondrotin sulphate,[7] are located in the specialised basal membrane to which the endothelial cells attach.

huCR-muFc/MR

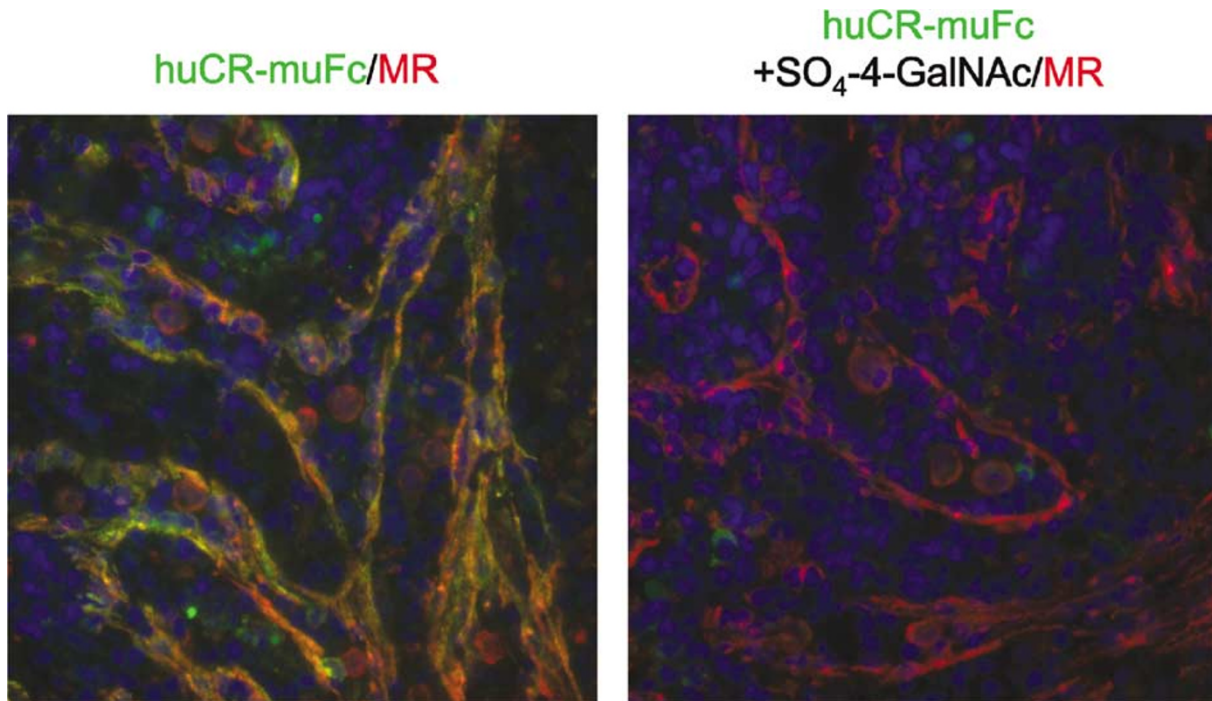## huCR-muFc +SO$_4$-4-GalNAc/MR

Figure 7 MR- and CR ligands colocalise in venous sinuses of splenic red pulp. Fluorescent labelling of CR ligands (in green) and MR (in red) in the presence or absence of SO$_4$-4-GalNAc demonstrates that there is a perfect colocalisation of the MR and ligands for the CR in venous sinuses.
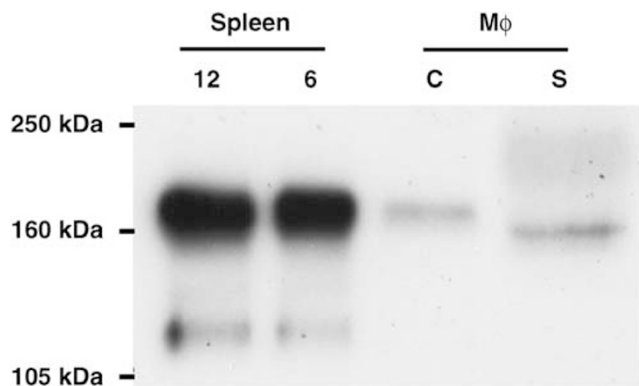

Figure 8 Splenic MR has the same rMW as cMR produced by human Mϕ. Protein lysates (12 and 6 μg) of human spleen were electrophoresed alongside lysate (14 μg) and supernatants (45 μl of 9 cm^2 dish containing 2 ml media with 2% autologous human serum) of cultured human monocyte-derived Mϕ and transferred to a nitrocellulose filter. Cell-associated and soluble MR were detected in Mϕ cell lysate and supernatant, respectively. Splenic MR comigrated with the cell-associated form of MR present in Mϕ.

The uniqueness of this structure is exemplified by the restricted expression of collagen XVII in the ring fibres together with the integrin subunit α6.[31] This pattern of expression suggests the formation of a firm hemidesmosome-like contact site between the intermediate filaments of the sinusoidal endothelial cells and the ring fibre matrix. Second, the presence of these CR ligands could be due to MR-mediated

uptake of sulphated ligands from the circulation. Detection of CR ligands was consistent but low probably due to low abundance or limited accessibility as the sulphated sugars recognised by our probe could already be interacting with the CR domain of the endogenous cell-associated MR. The ligand blot analysis shown in Figure 5b suggests that MR itself is not a CR ligand as no ligand with a rMW of 175 kDa was detected by our analysis. Intriguingly, a minor band with an approximate rMW of 70 kDa was observed. This mobility is similar to that displayed by glycosylated Lyve-1 in SDS-PAGE and raises the possibility of an interaction between MR and Lyve-1 in human spleen.

Absence of MR in Human Red Pulp Mϕ

In humans, MR expression in myeloid cells is supported by its detection in cultured Mϕ and dendritic cells *in vitro* and in myeloid populations in dermis and tonsils. Therefore, it was unexpected to find that red pulp CD68$^+$Mϕ in human spleen did not express this receptor.

Mannosylation has been used as a means to deliver glucocerebrosidase to putative Mϕ in Gaucher patients.[32] Our results indicate that, at least in normal spleen, it is unlikely that MR mediates enzyme uptake by red pulp Mϕ. These observations are in agreement with the low MR expression observed in Gaucher's cells,[33] which suggest that other mannose receptors and/or cells could be

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involved in the internalisation of mannosylated glucocerebrosidase.

In summary, our results provide evidence for an interaction between cell-associated MR with its sulphated sugar ligands in cells lining venous sinuses in human spleen. Venous sinusoidal cells have a phenotype intermediate between endothelial and haematopoietic cells and express low levels of CD31, thrombomodulin and the alpha chain of CD8. It is unclear if these cells provide any means of selectivity regarding the cellular and/or soluble blood components of host or pathogen origin that will be retained in the splenic parenchyma beyond the physical restriction determined by the size and or shape of the intercellular slits. The presence of MR and its ligands at this unique anatomical site suggests a role for this receptor in the interaction with mannosylated and/or sulphated blood components that could modify the adhesion properties of the endothelial cells and alter their filtration capacity.

Acknowledgements

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