Macrophage mannose receptor on lymphatics controls cell trafficking

Fumiko Marttila-Ichihara, 1 Raisa Turja, 1 Mari Miiluniemi, 1 Marika Karikoski, 1, 2 Mikael Maksimow, 1 Jussi Niemelä, 1 Luisa Martinez-Pomares, 3 Marko Salmi1 and Sirpa Jalkanen1

1MediCity Research Laboratory and Department of Medical Microbiology, Turku University and National Public Health Institute, FI-20520 Turku, Finland; 2 Turku Graduate School of Biomedical Sciences; 3School of Molecular Medical Sciences, Institute of Infection Immunity and Inflammation, Queen’s Medical Centre, University of Nottingham, NG7 2UH, United Kingdom

R.T. and Ma.M. contributed equally to this work.

Running title: Mannose receptor controls cell trafficking
Scientific heading: Vascular biology

Corresponding author: Sirpa Jalkanen, MediCity Research Laboratory, Tykistökatu 6A, 20520 Turku, Finland. Tel +358-2-3337006, fax:+358-2-3337000, e-mail:sirjal@utu.fi.
Macrophage mannose receptor (MR) participates in pathogen recognition, clearance of endogenous serum glycoproteins and antigen presentation. MR is also present on lymphatic vessels, where its function is unknown. Here we show that migration of lymphocytes from the skin into the draining lymph nodes through the afferent lymphatics is reduced in MR-deficient mice, whilst the structure of lymphatic vasculature remains normal in these animals. Moreover, in a tumor model the primary tumors grow significantly bigger in MR-/- mice than in the wild-type (WT) controls, whereas the regional lymph node metastases are markedly smaller. Adhesion of both normal lymphocytes and tumor cells to lymphatic vessels is significantly decreased in MR deficient mice. Ability of macrophages to present tumor antigens is indistinguishable between the two genotypes. Thus, MR on lymphatic endothelial cells is involved in leukocyte trafficking and contributes to the metastatic behavior of cancer cells. Blocking of MR may provide a new approach to controlling inflammation and cancer metastasis by targeting the lymphatic vasculature.
Introduction

Molecular mechanisms regulating lymphocyte entry from the blood into the lymphoid tissues during their recirculation are well characterized. In contrast, much less is known about the exit mechanisms of leukocytes from the peripheral tissues into the afferent lymphatics and from the lymphoid organs into efferent lymphatics, although lymphocyte and dendritic cell migration via the lymphatics is essential for controlling the nature and magnitude of immune responses. Moreover, lymphatics are important routes for metastasis of different types of cancers. For example, breast cancers and squamouscellular cancers in the head and neck area mainly spread via lymphatics. In vitro studies have suggested that MR is, in addition to macrophages, abundantly present on both afferent and efferent lymphatics. Furthermore, MR in the lymphatic tissues of human tissue sections is able to bind lymphocytes and certain types of tumor cells. However, lack of function-blocking antibodies against mouse MR and relevant gene-manipulated mice have prevented an evaluation of the role of MR in lymphocyte trafficking in vivo. Recent availability of MR-/-mice allowed us to study the role of lymphatic-associated MR.

Materials and methods

Animals

Specific pathogen free age and sex matched (6 - 16 weeks old) wild type (WT) and MR-/-mice (on the C57BL/6 background) were used in the experiments. Production of MR-/-mice has been described earlier. The experiments were approved by the Animal Care Committee of the University of Turku, permit number 1575/05 and conformed to the guidelines established by the European Union.
Flow cytometry

The phenotype of lymphocytes from WT and MR−/− mice was determined by flow cytometry. In brief, cell suspensions were prepared from the peripheral (axillary and inguinal) lymph nodes, mesenteric lymph nodes, Peyer’s patches, thymus and spleen by using mechanical teasing through a steel mesh. Erythrocytes were lysed from the spleen by a brief hypotonic lysis. Cells were stained with phycoerythrin (PE)-conjugated monoclonal antibodies against CD4, CD8, CD62L (Pharmingen). Biotinylated B220 antibody (TIB146, ATCC, Manassas, VA) followed by PE-conjugated streptavidin was used to detect B cells. Labelled cells were analyzed by FACScan (BD Biosciences).

Lymphocyte migration via lymphatic vessels

Lymphocyte migration into the draining lymph nodes was measured as described. In brief, cells from peripheral lymph nodes, mesenteric lymph nodes and spleen of WT mice were fluorescently labeled by a 30 min-incubation with 5-chloromethyl fluorescein diacetate (CMFDA, Molecular Probes). After washing, 5x10⁶ cells were injected subcutaneously into the left hind footpad of MR−/− mice and WT controls. After 13 h the recipient mice were sacrificed and lymphocyte suspensions were prepared from popliteal lymph nodes. Cells were then stained with alexa Fluor 647–conjugated anti–CD4 mAb, peridinin chlorophyll protein–Cyanine 5,5 (PerCP-Cy5,5)–conjugated anti–CD8 mAb and biotinylated B220 antibody followed by PE–conjugated streptavidin. Migration efficiency was determined as percentage of injected cells (CMFDA-labeled) within lymph node lymphocytes by flow cytometry. The 13 h migration time was pre-tested to be optimal as at
this time point the injected cells have migrated to and are dispersed throughout the draining lymph node but have not yet left it via the efferent lymphatics.

**Tumor cell migration via lymphatic vessels**

B16-F10-luc-G5 melanoma cells (Xenogen) were injected subcutaneously ($4 \times 10^5$ in 30 μl RPMI) into the left ear of 19 MR-/- and 19 WT mice. Primary tumor and metastatic growth were monitored twice a week using bioluminescence imaging as described previously. In brief, mice were anesthetized with 2.5% isoflurane (Becton Dickinson). A substrate for the luciferase (D-luciferin sodium salt, Synchem, Kassel, Germany; 150 mg/kg i.p.) was injected into mice 10 min before imaging. Photographic images were taken in the IVIS imaging station with a cooled CCD camera (IVIS; Xenogen, Alameda, CA). Signal intensities were quantified from regions of interest as photon counts using the Living Image software (Xenogen).

**Immunohistochemistry**

Acetone fixed frozen sections (5μm) of the ear and peripheral lymph nodes or lymph node metastases of the MR-/- (n=4) and WT (n=4) were stained with polyclonal rabbit anti-mouse LYVE (Relia Tech) and monoclonal anti-Langerin (CD207; eBioscience) or with negative control mAb or serum (anti-human CD44, Hermes-1 and normal rabbit serum). In addition, ears and lymph nodes of 3 MR-/- and 3 WT mice were stained with goat anti-mouse CCL21 (R&D Systems), rabbit anti-mouse S1P₁ (Cayman Chemical), rat mAb MECA32 (kind gift from E. Butcher, Stanford University, CA) and rat mAb against mouse CD169, clone 3D6, prepared in house. FITC-conjugated anti-rabbit Ig (Sigma), anti-goat
(Sigma) or anti-rat Ig (Sigma) diluted in PBS containing 5% normal mouse serum were used as second stage antibodies. The sections were analyzed using Olympus BX60 microscope. Langerin positive cells were counted in epidermis and dermis in the skin of the ears, in T cell areas of lymph nodes (since they are practically absent from the B cell follicles\textsuperscript{13}) and in primary tumors and metastases. MR expression was analyzed using MR5D3 antibody\textsuperscript{14} followed by Alexa-546 conjugated anti-rat Ig in immunofluorescence stainings and peroxidase-conjugated rabbit anti-rat Ig (Dako) in immunoperoxidase stainings. For peroxidase-conjugated secondary reagents, 3,3’-diaminobenzidine in PBS containing 0.03% hydrogen peroxide was used as a chromogen.

**Microlymphangiography**

The collecting lymphatic vessels were visualized by fluorescent microlymphangiography.\textsuperscript{15} In brief, 5 $\mu$l of FITC-dextran (MW 2,000,000; 8 mg/ml in PBS; Sigma) was injected into the subepidermal layer in the tip of both ears of 6 normal MR-/- and 6 WT mice. In addition, 6 MR-/- and 6 WT controls were inoculated with B16 melanoma cells into the ear. After 2 weeks, the animals were used for microlymphangiography as explained above. The ears were examined microscopically with an Olympus BX60 equipped with fluorescence light source (Olympus U-RFL-T). The vessel diameters were analyzed using ImageJ image analysis program (NIH, public domain). The vessel densities were calculated as % of the FITC positive area per field with the same program.
Lymphocyte proliferation assay

The antigen was prepared by lysing B16-luc cells (2x10^6 cell/ml in PBS) by five freezing-thawing cycles followed by sonication. 0.5 ml B16-luc cell lysate was injected intraperitoneally into 8 MR-/- and 8 WT mice. Four MR-/- and four WT mice were left as controls. After 7 d immunized and non-immunized mice were sacrificed and peritoneal macrophages were harvested. Spleens of the immunized mice were collected for isolation of T-cells. Spleens were homogenized and red cells were lysed using hypotonic saline. T cells (0.2x10^6) from immunized MR-/- and WT mice were co-cultured in 10:1 ratio with MR-/- and WT macrophages in round-bottom 96-well plates. Cultures containing T cells and macrophages from non-immunized MR-/- and WT mice served as baseline controls for proliferation. Co-cultures were incubated in complete medium (RPMI 1640 supplemented with 10 % FCS, 20 mM L-glutamine, 5x10^{-5} M 2-mercaptoethanol, penicillin/streptomycin) for 3 d and pulsed with ^3H-thymidine (1 μCi [0.037 MBq] per well) for the final 6 h. Cells were harvested using semi-automated plate harvester (Tomtech MACH III; Fisher Scientific, Hampton, NH) and counted with the 1450 Microbeta counter (Wallac).

In vitro adhesion and migration assays

Adhesion assays were performed on frozen sections of lymph nodes of MR-/- and WT mice as described. Briefly, mixture of lymphocytes isolated from mesenteric lymph nodes of WT mice and B16 melanoma cells were incubated for 15 min in static conditions, followed by 5 min of rotation at 60 rpm and then again 15 min without rotation at 7°C. The adherent cells were fixed in 1% glutaraldehyde. Binding of melanoma cells and lymphocytes (easily discriminated by their size) to lymphatic sinuses and high endothelial venules (HEV) was
counted and the results are expressed as number of cells bound/lymphatic sinuses or HEV (mean ± SEM).

**Detection of MR ligands in tumor cell lines**

Binding of MR-Fc chimeras to different murine tumor cell lines (F9 teratocarcinoma, EL-4 T lymphoma, 38C13 B lymphoma, LLC-1 Lewis lung cell carcinoma, B16 melanoma, SP2/0 myeloma, MC57G fibrosarcoma, TC-1 lung epithelial cell tumor) was also tested. The Fc chimeras CR-Fc, CRD4-7-Fc and CR-FNII-CTLD1-3-Fc\(^{16,17}\) (10 μg/ml in RPMI) were incubated with 10\(^6\) tumor cells. After three washes the bound chimeras were detected with FITC-anti-human Ig containing 5% normal mouse serum. The cells were analyzed with FacsCalibur (Becton Dickinson).

**Cell migration assays**

For trans-well migration assays cells from peripheral and mesenteric lymph nodes of MR -/- and WT mice were fluorescently labeled with carboxyfluorescein diacetate N-succinimidyl ester (CFSE, Molecular Probes, Oregon). Different number of cells (from 4 to 25x10\(^4\)) were resuspended in RPMI medium and placed into the upper chamber of transwell (96 wells, 3 μm pores, Neuro Probe, MD). RPMI with or without murine CCL21 (100 ng/ml, R&D), or with 50% of serum of 4 MR -/- or 4 WT mice was added into the lower chamber. After 2 h at 37°C, the upper chamber was removed and the number of migrated cells in the lower chamber was determined using Tecan Infinite M200.
Statistics

Data were analyzed by the parametric Student's t-tests. Growth curves were analyzed by repeated measures for analyses of variance. Statistical significance was set at p<0.05.

Results

MR on lymphatics regulates the traffic of both B and T cells into the draining lymph nodes

To evaluate the role of MR in lymphocyte migration via afferent lymphatics, we quantified the migration of CMFDA-labeled WT lymphocytes to popliteal lymph nodes following subcutaneous injection into the footpads of both MR/−/− and WT mice. We found that the absence of MR significantly impaired the trafficking of CD4 and CD8 positive cells and B cells into the draining lymph nodes (Figure 1A and B). In contrast, short-term in vivo homing experiments analyzing extravasation of intravenously administered lymphocytes from the blood into several lymphatic organs (peripheral lymph nodes, mesenteric lymph nodes, Peyer's patches and spleen) revealed no significant differences between the genotypes (Figure 1C). These results are compatible with the fact that blood vessels are devoid of MR (Figure 1D) and show that lymphocyte extravasation is not markedly affected in these mice. Moreover, the proportions of CD4 positive and CD8 positive T cells and B220 positive B cells in the lymphoid organs were virtually identical in MR-deficient and WT animals and even though L-selectin has been shown to be one of the ligands of MR there were no differences in the number of cells expressing this lymphocyte homing...
receptor (Table 1). These data indicate that MR is involved in the trafficking of T and B cells through the afferent lymphatics but not in lymphocyte homing via blood vessels.

**MR is responsible for tumor metastasis via lymphatics**

Many tumors spread via the lymphatic vessels. To study the role of MR in the metastatic behavior of cancer cells we employed an *in vivo* model that measures local growth and lymphatic spreading of tumor cells. This model involves the injection of melanoma cells subcutaneously into the ear which results in metastatic spread to the draining lymph nodes in the neck. By using luciferase-expressing melanoma cells, the growth of primary tumor and metastatic lesions could be assessed in real time in living animals using bioluminescence imaging (Figure 2A). In WT animals the luciferase signal (that correlates to the number of living tumor cells) from the primary tumor increased on average 8.5 fold during the 2-wk follow-up. Strikingly, we found an average increase of 89-fold in the signal in MR-deficient animals, indicating much faster growth of the primary tumors (Figure 2B). In contrast, the metastatic lesions in the draining lymph nodes were much smaller in the MR/- animals than in the WT controls at the 2 week time point (Figure 2C). Similarly the ratio between the sizes of the lymph node metastases and primary tumors was significantly smaller in MR/- than in the control mice (Figure 2D). Importantly, the overall growth of the tumor foci (primary + metastasis) was not higher in MR/- than in control mice even though the location of the growth was markedly different (Figure 2E). Collectively these data suggest that the lack of MR leads not only to faster growth of the primary tumor but also impairs the formation of metastatic lesions in the draining lymph nodes.
To study whether lymphocytes of MR-/− and control mice respond differently to CCL21 chemokine or whether sera from these animals have different capacity to attract lymphocytes we performed trans-well migration assays. In these assays we did not observe any differences between the lymphocytes or sera of MR-/− and WT mice (data not shown). These data support the idea that adhesion defects rather than chemotactic alterations are behind the impaired migration of lymphocytes and melanoma cells into the draining lymph nodes.

**Tumors of MR-/− and WT mice contain comparable number of dendritic cells**

One possible reason for the aberrant growth pattern of the tumors in MR-/− mice could be impaired migration of dendritic cells from the skin to the draining lymph nodes thereby reducing presentation of tumor antigens to lymphocytes. Therefore, we tested whether the number of dendritic cells originating from skin is altered in MR deficient mice. Non-challenged MR-/− mice and their controls had comparable numbers of dendritic cells in the epidermis and dermis of the ears when detected with anti-Langerin antibody (Figure 3A). Most Langerin positive cells were located in epidermis but occasionally they were also found in dermis. However, about 30% fewer Langerin positive cells were seen in the draining lymph nodes of MRKO mice as compared to those of WT animals, although the difference did not reach statistical significance (Figure 3B). Tumors (primary and metastases) of both genotypes had very few Langerin positive cells and these were randomly dispersed throughout the tumors in comparable numbers (Figure 3B, data from metastases are shown). Thus, the numbers of dendritic cells present in primary and
metastatic tumor foci cannot explain the aberrant growth pattern of B16 melanoma in MR-/- mice.

**Macrophages of MR-/ are able to normal presentation of tumor antigen**

As macrophage mannose receptor is present on macrophages and dendritic cells and could be potentially involved in anti-tumor immune response we analyzed the capacity of peritoneal macrophages to present tumor antigens. After *in vivo* immunization of MR-/ and WT mice *in vitro* proliferation assays were performed using different combinations of macrophages and T cells isolated from these mice. These assays showed that lack of MR does not affect the ability of *in vivo*-primed macrophages to present tumor antigens to T cells (Figure 3C).

**Normal density and morphology of lymphatics in MR deficient mice**

Since MR is present on the lymphatic endothelium, its deletion could affect the structure or fluid-collecting function of the lymphatic vasculature. Immunostaining with LYVE-1, a lymphatic endothelium selective marker, showed that the number and overall morphology of lymphatic vessels were similar in WT and MR-/ animals (Figure 4A). The expression patterns of sphingosine 1 phosphate receptor 1 (S1P₁) and CCL21, two molecules involved in lymphatic migration, were also indistinguishable between WT and MR-/ mice (Supplemental Figure 1). In addition expression of CD169/sialoadhesin, identifying macrophages in subcapsular sinus, was similar in both genotypes (Supplemental Figure 1).

Microlymphangiography with subcutaneously injected, high molecular weight fluorescent dextran also revealed no detectable differences regarding morphology,
diameter, location and density of lymphatic vessels between WT and MR-/- animals (Figure 4B-D). In tumor bearing ears, the density of the lymphatics was slightly increased. Practically all lymphatics were peritumoral as no FITC-dextran was visualized within the tumors, but again no differences were evident between the two genotypes (Figure 4B, C). Since the absence of MR did not have marked consequences to the morphology or phenotype of lymphatics, these data support the idea that the deficient trafficking of cells in afferent lymphatics in MR-/- animals is caused by lack of the binding between MR and its counter-receptor on migrating cells.

**Adhesion of tumor cells and lymphocytes to lymphatic vessels is compromised in MR-/- mice**

To directly test, whether lack of MR affects the binding of lymphocytes and tumor cells to lymphatics we performed *in vitro* adhesion assays. Although both afferent and efferent lymphatics are MR positive (Figure 5A and B), afferent lymphatics are very narrow and it is practically impossible to measure adhesion to them. Therefore, we analyzed lymphocyte and tumor cell binding to efferent lymphatic sinuses in lymph nodes. Both, tumor cells and lymphocytes avidly adhered to efferent lymphatic sinuses (Figure 5B and C). In contrast, tumor cells showed poor binding to HEV, while small lymphocytes bound relatively well to HEV in these assay conditions, which have been optimized to support binding to lymphatic sinuses. Lack of MR significantly reduced binding of both tumor cells and lymphocytes to lymphatic sinuses, but did not affect binding to HEV (Figure 5C). These data suggest that in the absence of MR the cells cannot efficiently bind to lymphatic sinuses and thus would have impaired capacity to leave the tissues.
To analyze how commonly tumor cells possess the ability to bind MR, we tested binding of MR-Fc chimeras to eight different types of tumor cell lines. Five lines (MC57G, EL-4, SP2/0, F9 and B16) showed reproducible staining with the chimera containing CRD4-7 region (stainings of F9 and MC57G are shown as examples in Figure 5D), whereas very weak or no binding at all was seen to the other three lines tested by any of the chimeras. Thus, the possibility to interact with MR is relatively common among tumor types and is not restricted to melanoma.
Discussion

This study demonstrates the importance of MR in the trafficking of different lymphocyte subsets from the periphery via the afferent lymphatics into the draining lymph nodes. Moreover, our results clearly suggest that tumor cells cannot efficiently metastasize to the local lymph nodes in the absence of MR on afferent lymphatics and consequently, tumors reach bigger sizes at the primary site of injection. In spite of being involved in Ag presentation and clearance MR does not seem to play a role in limiting the growth of the tumor as the total tumor burden (primary + metastases) does not statistically differ between MR-deficient and WT animals.

MR is present on both afferent and efferent lymphatics. Lymphatic sinuses belong to the efferent arm of the lymphatic system and are the exit sites for lymphocytes from the nodes. They are also considered as sieves for arresting cellular debris and microbes. This is well in line with the fact that, especially in the mouse, the lymph node sinuses contain an abundant MR positive macrophage population. Theoretically, it would be possible that these medullary MR positive macrophages are also able to arrest lymphocytes and cancer cells. Our studies favor the idea that MR can serve as an adhesion molecule during cell migration to lymph nodes. We have shown that both lymphocytes and cancer cells bind to lymphatic sinuses in an MR-dependent fashion in vitro. Moreover, in vivo we find no evidence for altered chemotaxis, cell motility or lymph node entrapment in MR-/- mice. Theoretically, however, it remains possible that some non-adhesion dependent function could explain the observed phenotype. In any case, we believe that cell migration to lymphatics may be a multi-step cascade reminiscent of that seen in the blood vessels and MR is only one step in the continuum of adhesive and chemoattractive events/see below).
Cells may use multiple receptors for binding to MR on lymphatic endothelial cells. L-selectin, which can bind to MR expressed on efferent lymphatic sinuses in vitro,\(^8,^{20}\) may also be utilized by L-selectin positive lymphocytes when they leave the peripheral tissues via afferent lymphatics. However, our phenotypic analysis of lymphoid tissues showed no marked differences in L-selectin expression between the WT and MR-/- animals (Table 1). These data suggest that additional receptors must exist or that lymphocytes utilizing MR-dependent and MR-independent lymphatic trafficking pathways express similar levels of L-selectin. Moreover, L-selectin negative lymphocytes and tumor cells must express an additional receptor for MR. Since MR has three main types of binding domains (a cysteine-rich domain, a fibronectin type II domain and eight C-type lectin-like domains), there are many possibilities for the counter-receptor. Sulfated glycoproteins binding to the cysteine-rich domain, and molecules with terminal mannose, fucose, N-acetylglucosamine or glucose residues binding to carbohydrate recognition domains remain the strongest candidates.\(^8,^{21-24}\)

MR belongs to pattern recognition molecules and binds various bacteria and yeast cells containing terminal mannose or N-acetylglucosamine on their cell surface. Numerous studies have reported its scavenger function during pathogen elimination, and it is thought to serve a homeostatic function by clearing endogenous glycoproteins.\(^{25-27}\) On the other hand, MR on monocyte-derived dendritic cells have been demonstrated to mediate uptake and presentation of mannosylated antigens.\(^{28,29}\) Interestingly the MR-/- mice do show impaired clearance of proteins having accessible mannose and N-acetylglucosamine residues, but they mount normal immune response to the pathogens tested.\(^{10}\) These data when taken together with our study showing normal antigen presentation of tumor antigens
in MR−/− mice, may indicate that MR is not required for antigen presentation in vivo or that its function can be compensated by other professional molecules capable of antigen presentation.

Lymphangio genesis during development and in pathological conditions in adults has been dissected in great detail during the past few years, and many of the molecules involved have been thoroughly characterized. In contrast, the molecular complexity behind the regulation of cell movement, especially that of lymphocytes, into the lymphatics remains largely unresolved although the importance of lymphatics as an active element in normal leukocyte trafficking and tumor spread is widely accepted.

Recently, several molecular interactions have been reported to contribute to dendritic cell trafficking through afferent lymphatics. Regarding lymphocyte migration within lymphatics, it is known that, besides attracting dendritic cells, chemokine CCL21 present on lymphatic endothelium guides CCR7 positive lymphocytes into the afferent lymphatics and hence to the draining lymph nodes. SIP1 expressed in many different cell types participates in lymphocyte exit from the lymph nodes and regulates emigration of thymocytes from the thymus. Current studies suggest that SIP1 controls lymphocyte trafficking by regulating endothelial permeability and/or suppressing lymphocyte chemotaxis in SIP gradients. When compared to MR, the molecules so far described to mediate either lymphocyte or dendritic cell trafficking within the lymphatics have notable differences. They are either chemotactic molecules such as SIP1, CCL21 and CXCL12 and/or expressed on a wide variety of other cell types in addition to lymphatic endothelium (e.g. ICAM-1 and VCAM-1). Most importantly, all of them are also involved in leukocyte extravasation through the vascular endothelium.
Selective expression of endothelial MR on lymphatics and its absence on blood vasculature places MR in a unique position among the homing-associated molecules, most of which only operate in the blood vasculature or are universally involved in cell trafficking.\textsuperscript{1,2,40} Therefore, while therapeutic intervention aiming at blocking the function of homing-associated molecules such as ICAM-1 and VCAM-1 could potentially affect normal cell trafficking and immune functions, inhibition of MR could selectively reduce leukocyte migration from the periphery into the draining lymph nodes during inappropriate inflammatory reactions. Furthermore, similar strategies could also be effective in reducing metastatic seeding via the lymphatic vasculature, for instance as an adjunctive therapy during cancer surgery.
Acknowledgments

This work was supported by the funding from the Academy of Finland, the Finnish Cancer Organizations, the Sigrid Juselius Foundation, the Arvo and Inkeri Suominen Foundation and the Technology Development Center of Finland.

We thank Michel Nussenzweig for donating the MR-/- mice, Tero Vahlberg, Kaisa Auvinen, Suvi Nevalainen, Jouko Sandholm, Maritta Pohjansalo, Sari Mäki and Laila Reunanen for advice and technical help, Christopher Bayliss for revising the language and Anne Sovikoski-Georgieva for secretarial help.

Authorship

Contribution: F.M.-I., R.T., Ma.M., M.K., Mi.M. and J.N. performed the experiments and contributed to the writing of the manuscript. L.M.-P., provided key reagents and contributed to the writing, M.S. designed the work and wrote the manuscript and S.J. performed the experiments, designed the work and wrote the manuscript.

Conflict-of-interest disclosure: The authors have no conflicting financial interest.
References


23. Leteux C, Chai W, Loveless RW, et al. The cysteine-rich domain of the macrophage mannose receptor is a multispecific lectin that recognizes chondroitin sulfates A and
B and sulfated oligosaccharides of blood group Lewis(a) and Lewis(x) types in addition to the sulfated N-glycans of lutropin. J Exp Med. 2000;191:1117-1126.


Figure legends

Figure 1. Lack of mannose receptor affects lymphocyte traffic into the draining lymph nodes but not lymphocyte homing to lymphoid organs from the blood. (A,B) CMFDA labeled lymphocytes were injected into the footpads of MR-/- mice and their controls. The input population and lymphocytes from the draining the popliteal lymph node were stained with antibodies against CD4, CD8 and B cells and the percentage of CMFDA positive CD4, CD8 and B cells was analyzed with a flow cytometer. (A) Representative FACS blots of injected cells (input) and migrated cells recovered from popliteal lymph nodes of one KO and one WT mouse. The blots obtained from a non-injected contralateral lymph node of a WT mouse are shown as a control (Control WT). The percentages of CMFDA positive cells expressing the indicated marker are shown in the boxes. (B) Combined results (mean ± SEM) of all mice analyzed are shown as relative migration index (number of migrated cells in WT mice is 1.0 by definition). (C) Homing of intravenously injected, CMFDA labeled lymphocytes after 4 h recirculation time into the indicated organs. The values shown are mean percentages (± SEM) of the homed cells recovered from individual organs. (D) Staining of serial sections with MECA-32 mAb (a pan vascular endothelial marker against PV-1 antigen), anti-MR mAb and anti-CD31. Arrows point to some of the blood vessels, which are positive for MECA-32 and CD31 and negative for MR. Thick arrows point to lymphatic sinuses positive for MR and CD31. Staining with a negative control antibody is shown in the insert. Bar 50 μm.

Figure 2. Lack of mannose receptor alters the behavior of B16 melanoma. (A) Representative examples of mice after subcutaneous injections of B16 melanoma cells
bearing the luciferase-containing construct into the ears. Arrows point to the signals from the primary tumors (white) and metastatic foci (yellow) in the neck lymph nodes. (B) The size of the primary tumors during the 14-day follow-up measured based on the luciferase signals. (C) The size of the lymph node metastases at the end of the experiment. (D) The ratios between the lymph node metastases and primary tumors of WT and MR-/- mice. (E) Total luciferase counts of primary tumor and lymph node metastases in MR-/- and WT mice at the end of the experiments (14 days). All values shown are mean ± SEM.

Figure 3. Number of dendritic cells and presentation of tumour antigens by macrophages are normal in MR-/- mice. (A) Langerin positive cells were counted in the ears and lymph nodes in non-tumor bearing and tumor bearing MR-/- and WT mice (n=4 in all groups). In tumor bearing mice the Langerin positive cells were counted in non-tumor containing area. The values are mean ± SEM/mm of epithelial surface of the ears. (B) Langerin positive cells in lymph nodes (LN) of non-tumor bearing mice and in lymph node metastases of mice with tumors. The values are mean ± SEM/ mm$^2$ for the lymph nodes/metastases. (C) Ability of macrophages (MO) to present tumor antigen was measured using proliferation assays. Different combinations of macrophages and T cells from MR-/- and WT mice were used as indicated in the figure. The results are expressed as mean ± SEM.

Figure 4. Lymphatics are anatomically and phenotypically normal in MR-/- mice. (A) Examples of expression of LYVE-1 in the ears and gut of normal MR-/- mice and their WT controls. Insets are negative control stainings with normal rabbit serum as the first stage
antibody. Bar 50 μm (B) FITC-dextran was used to visualize lymphatics in the ears of healthy and tumor bearing MR-/– and WT mice. Lymphatics are almost exclusively peritumoral in the tumor-bearing ears. A dashed line outlines the tumor. Bar 100 μm. (C) Density and (D) diameter of lymphatics in MR and WT mice without and with tumors. Values shown are mean ± SEM.

Figure 5. Binding of lymphocytes and cancer cells to lymphatic sinuses is compromised in MR-/– mice. (A) Double staining of the WT ear with an anti-MR antibody (MR5D3, red color, left) and anti-LYVE-1 antibody (green color, middle) shows that some of the afferent lymphatics are positive for both markers (arrows pointing out yellow lymphatics in the merge, right). Many MR+ LYVE- macrophages are also visible. Negative control staining with class-matched negative control antibodies is shown in the inset. (B) Lymphatic sinuses (dark brown) are MR positive in WT mice (arrow, left) while MR-/– mice completely lack the molecule (arrows, middle). Lymphocytes (small round cells) bind well to HEV, whereas tumor cells (big cells) show efficient binding only to lymphatic sinuses. The basement membrane outlining an HEV is marked with ……… and a lymphatic sinus is marked with ------- in this dark field microscopic micrograph. Three small lymphocytes binding with 9 tumor cells to the lymphatic sinus are pointed out by arrows. Because the adherent cells are lying on the top of the tissue section, the focus of the photograph is a compromise between the tissue and adherent cells. (C) Quantification of the binding of tumor cells and lymphocytes to lymphatic sinuses and HEV in lymph nodes of MR-/– and WT mice. The results are expressed as number of cells bound to HEV and lymphatic sinusoids (mean ± SEM, n = 6). Bars for A and B (right) 50 μm and B (left and
middle) 0.25 mm. (D) FACS histograms of F9 and MC57G tumor cell lines stained with a negative control (huIg) and different MR-Fc chimeras.
<table>
<thead>
<tr>
<th>Location</th>
<th>MR +/+</th>
<th>MR +/-</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thymus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>97±0.2%</td>
<td>97±0.3%</td>
</tr>
<tr>
<td>CD8</td>
<td>92±0.3%</td>
<td>92±0.7%</td>
</tr>
<tr>
<td>B220</td>
<td>0.4±0.2%</td>
<td>1±0.2%</td>
</tr>
<tr>
<td>CD62L</td>
<td>91±2%</td>
<td>92±1%</td>
</tr>
<tr>
<td><strong>Peripheral lymph node</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>30±2%</td>
<td>31±1%</td>
</tr>
<tr>
<td>CD8</td>
<td>30±2%</td>
<td>28±0.4%</td>
</tr>
<tr>
<td>B220</td>
<td>29±2%</td>
<td>39±7%</td>
</tr>
<tr>
<td>CD62L</td>
<td>83±2%</td>
<td>86±1%</td>
</tr>
<tr>
<td><strong>Mesenteric lymph node</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>29±2%</td>
<td>29±1%</td>
</tr>
<tr>
<td>CD8</td>
<td>24±2%</td>
<td>23±1%</td>
</tr>
<tr>
<td>B220</td>
<td>37±3%</td>
<td>39±2%</td>
</tr>
<tr>
<td>CD62L</td>
<td>83±1%</td>
<td>85±1%</td>
</tr>
<tr>
<td><strong>Peyer’s patch</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>10±0.5%</td>
<td>10±1%</td>
</tr>
<tr>
<td>CD8</td>
<td>2±0.3%</td>
<td>2±0.2%</td>
</tr>
<tr>
<td>B220</td>
<td>83±1%</td>
<td>81±1%</td>
</tr>
<tr>
<td>CD62L</td>
<td>44±3%</td>
<td>45±4%</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>12±2%</td>
<td>14±2%</td>
</tr>
<tr>
<td>CD8</td>
<td>4±1%</td>
<td>5±1%</td>
</tr>
<tr>
<td>B220</td>
<td>74±3%</td>
<td>70±2%</td>
</tr>
<tr>
<td>CD62L</td>
<td>71±5%</td>
<td>79±3%</td>
</tr>
</tbody>
</table>

Values are mean±SEM. None of the values is statistically different between the groups except B220 in thymus (p< 0.05). N=8 in each group.
Figure 1
Figure 3
Figure 5
Macrophage mannose receptor on lymphatics controls cell trafficking

Fumiko Marttila-Ichihara, Raisa Turja, Mari Miiluniemi, Marika Karikoski, Mikael Maksimow, Jussi Niemela, Luisa Martinez-Pomares, Marko Salmi and Sirpa Jalkanen