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Species-specific restriction of cell surface expression of mouse MARCO glycoprotein in murine cell lines ☆

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Abstract

The MARCO (macrophage receptor with collagenous structure) glycoprotein belongs to the scavenger receptor type family of pattern-recognition molecules produced by a subset of marginal zone macrophages in the spleen. Stimulation with LPS leads to its appearance on macrophages located at other tissue compartments. In the present work, we report its in vitro expression by various cell lines using transient and stable (lentiviral) gene delivery aimed at investigating the signaling properties of this receptor and its analysis using a novel rat monoclonal antibody against the SRCR-domain of mouse MARCO. When trying to establish stable mouse MARCO-transfectants using lentiviral transduction and other methods, we consistently found that MARCO accumulated intracellularly in various murine host cells. In contrast, such a phenomenon was not observed in non-murine cell lines. Our observations indicate the presence of an unexpected limitation of the in vitro expression of mouse MARCO glycoprotein in murine cell lines. We believe that the failure to express MARCO on the cell surface of the many murine cell lines is likely due to the absence of endoplasmic reticulum molecular chaperones needed for the correct folding and assembly of the trimeric MARCO molecule.
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The ability of the adaptive immune system to establish efficient immune responses requires the presence of organized lymphoid tissues, with a highly regulated distribution of its various stromal and hemopoietic cellular elements [1]. A substantial bulk of mobile leukocytes is continuously recirculating between various lymphoid organs, while the sessile stromal cells and a smaller fraction of hemopoietic cells remain stationed in their tissue environment. This latter group includes various macrophage cell types and a smaller subset of B cells comprising the marginal zone (MZ) of the spleen both in human and rodents [2]. These cells capture blood-borne pathogens, and launch T-independent immune responses, or to process these antigens towards the follicles for initiating T-dependent reactions. During this communication between various splenic compartments a considerable cellular re-distribution of MZ macrophages and B cells has been demonstrated to take place, regulated by various soluble factors, including chemokines and other compounds [3].

Scavenger receptors (SRs) constitute a diverse family of cell surface molecules, comprising eight different groups of transmembrane glycoproteins [4]. These membrane receptors expressed primarily by macrophages and endothelial subsets in the lymphoreticular tissues and elsewhere recognize a broad range of foreign polyanionic ligands, such as

☆ *Abbreviations:* AcLDL, acetylated low-density lipoprotein; AP, alkaline phosphatase; BSA, bovine serum albumin; cPPT sequence, central polypurine tract; FITC, fluorescein-isothiocyanate; GFP, green fluorescent protein; HRP, horseradish peroxidase; LPS, lipopolysaccharide; LTR, long terminal repeat; MAdCAM-1, mucosal addressin cell adhesion molecule-1; MARCO, macrophage receptor with collagenous structure; PBS, phosphate-buffered saline; NBT/BCIP, nitro-blue tetrazolium/bromchloro-indolyl-phosphate; SIN, self-inactivating long terminal repeat; SRCR, scavenger receptor cysteine-rich domain; TU, transforming unit; WPRE, woodchuck hepatitis virus post-transcriptional responsive element.

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microbial cell wall components of certain structural motifs (PAMPs) found in Gram-negative and Gram-positive bacteria and fungi [5–7]. As endogenous ligands, they also bind lipoproteins and modified plasma proteins, as well as phospholipids of damaged cell membrane constituents, heat shock proteins, and β-amyloid fibrils [8–10]. Furthermore, members of the SR-A family (including SR-AI, SR-AII, SR-AIII, MARCO, and SRCL, respectively) may be involved in mediating the adhesion of activated B cells, and also in the binding of macrophages to the extracellular matrix [11,12]. Their absence in KO mice led to the aberrant formation of MZ, manifested in the reduced number and scattered distribution of MZ macrophages expressing SIGN-R1, a C-type lectin-like membrane glycoprotein [13]. In addition, their contribution in various diseases associated with disturbed metabolism such as in hyperlipidemias and diabetes has further emphasized their role in tissue homeostasis and regeneration, thus expanding their role beyond immunological functions [4,14,15].

The engagement of class A SR family members by their various ligands induces a broad array of cellular responses, including the tyrosine phosphorylation of phospholipase C-γ1 (PLC-γ1) and phosphatidylinositol 3-kinase (PI 3-kinase), the activation of signaling pathways involving protein kinase C (PKC), heterotrimeric $G_{(i/o)}$ proteins, mitogen-activated protein kinases (MAPKs), caspases, and cytokine secretion [16,17]. As the macrophages possess an extensive set of other non-SR-type receptors capable of binding similar microbial ligands (such as TLRs and CD14), the dissection of signaling events elicited by the engagement of various members of the SR group requires the use of monoclonal antibodies directed against individual SRs, and also cells expressing SRs as target antigens [18].

In this paper, we report the production, epitope analysis and functional characterization of a novel rat monoclonal antibody against the mouse MARCO molecule, as well as the constitutive expression of this glycoprotein using lentiviral vectors in a range of various human and murine cell types. Our results indicate that while the lentiviral approach can successfully be applied to express MARCO, its cell-surface display is severely limited in murine cells with a strong bias for intracellular retention, which may indicate a strict regulation of expression.

Materials and methods

Mice. Inbred female BALB/c mice aged between 8 and 12 weeks were obtained from the University's SPF Animal Facility and were kept under conventional conditions with rodent chow and water ad libitum. For LPS effects, the mice were injected intraperitoneally with 50 μg LPS (serotype O55:B5, purified from *Escherichia coli* and dissolved at 0.5 mg/ml in PBS), followed by the sacrifice of the animals 16 h later. When studying the in vivo effects of the IBL-12 mAb for the mobilization of the marginal zone B cells, 100 μg protein G-purified IgG was administrated intravenously and the mice were sacrificed 2 h later. The spleen was removed from mice killed by cervical dislocation, in accordance with the guidelines set out by the Committee on Animal Experimentation of the University of Pécs.

Cell lines. J774.1 and $P388D_1$ macrophage cells, EL-4 T-lymphoma cells, WEHI-3b myelomonocytic cells, and NIH 3T3 and L-929 fibroblasts (all murine) and diploid Jurkat human T-cell leukemia were obtained from ATCC. PC12 rat pheochromocytoma cells were generously provided by Dr. József Szeberényi (Department of Medical Biology, University of Pécs). U-937 human macrophage cell line was kindly provided by the Regional Laboratory of Virology, Baranya County Institute of State Public Health Service, Pécs. The cells were maintained in Dulbecco's MEM containing 10% FCS.

Hybridoma production and monoclonal antibodies. The IBL-12 (rat IgG1/κ) hybridoma was produced in our lab as part of an extensive project for mAb production against mouse splenic stromal components using intact white pulp as antigen by standard PEG-mediated fusion [19] and references therein. For multiple labeling the IBL-12 mAb was purified on a protein G affinity column using FPLC, and conjugated with *N*-hydroxy-sulfo-succinimidobiotin ester (Sigma–Aldrich Kft, Budapest) or FITC (Sigma–Aldrich). The unlabeled rat anti-mouse MAdCAM-1 antibody (clone MECA- 89 from BD Pharmingen), ED31 anti-MARCO antibody (hybridoma supernatant kindly provided by Dr. Georg Kraal [20]), or IBL-13 mAb was detected using PE-conjugated goat anti-rat IgG (BD Pharmingen) mAb. The binding of the biotinylated IBL-12 mAb was visualized with FITC-streptavidin (DAKOCytoMation, Budapest). The residual free binding sites of the PE-labeled anti-rat IgG were saturated using 20% normal rat serum in PBS prior to the addition of the biotinylated IBL-12 mAb. For flow-cytometry the IBL-12 mAb was used as an undiluted hybridoma supernatant containing 0.1% Na-azide. The bound antibody was detected by PE-labeled goat anti-rat IgG. As control, normal rat IgG purified from rat serum was used at the concentration of 10 μg/ml.

The immunohistochemical detection of MZ B cells in mice treated with the IBL-12 mAb was performed with FITC-conjugated rat anti-mouse B220/CD45RA mAb (clone RA3-6B2, BD Pharmingen), visualized by HRP-conjugated sheep anti-fluorescein IgG Fab (Roche) in conjunction with biotinylated anti-MAdCAM-1 (BD Pharmingen) antibody and streptavidin-AP.

For the staining of IgM-producing cells goat anti-mouse IgM peroxidase conjugate (Zymed) was used in combination with a three-step indirect immunohistochemical detection of marginal zone macrophage subsets using streptavidin-AP as tracer.

For the flow-cytometric quantification of MZ B cells and follicular B cells in the spleen of IBL-12-treated mice, a cocktail of FITC-conjugated anti-B220, PE-labeled anti-CR1/2 (CD35/CD21, 7G6 mAb), and biotinylated anti-CD23 mAb (clone B3B4) was used, followed by incubation with streptavidin-CyChrome, in the presence of 2.4G2 mAb (all purchased from BD Pharmingen) to inhibit FcγRII-mediated non-specific binding.

Immunohistochemistry, immunofluorescence, and flow-cytometry. The initial screening of hybridoma cultures on acetone-fixed frozen sections from spleen was performed as previously described [19]. For IgM detection, the slides were first processed for anti-marginal zone macrophage mAb staining with AP development, followed by endogenous peroxidase inhibition and incubation of sections with HRPO-conjugated goat anti-mouse IgM.

For dual immunofluorescence, the slides were first incubated with unlabeled monoclonal antibodies (anti-MARCO/ED31 or IBL-13), then developed with PE-conjugated goat anti-rat IgG. After thorough washing the residual binding sites were blocked with diluted rat serum for 20 min, followed by the addition of biotinylated IBL-12 or other secondary rat mAb, detected with FITC-streptavidin conjugate.

When staining for intracellular MARCO in transfected cells, the cells were cytospinned (Shandon cytospin), allowed to dry at room temperature, and fixed in chilled acetone. After blocking with 5% BSA the immunofluorescent staining was performed using IBL-12 supernatant as first-layer antibody, followed by PE-conjugated goat anti-rat IgG. To determine the frequency of positive cells, the nuclei were counterstained with Hoechst 33342. The specimens were viewed under an Olympus BX61 fluorescent microscope. The acquisition of digital pictures with a CCD camera and the morphometric image analysis were performed using the analySIS software.

The analysis of surface MARCO expression by cell lines and the frequency of MZ and follicular B cells were determined by flow-cytometry. Cell lines transfected with lentiviral constructs or unmanipulated controls were collected in PBS containing 0.1% BSA and Na-azide by centrifugation at 800*g* for 10 min at 4 °C. The pellet was resuspended in cold PBS–BSA and adjusted to 5×10^6 cells/ml. From this cell suspension, 50 μl was added to 200 μl of undiluted IBL-12 hybridoma supernatant and incubated for 20 min on ice with repeated vortexing. After washing, the antibodies were detected with PE-conjugated anti-rat IgG mAb. Prior to intracellular labeling the cells were harvested, washed once in PBS and then centrifuged. The cell pellet was resuspended in 200μl of 4% paraformaldehyde solution then incubated for 30 min on ice. The fixated cells were then washed once in PBS and twice in PBS containing 0.1% saponine followed by the addition of 4 μg/ml IBL-12 IgG in PBS-10, 1% saponine. Following incubation for 30 min on ice, the cells were washed twice with PBS/saponine. The secondary antibody (PE-conjugated anti-rat IgG mAb) was used at 1/200 dilution in PBS/saponine. Controls for the cytoplasmic MARCO labeling included normal unlabeled rat IgG at 10 μg/ml as well as untransfected parental cells. After an incubation of 30 min on ice, the cells were washed twice in PBS/saponine and once in PBS buffer. A final fixation was performed using 1% paraformaldehyde before analyzing the cells by flow-cytometry. The labeling procedure for all flow-cytometric analyses was repeated in at least three independent experiments.

Transient MARCO expression and ligand-binding studies. The pcDNA3 mammalian expression vector encoding the full-length murine MARCO [21] was transfected into PC12 rat phaeochromocytoma cells, NIH 3T3 murine fibroblasts, and Chinese hamster ovary (CHO) cells. Plasmid DNA was prepared by Endotoxin-free Ultrapure Plasmid Purification Kit (V-gene). For transfection, 10 μg plasmid DNA was introduced onto adherent cells on 10-cm plates using the calcium-phosphate method. Binding of DiI-labeled AcLDL (Molecular Probes; used at a concentration of 3 μg/ml) and FITC-labeled *E. coli* (Molecular Probes) was tested as described [22], except that the test solutions contained also 5 μg/ml IBL-12 or an irrelevant control rat mAb. Cells were also preincubated for 15 min in a humidified atmosphere with 5% CO_2 at 37 °C in the antibody-containing solution before adding the test solution.

Lentiviral plasmid constructs. All transfer plasmids were generated from the pWPTS plasmid family [23] containing the EF1α promoter. Monocistronic constructs were embedded in the viral LTR frame with a SIN element, a cPPT sequence, followed by an intronless EF1α promoter, the transgene of interest and a WPRE element. For the bicistronic vector, sMARCO (Igκ leader followed by a fragment encoding the extracellular part of mouse MARCO) was inserted before IRES, while GFP was placed downstream of this segment [24,25]. Other elements of the vectors were not affected.

Production of lentiviral vectors. Late second generation lentiviral vectors were produced as published previously [26]. Briefly, an envelope construct (pMD.G), a packaging plasmid (R8.91), and a transfer plasmid (pWPTS based constructs) were transiently co-transfected by calcium-phosphate method into 293T cells pretreated with chloroquine (1 μM final concentration). Following an overnight incubation and medium change, the supernatant of the virus producer cells was harvested 24 h later, centrifuged (2000 rpm, 10 min, 4 °C), and filtered (0.45 μm pore-size PVDF-coated filters) to eliminate rough cellular debris. The supernatants were aliquoted and stored at −80 °C until utilization.

Transfection of cell-lines with lentivectors. Target cells were transduced by spinoculation [27]. Briefly, viral supernatants were thawed and spun in 24-well plates (3600 rpm, 60 min, 4 °C). The supernatant was discarded and 20,000 cells were carefully layered into the wells in 2 ml fresh medium (Dulbecco's MEM with 10% FCS). Cells were first analyzed 3 days following transfection. Viral supernatants were similarly titrated on Jurkat cells, viral titers were approx. 10^5 TU/ml for all preparations. The amount of viral supernatant necessary for transfection varied depending on the type of target cells. For most cells (Jurkat, WEHI-3b, EL-4, P388D_1, and L-929) MOI = 1 was sufficient (MOI: multiplicity of infection or virus/cell ratio), for others (J774.1, U-937) MOI = 10 was necessary, perhaps due to phagocytosis-mediated viral degradation.

Results

IBL-12 mAb reacts with the mouse MARCO macrophage surface antigen

Our initial screening of approximately 950 rat hybridoma cultures [19] revealed two samples reactive with marginal zone macrophages, showing a restricted labeling pattern against two distinct subsets located at the boundary between the white pulp and the red pulp (Figs. 1A and B). Of these, the IBL-12 mAb-positive region appeared to overlap with the peripheral rim of white pulp containing the IgM^{hi} positive marginal zone B-cell area, whereas the IBL-13-positive cells positioned more centrally in the white pulp seemed to form a loose boundary between the IgM^{med} follicular B cells and the MZ B cells (Figs. 1C and D). Furthermore, upon LPS administration, a strong upregulation of IBL-12 reactivity was evident in the red pulp, whereas the IBL-13-positive cells migrated into the follicular area of the white pulp (Figs. 1E and F). Taken together, these morphological observations indicated that the antigen recognized by the IBL-12 mAb may be related to, or identical with, MARCO, a previously described member of the scavenger receptor family expressed by marginal zone macrophages [28,29]. Also, when staining spleen sections first with the anti-MARCO mAb ED31 as a reference antibody, followed by that with IBL-12, a near-complete overlapping of staining patterns was detected. This finding confirmed our hypothesis that the two markers are expressed on the same cells. The simultaneous dual labeling of the cells also indicated that the mAbs are probably directed against different epitopes displayed on the same cell (Figs. 1G–I).

To confirm the MARCO-specificity of the IBL-12 mAb, PC12 rat pheochromocytoma cells were transiently transfected with a eukaryotic expression plasmid encoding full-length murine MARCO. The transfected cells reacted with the IBL-12 mAb, whereas the mock-transfected controls remained negative (Fig. 2A). Furthermore, similarly to previous studies employing ED31 [30], upon in vivo administration of the IBL-12 mAb into young adult mice a near-complete mobilization of MZ B cells could be observed, without their elimination [Supplemental material]. These results indicate that similar in vivo effects can be generated by using two different mAbs directed against non-overlapping epitopes of the MARCO antigen.

IBL-12 mAb binds to a conformational epitope in the SRCR domain of mouse MARCO and affects its ligand-binding activity

To localize the IBL-12-binding epitope on MARCO, we utilized various truncated forms of this receptor. We found that the antibody did not recognize transient CHO-transfectants expressing a C-terminally truncated form of MARCO lacking the SRCR domain (a stop codon introduced after the codon for serine 419, the last residue of the collagenous domain), indicating that the epitope resides

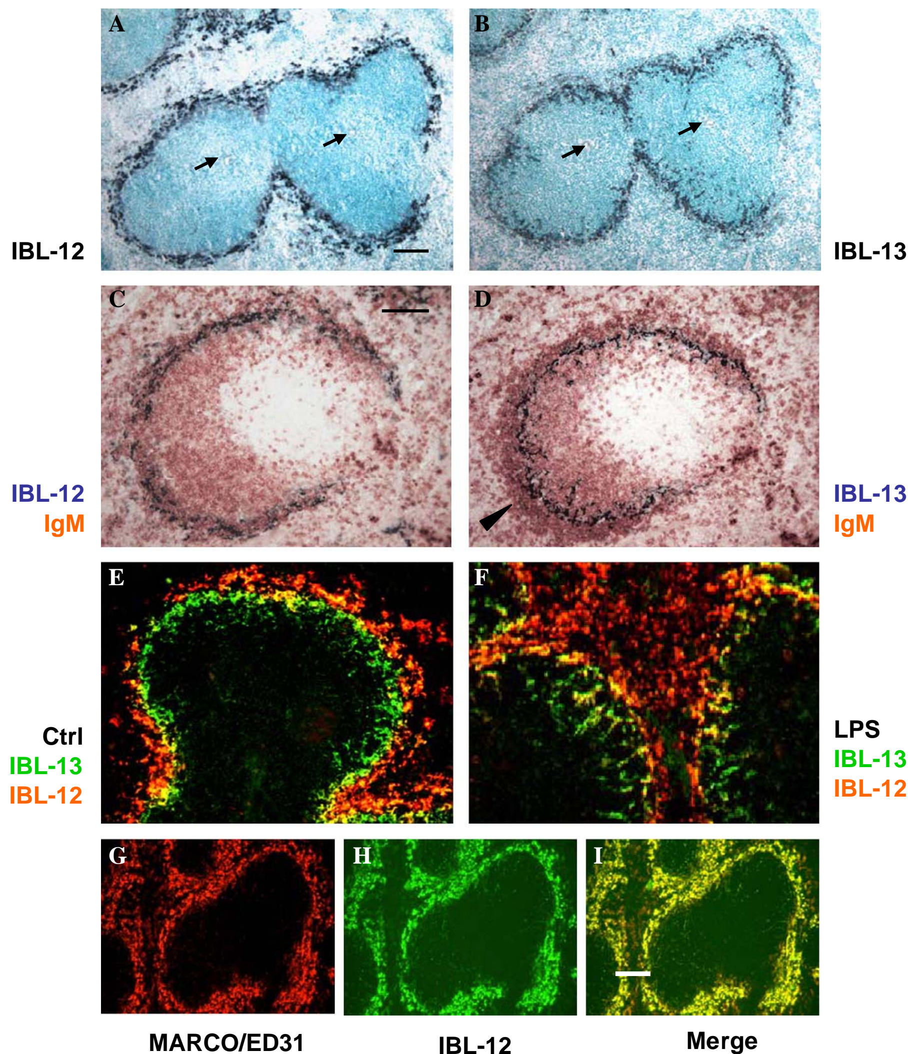

Fig. 1. Marginal zone macrophage heterogeneity revealed by IBL-12 and IBL-13 mAbs. Spleen sections from young adult mice (adjacent pairs in A,B and C,D) were reacted with the reagents as indicated. Arrows in A and B point to identical central arterioles. The arrowhead in D indicates the IgM^{hi} marginal zone B cell compartment. For LPS effect (F, for control see E) the mice received 50 μg *E. coli* LPS ($n = 5$). The size bars correspond to 100 μm. The illustrations are representative of experiments and stainings performed on at least three separate occasions. The initial reaction of mouse spleen section with ED31 mAb (developed with PE anti-rat IgG) still permitted the subsequent binding of FITC-conjugated IBL-12 mAb ($n = 4$). The reaction with various anti-MARCO antibodies viewed under different rhodamine (G) and FITC (H) filters was merged in the picture I. Controls included irrelevant rat antibodies in place of either the first or second anti-MARCO mAb (not shown). Size bar corresponds to 100 μm.

in the SRCR domain. In line with this notion, the antibody recognized a MARCO variant whose extracellular part is composed of only the SRCR domain (deletion of residues 79–420). Identical results were obtained with living cells incubated on ice with the antibody before fixation, or if cells were fixed before antibody staining.

In support of the finding that incubation of sections with ED31 did not block the subsequent binding of IBL-12, further studies with transfected cells indicated that the two anti-MARCO SRCR domain antibodies do not share the same epitope. For example, unlike ED31, IBL-12 recognized a point mutant of MARCO containing an alanine residue instead of arginine 433. On the other hand, binding of IBL-12, but not that of ED31, was abolished when either arginine 431 or 468 was replaced by an alanine residue (data not shown). It is worth mentioning that if using the crystal structure of the SRCR domain of Mac-2 BP as a template for the structure of the MARCO SRCR domain [31], these three residues are close to each other in the tertiary structure, although arginine 468 is far away from arginines 431 and 433 in the primary structure. These two latter residues are located at the N-terminus of the β-strand B, whereas arginine 468 is located at the β-strand D (Fig. 2B), domains of which are separated by a β-strand formed by the very last residues of the SRCR domain (β-strand F, [32–34]). Indeed, the replacement of the last 11 residues of the SRCR domain of MARCO by a corresponding sequence of a related scavenger receptor Tesr [35] abolished the binding of IBL-12, but not that of ED31.

We examined whether IBL-12 affects AcLDL and bacterial binding to the transient transfectants expressing full-length MARCO. Interestingly, it dramatically affected the

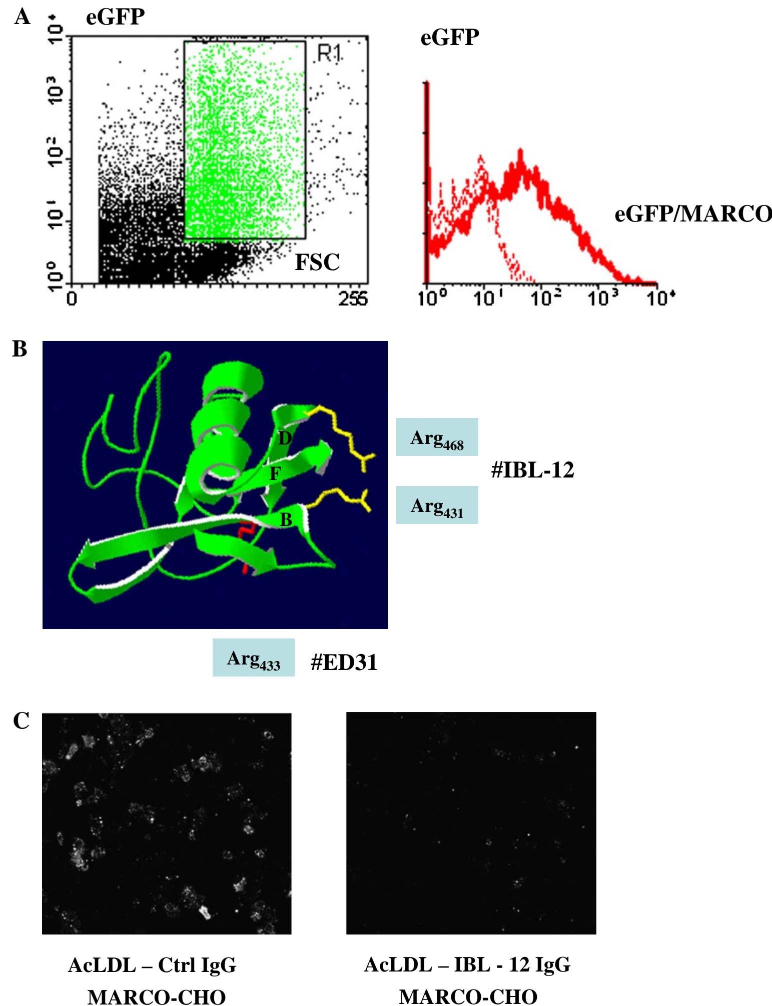

Fig. 2. Characteristics of the MARCO epitope recognized by IBL-12 mAb. (A) PC12 cells were co-transfected using Ca-phosphate mediate precipitation of pcDNA3-GFP-plasmid and full-length MARCO plasmid. The GFP-positive cells were gated (rectangle) and analyzed for MARCO expression (thick histogram; pcDNA3.1-GFP plasmid control is shown in dashed line). (B) A structural diagram indicates the essential arginine residues within the MARCO SRCR domain for recognition by IBL-12 mAb (yellow); the Arg433 necessary for ED31 binding is indicated as red. The nomenclature of β-strands (indicated as letters B, D, and F) is based on the crystal structure of Mac-2BP [31]. The addition of IBL-12 mAb interferes with the binding of DiI-labeled Ac-LDL by transient CHO-transfectants expressing full-length mouse MARCO (panel C, 10× objective magnification). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

binding of AcLDL (Fig. 2C), whereas there were no marked effects on bacterial binding (data not shown). An irrelevant rat monoclonal antibody affected neither binding of AcLDL nor bacteria (Fig. 2C, and data not shown). In further experiments, we found that in vivo administration of IBL-12 into normal mice did not prevent the subsequent accumulation of intravenously injected Gram-negative bacteria in the marginal zone. It appears, therefore, that bacterial and AcLDL binding is mediated by two distinct regions within the SRCR domain of MARCO [22].

Species-specific intracellular retention of the MARCO protein in mouse cell lines

To gain further insight into the possible cellular functions of MARCO, we attempted to express full-length mouse MARCO in murine NIH 3T3 cells using Ca-phosphate precipitation of the MARCO plasmid followed by neomycin selection. We found that the MARCO-positive cells of the G418-resistant colonies displayed the antigen inside the cells and not on the cell surface (data not shown). We also attempted to use various murine macrophage cell lines ($P388D_1$ and J774.1), but we could not isolate G418-resistant cells from either one of these. Therefore, we chose to use a more long-lasting delivery of MARCO constructs using a lentiviral vehicle. Using GFP as a reporter protein, we found that the EF-1 promoter was more efficient than the CMV promoter in these macrophage lines (data not shown). In parallel, we also employed the Jurkat human T lymphoblast cell line as an irrelevant host to express murine MARCO using the same lentiviral system. We found that approximately 25% of the transfected J774.1 cells became GFP-positive at MOI = 10, while a considerably higher proportion (65%) of Jurkat T cells expressed MARCO already at MOI = 1. Examination of J774.1 and Jurkat cells transduced with a lentivirus expressing

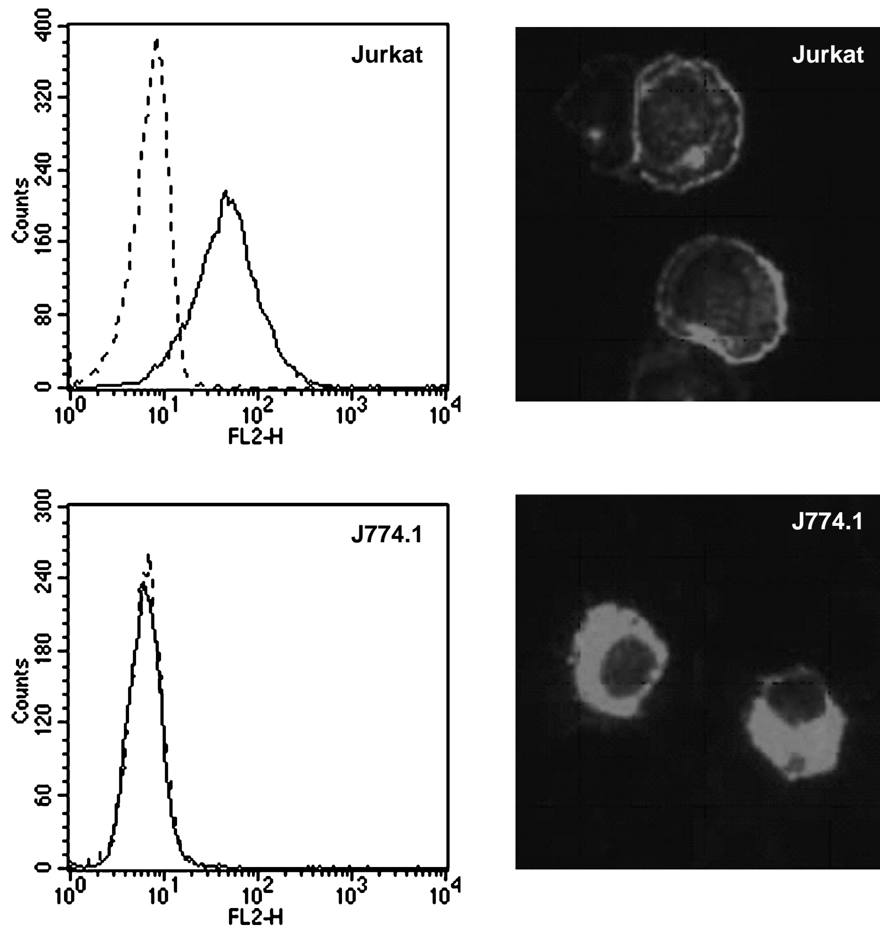

Fig. 3. Ectopically expressed mouse MARCO is transported to the cell surface in the human Jurkat cells, whereas it is retained intracellularly in the murine J774 cell line. The histograms indicate the flow-cytometric analyses of cell surface MARCO expression using IBL-12. The continuous lines represent the MARCO-transfected cells, whereas the dotted ones represent control cells. For the immunofluorescent microscopic analyses shown in the right panels, cytospinned cells were first permeabilized with saponin before staining with IBL-12.

MARCO under the EF-1 promoter indicated that MARCO was retained intracellularly in the murine J774.1 cells, whereas it was expressed predominantly on the cell surface of the human Jurkat cells (Fig. 3). Similar lentiviral transfection procedures using other murine cell lines (EL-4 thymoma cells, WEHI-3B myelomonocytic cells, and P388D_1 macrophages) also failed to yield detectable surface expression of MARCO, whereas in U-937 human monocyte cells stable surface expression could be achieved. Moreover, stimulation of neither the parental J774.1 nor the intracellular MARCO-positive transgenic J774.1 cells with LPS, CpG, or their combination resulted in a detectable surface display of MARCO, despite the robust morphological effect (appearance of pseudopodia) elicited by these compounds (not shown). Our results with the various cell lines are summarized in Table 1.

L-929 cells efficiently express both the cell-surface and soluble forms of MARCO

One notable exception to the above-described results with the murine cell lines was the L-929 fibroblastic cell line, in which lentiviral transduction resulted in the cell surface expression of MARCO. Furthermore, the transduction efficiency of MARCO and GFP was very similar when these two proteins were expressed from monocistronic vectors. In order to verify that the compartmentalization of transgenic proteins is intact in L-929 cells when using lentivectors, we also transduced the cells with a lentivirus expressing a truncated form of MARCO lacking the intracellular and transmembrane domains of the molecule (sMARCO). In this case, we utilized a bicistronic vector expressing both sMARCO and GFP, of which the latter one facilitated an easy identification of the transduced cells. As expected, sMARCO was not detectable on the surface of transfected L-929 cells. However, when the cells were stained after fixation and permeabilization, sMARCO was readily detected in the cells co-expressing GFP. Stable clones of these transgenic L-929 cells were isolated by limiting dilution (Fig. 4A).

MARCO-expressing L-929 cells exhibit AcLDL binding, which is affected by the IBL-12 mAb

To determine whether the transgenic MARCO expressed following lentiviral transduction is functional

Table 1
Summary of experiments performed to create murine cell lines expressing membrane-bound mouse MARCO

Cell line	Species	Tissue	Transfection/promoter	MARCO expression
PC12	Rat	Adrenal	Transient/CMV	Surface
U-937	Human	Macrophage	Stable/EF1	Surface
Jurkat	Human	T lymphocyte	Stable/EF1	Surface
NIH 3T3	Mouse	Fibroblast	Transient/CMV	Intracellular
J774.1	Mouse	Macrophage	Stable/EF1	Intracellular
$P388D_1$	Mouse	Macrophage	Stable/EF1	Intracellular
EL-4	Mouse	T lymphocyte	Stable/EF1	Intracellular
WEHI-3b	Mouse	Myelo/monocytic	Stable/EF1	Intracellular
L-929	Mouse	Fibroblast	Stable/EF1	Surface

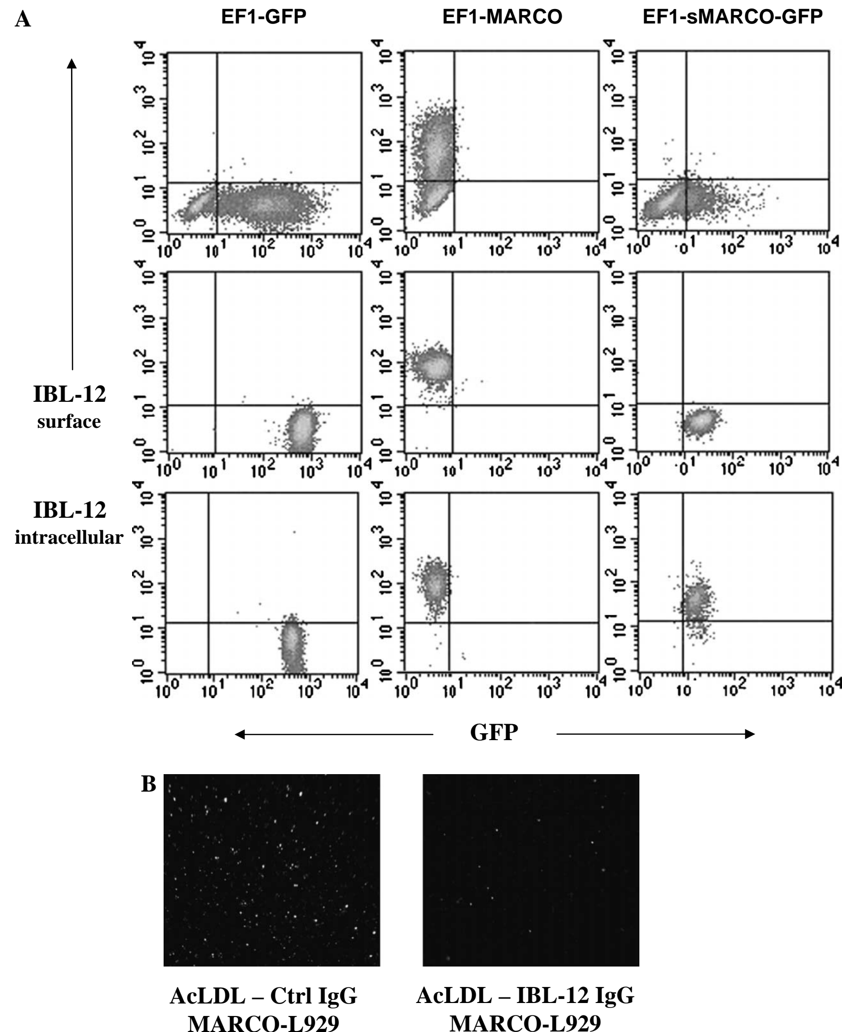

Fig. 4. Flow-cytometric analyses of transfected L-929 cells, and the functional examination of the cells expressing full-length MARCO. (A) FL1/GFP fluorescence is shown on the horizontal, FL2/MARCO expression on the vertical axis. Notes on the top indicate the vector constructs. The top row shows primary cultures after transfection, the middle and bottom rows illustrate sublines cloned from the primary cultures. The top and middle rows demonstrate cells stained for surface MARCO, whereas samples in the bottom row were permeabilized, thus allowing the detection of intracellular sMARCO as well. Results are representatives of three independent analyses. (B) The stable L-929 transfectants expressing full-length MARCO exhibit AcLDL binding, which can be blocked by the IBL-12 mAb, but not with a control mAb, evidenced as the reduction of fluorescent signal.

on the cloned L-929 transfectants, we tested the binding of AcLDL to these cells. We found a readily detectable staining above control (untransfected samples), which could almost completely be inhibited by the addition of the IBL-12 mAb (Fig. 4B). These data indicate that MARCO expressed by transfected L-929 fibroblasts is properly processed to achieve functional activity in its trimeric form.

Discussion

The marginal zone in both man and rodents is an important compartment of the spleen. It serves as a major exit area for recirculating lymphocytes and also as a possible site for blood-borne pathogens to be recognized by a distinct set of cells, including lymphoid and myeloid cells. Of these, various populations of marginal zone macrophages represent those elements that possess non-clonally rearranged receptors with restricted selectivities against microbial products. Their heterogeneity and diverse functional commitments are important factors in linking the adaptive and innate immune mechanisms for the generation of efficient immune responses against blood-borne microbial agents.

As a continuation of our work aimed at dissecting the stromal components of the spleen [19], we isolated two rat mAbs against two different MZ macrophage subsets. Of these, IBL-12 was found to be directed against the SRCR-domain of MARCO expressed by macrophages in the outer layer of the MZ, whereas IBL-13 identified another subset of MZ macrophages, positioned more proximally to the white pulp. As an indication of their differential responsiveness upon encounter with bacterial products, LPS administration, as reported previously, resulted in the upregulation of MARCO antigen in the red pulp macrophages [20], whereas the IBL-13 positive cells appeared to migrate into the white pulp. A similar phenomenon has been described for a marginal zone macrophage/dendritic cell subset binding the cysteine-rich domain of mannose receptor (CR [36]), but we could not detect any significant reactivity of these cells with IBL-13 mAb when spleen sections were co-stained with IBL-13 mAb and the CR-Ig fusion protein. We conclude that IBL-13 mAb reacts with a subset of marginal zone macrophages distinct from both the IBL-12 positive and the CR-Ig positive populations, but resembling the MOMA-1 positive macrophages [2,36].

Having established that IBL-12 recognizes the SRCR domain of MARCO, we wanted to examine whether it affects the ligand-binding activity of this cell-surface glycoprotein. Of significant interest, IBL-12 had profound effects on AcLDL binding to MARCO-expressing transfectants. Notably, when trying to establish stable MARCO-transfectants, we consistently found that MARCO accumulated intracellularly in various murine host cells. In contrast, such a phenomenon was not observed in non-murine cell lines. A similar divergent expression pattern has been reported for SR-A. That is, upon exposure to LPS, SR-A was downregulated in human macrophages, whereas its expression increased at both the mRNA and protein levels in murine macrophages [37]. In our study, the L-929 fibroblastic cell line was the only murine cell line, in which MARCO was not retained inside the cells, but was transported to the cell surface. We did not examine the subcellular localization of MARCO in the cell lines where it was retained intracellularly, but it is very likely that the protein was retained in the endoplasmic reticulum, a cell compartment known to possess a quality control system. Thus, we believe that it is quite possible that the differential behavior of MARCO in the different cell lines is due to the presence or absence of endoplasmic reticulum molecular chaperons needed for the correct folding and assembly of the trimeric MARCO molecule.

Upon ligand binding, mouse MARCO may activate downstream signaling when expressed in the permissive context of murine cells. As several components (bovine serum-derived, or cellular, [38]) present in the culture medium may act as ligands for recombinant mouse MARCO, it is also possible that they may elicit some cellular response in murine cells, leading to the retention of MARCO. The details of MARCO-induced signaling events are largely unknown, partly due to the lack of stable MARCO-expressing cells. Its induction requires rather potent stimulants such as LPS or CpG which, according to the literature, usually does not exceed low-to-moderate level increase. In primary cell cultures of mouse peritoneal macrophages, the antibody-mediated engagement of MARCO stimulated the production of IL-12, whereas the ligation of SR-AI/II suppressed its production [39]. For SR-A-mediated stimulation, the early internalization of the receptor is an important element of the signaling, followed by subsequent intracellular redistribution of ligand-bound receptor. It is not yet known how the trimeric structure of MARCO glycoprotein is implicated in transmitting stimulatory or other signals neither how it relates to the formation of IBL-12 epitope, but we hypothesize that latter is independent from the actual effect or occurrence of ligand-induced signaling, as both surface and intracellular (transmembrane or soluble) MARCO could be detected by employing IBL-12 mAb in various immunological methods.

In summary, our study shows that murine host cells display a consistent inhibition of in vitro mouse MARCO expression by lentiviral and other means of transfection, whereas non-murine cells can readily be transformed for the surface display of this molecule. These cells can be used for searching for potential MARCO-ligands by high throughput technologies, and can also serve as valuable tools for identifying important regulatory elements directing the cellular distribution and signaling by this receptor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.01.083.

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