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The scavenger receptor MARCO is involved in *Leishmania major* infection by CBA/J macrophages

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SUMMARY

CBA/J mice are resistant to Leishmania major *infection but are permissive to* L. amazonensis *infection. In addition, CBA/J macrophages control* L. major *but not* L. amazonensis *infection* in vitro. *Phagocytosis by macrophages is known to determine the outcome of* Leishmania *infection. Pattern recognition receptors (PRR) adorning antigen presenting cell surfaces are known to coordinate the link between innate and adaptive immunity. The macrophage receptor with collagenous structure (MARCO) is a PRR that is preferably expressed by macrophages and is capable of binding Gram-positive and Gram-negative bacteria. No research on the role of MARCO in* Leishmania*–macrophage interactions has been reported. Here, we demonstrate, for the first time, that MARCO expression by CBA/J macrophages is increased in response to both* in vitro *and* in vivo L. major *infections, but not to* L. amazonensis *infection. In addition, a specific anti-MARCO monoclonal antibody reduced* L. major *infection of macrophages by 30%–40%* in vitro. *The draining lymph nodes of anti-MARCO-treated mice displayed a reduced presence of immunolabelled parasite and parasite antigens, as well as a reduced inflammatory response. These results support the hypothesis that MARCO has a role in macrophage infection by* L. major in vitro *as well as* in vivo.

Keywords Leishmania major*, macrophage, MARCO*

Disclosures – none of the authors has potential conflicts of interest, including relevant financial interests in any company or institution that might benefit from this publication.
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INTRODUCTION

Leishmania are obligate intracellular parasites found in many parts of the world and cause diseases such as cutaneous, mucocutaneous and visceral leishmaniases. These parasites mainly infect macrophages and dendritic cells. Experimental mouse models have been extensively used to characterize cell-mediated immunity to *Leishmania* (1). In order to understand the mechanisms involved in resistance or susceptibility to *Leishmania* infection, several studies using different strains of mice have been performed. Most of these studies used inbred mouse strains resistant or susceptible to *L. major* infection (2,3).

Innate immune responses are supposed to be the determinants for the outcome of *Leishmania* infection (4), and macrophages are one of the primary targets of *Leishmania*. The first event in the *Leishmania*–macrophage interaction consists of parasite recognition by several macrophage surface receptors, followed by parasite internalization (5). Macrophages are also involved in the control of *Leishmania* infection, as parasite engagement to host cell receptors may lead to the activation of innate killing mechanisms (6). Furthermore, the progression of *Leishmania* infection is determined by adaptive T-helper cell responses orchestrated by MHC-restricted antigen presentation and cytokines produced by host cells during the innate response (7). Interestingly, we have previously shown that CBA/J mice, known to be resistant to *L. major* infection, are susceptible to *L. amazonensis* infection, constituting a model for the study of mechanisms related to *Leishmania* infection without interference from the genetic background of the mice (8). Additionally, we have demonstrated that CBA/J macrophages control *L. major* infection, although they are permissive to *L. amazonensis* infection *in vitro*. These data reinforce the idea that macrophages participate in the

determination of *Leishmania* infection outcome (9), and offer a model for the identification of molecules related to *Leishmania* infection.

Non-opsonic receptors on the surface of antigen presenting cells are characterized by their broad ligand specificities, and are considered to have evolved as pattern recognition receptors (PRR) (10,11) that coordinate the link between innate and adaptive immunity (12,13). The PRR family is comprised of mannose receptor, CD14, toll-like receptors (TLR), and scavenger receptors (SR) (10). The class A SR are collagenous transmembrane glycoproteins with cysteine-rich domains, and include type I and type II isoforms (SR-A) as well as the macrophage receptor with collagenous structure (MARCO) (10,14). MARCO is a distinct type-A SR that contains a longer collagenous domain and lacks the coiled coil domain of classical SR-A molecules (14). This receptor is able to bind Gram-positive and Gram-negative bacteria (13,15–17) and is constitutively expressed by subpopulations of macrophages, including spleen marginal zone macrophages and freshly harvested peritoneal macrophage populations (14,18).

Recently, the influence of other PRR on *Leishmania* macrophage infection has been demonstrated (19,20). However, there are no reports regarding the role of MARCO in innate and adaptive immune responses to *Leishmania* infection. In the present study, we investigated the contribution of MARCO to *Leishmania* infection and demonstrated for the first time that MARCO plays a role in *Leishmania major* but not *L. amazonensis* infection.

MATERIALS AND METHODS

Animals

All animal experiments were performed according to the standards of the Oswaldo Cruz Foundation guidelines for animal experimentation, and the Committee of Ethics on Animal Experimentation (CEUA-CPqGM/FIOCRUZ). Inbred 6–12 week-old CBA/J mice were obtained from the Animal Facilities Center of FIOCRUZ (Rio de Janeiro, Brazil) and the Animal Facility of CPqGM/FIOCRUZ, and maintained under specific pathogen-free conditions.

Parasites

Leishmania amazonensis (MHOM/Br88/Ba-125) and *L. major* (MHOM/RI/–/WR-173) were provided by Dr Aldina Barral from the Laboratory of Immunoparasitology at CPqGM/FIOCRUZ. Fresh *L. amazonensis* or *L. major* promastigotes were derived from isolated amastigotes obtained from the lymph nodes of C57BL/6 resistant mice, resuspended in Novy–Nicolle–MacNeal blood agar, and then transferred to Schneider's complete medium (Sigma Chemical Co., St Louis) for a maximum of six passages. For macrophage experiments, the promastigotes were expanded for 3–5 days in Schneider's complete medium until they reached stationary phase, then washed with saline as previously described (8) and adjusted to the desired concentrations indicated in the results.

Macrophage culture

Thioglycolate-induced peritoneal exudate cells were harvested from the peritoneal cavity of CBA/J mice after 3–4 days of intra-peritoneal injections of 2·5 mL of 3% thioglycolate medium (Sigma Chemical Co.). These elicited macrophages were obtained and used as previously described (9). Briefly, macrophage suspensions in DMEM complete medium were cultivated at a concentration of 1×10^6 cells/mL plated in a volume of 2 mL in 6-well plates, or 2×10^5 cells/mL plated in a volume of 1 mL in 24-well plates, both at 37°C in 5% CO_2/95% humidified air. After 24 h, nonadherent cells were removed by washing three times with RPMI 1640 supplemented with 25 mM HEPES before infection.

MARCO detection in infected cells by flow cytometry assay (FACS)

After *L. amazonensis* or *L. major* stationary phase promastigotes were added to the cultures in 6-well plates (10^6 cells per well) at a ratio of 10 : 1 in 37°C 5% CO_2/95% humidified air, the macrophages were cultivated for an additional period of 6 and 24 h. At the same time as infection, parallel cultures of infected macrophages were treated with rIFN-γ (100 UI/mL) and/or rTNF-α (100 UI/mL) as positive controls. Both rIFN-γ and rTNF-α were purchased from BD Biosciences Pharmingen (Franklin Lakes, NJ). Stimulated cells were cultivated for an additional 24 h, and then detached from the culture plates using a cell scraper. MARCO expression was determined by flow cytometry after labelling cells with a hybridoma supernatant containing a specific anti-mouse MARCO monoclonal antibody (mAb), ED31 (Serotec, Oxford, UK). After secondary antibody labelling with phycoerythrin-conjugated anti-rat IgG (Sigma Chemical Co.), the labelled cells were detected using a FACScan flow cytometer (Becton & Dickinson, Franklin Lakes, NJ). Positive control cells incubated with rIFN-γ (100 UI/mL) plus rTNF-α (100 UI/mL) induced a 95% enhancement of MARCO expression by elicited macrophages (data not shown).

In vitro MARCO blocking

For *in vitro* blocking, 2×10^5/mL elicited peritoneal macrophages were treated with the specific anti-MARCO mAb

ED31 (1 : 2) for 30 min at 37°C in 5%CO_2/95% humidified air, followed by *L. amazonensis* or *L. major* addition to the cultures at a ratio of 10 : 1. After 1·5, 3, 6, 12 or 24 h of infection, the cells were fixed in ethanol for 10 min at room temperature, and then stained with haematoxylin and eosin (H&E). Nonspecific IgG-treated cells were used as controls. The percentage of infected cells was estimated by counting at least 200 macrophages, and the results were expressed as the percentage of cells compared to the IgG-treated control cells. The data represent an average ± SE of replicates from one to five experiments performed in triplicates.

MARCO detection in *Leishmania*-infected mice by immunohistochemistry

Infection with *L. major* or *L. amazonensis* was accomplished by subcutaneously injecting 5×10^6 promastigotes in 25 µL into the left hind footpad of CBA/J mice. Control mice were injected with the same volume of saline. After 1, 3 (not shown) or 7 days of infection, the mice were sacrificed. Draining lymph nodes and spleens were fixed with acetone for 30 min and cryopreserved. To detect the expression of the MARCO receptor, immunohistochemistry was performed using 3 µm cryostat sections of the organs obtained from infected and saline-injected CBA/J mice. Immunohistochemistry was performed on all sections at the same time. The indirect immunoperoxidase technique was applied using the specific rat anti-mouse MARCO mAb ED31 and a biotinylated goat anti-rat IgG (DAKO North America, Inc., Via Real Carpinteria, CA). Both anti-MARCO ED31 (1 : 3) and goat anti-rat IgG (1 : 100) antibodies were diluted in 1% PBS/BSA. As a negative control, the specific antibody was replaced by a nonrelated rat IgG. Sections were counterstained with haematoxylin. Qualitative evaluations were performed considering two parameters; the frequency of cells expressing MARCO and the intensity of anti-MARCO labelling in these cells in draining lymph nodes and spleens of infected mice.

In vivo MARCO blocking

For *in vivo* blocking, CBA/J mice were treated twice by intravenous injection with 125 µg/250 µL of anti-MARCO ED31 at days zero and three after infection. Six hours after the first dose, the mice were infected by injecting 5×10^6 stationary phase *L. major* promastigotes subcutaneously into the left hind footpad. Ten days after infection, the mice were sacrificed. Infected footpads and popliteal draining lymph nodes were removed and fixed in 10% formaldehyde. Nonspecific IgG-treated mice were used as controls.

To demonstrate the presence of parasites and parasite antigens in the hind footpads and draining lymph nodes, immunohistochemistry for *Leishmania* was performed on 3 µm-thick sections obtained from formalin-fixed and paraffin-embedded tissues, as previously described (8). The indirect immunoperoxidase technique was applied using a rabbit polyclonal antibody against *Leishmania* (1 : 100) (21) and biotinylated goat anti-rabbit IgG (1 : 500) (DAKO, Carpinteria, CA) as previously described (8). The images were analysed using Image Pro-Express version 6·0 (Media Cybernetics, Bethesda, MD).

Histological analyses

To characterize the histological changes in draining lymph nodes due to anti-MARCO treatment, sections stained with H&E or subjected to immunohistochemistry for *Leishmania* detection ($n = 4$, per group) were analysed in a blinded fashion to qualitatively characterize the alterations observed in each section. The parameters analysed were the maintenance of lymph node architecture, presence of parasite, presence of parasite antigen and histiocytosis. The lymph node architecture was classified as organized, mildly disorganized and heavily disorganized. The alterations in the other parameters were classified as discrete, moderate and intense depending on the frequency of events and the intensity of anti-*Leishmania* staining.

RESULTS

Leishmania major infection induces up-regulation of the SR MARCO by elicited peritoneal CBA/J macrophages *in vitro*

In order to evaluate whether *L. major* or *L. amazonensis* differentially modulates MARCO expression in CBA/J macrophages, we used FACS analyses to compare MARCO expression on the surfaces of uninfected, *L. major* – and *L. amazonensis*-infected macrophages. First, we determined the percentage of uninfected elicited peritoneal macrophages that expressed MARCO, where the basal expression in uninfected cells was 13·70 ± 4·6 ($n = 5$). In response to *L. major* infection, MARCO expression was enhanced by 60% (24·25 ± 4·4, $n = 5$, $P < 0·05$, Newman–Keuls), compared to an enhancement of only 18% in response to *L. amazonensis* infection (17·93 ± 3·1, $n = 5$, $P > 0·05$, Newman–Keuls). These differences are statistically significant ($P = 0·0279$, ANOVA). We also evaluated the intensity of MARCO expression (median of fluorescence intensity, MFI), and the variations observed were similar among groups (macrophages = from 15·50 to 42·47; *L. major* = from 14·36

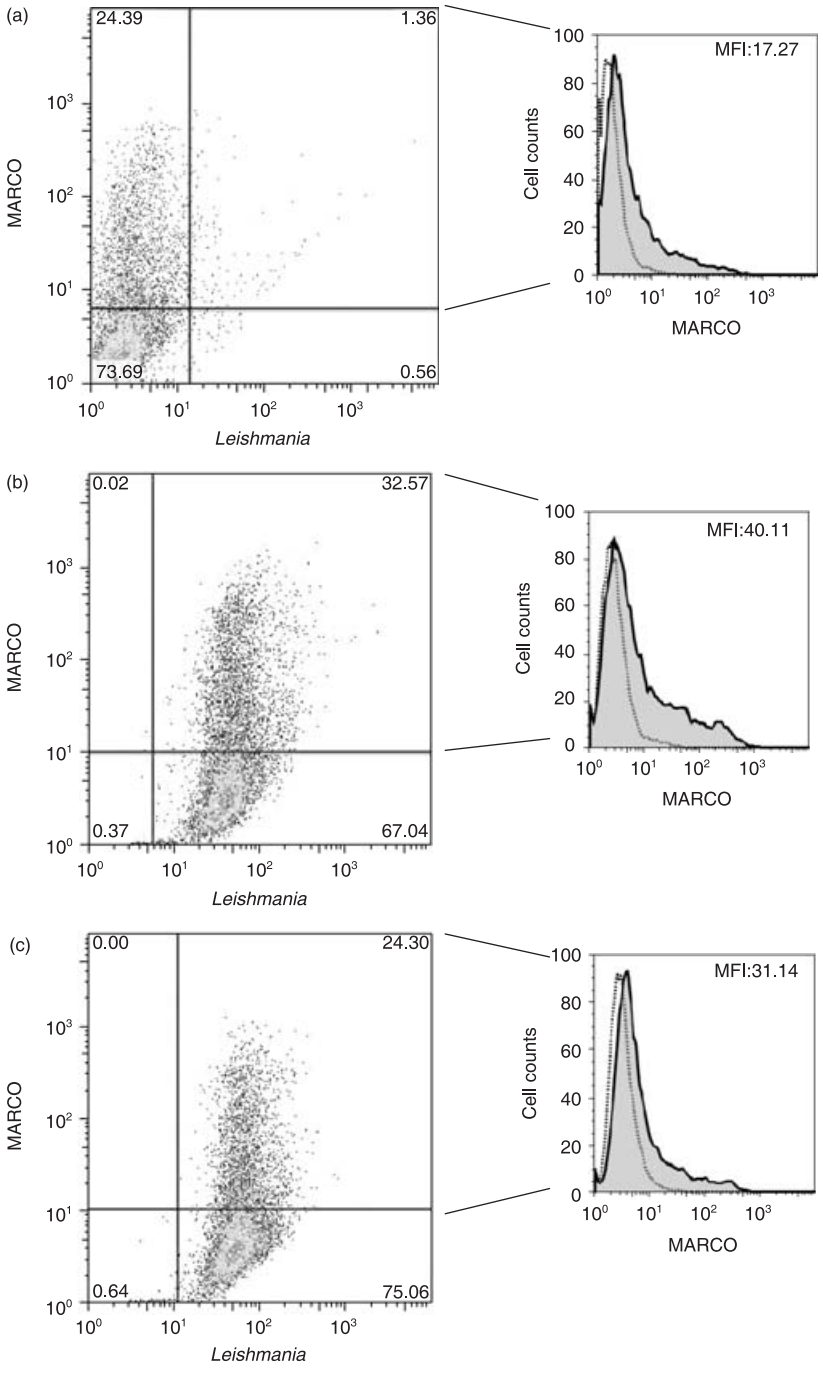

Figure 1 *L. major* infection induced higher MARCO expression by elicited macrophages *in vitro* in comparison to *L. amazonensis* infection. MARCO expression was determined in elicited peritoneal macrophages infected with stationary phase promastigotes at a 10 : 1 ratio. Uninfected macrophages were used as a negative control. After 24 h of infection, cells were stained with monoclonal anti-MARCO and anti-*Leishmania* antibodies and fluorescence was detected using FACS. Scatter plots show MARCO-positive cells vs. *Leishmania*-infected cells in control (a), *L. major* (b), or *L. amazonensis*-infected macrophages (c). The percentage of labelled cells is indicated in each quadrant. *Leishmania major* induced a significant increase in the percentage of MARCO-positive cells compared to the control and *L. amazonensis*-infected macrophages. Among the MARCO-positive cells, no differences in the percentage of *L.*-infected cells were observed between *L. major* and *L. amazonensis* infection. Inserts represent MFI histograms of MARCO expression in control, *L. major*, or *L. amazonensis*-infected macrophages, which were not significantly different. One representative experiment out of five is shown.

to 55·15; *L. amazonensis* = from 23·75 to 37·09; $P = 0{\cdot}57$, ANOVA). Figure 1 illustrates representative Scatter plots of one experiment that show the differences observed in the percentages of MARCO-positive cells. Differences in MARCO expression were dependent only on the percentage of infected cells, not on MFI values.

Anti-MARCO-specific mAb ED31 reduces *L. major*, but not *L. amazonensis* uptake into elicited peritoneal macrophages

To evaluate the actual contribution of MARCO in CBA/J macrophages during *L. major* infection, a specific neutralizing

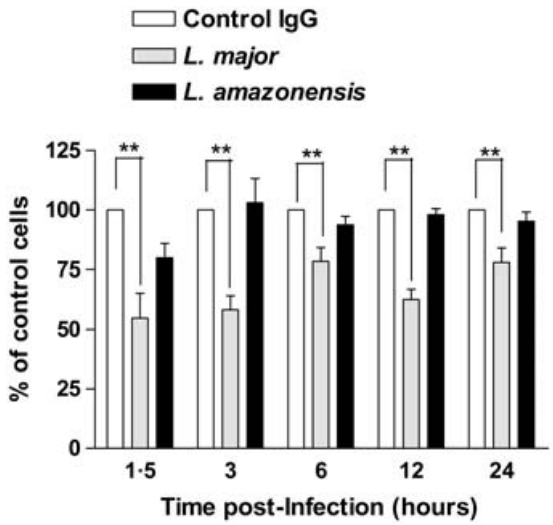

Figure 2 MARCO neutralization inhibits *L. major*-induced macrophage infection *in vitro.* Elicited macrophages were cultivated and treated with mAb ED31 for 30 min, followed by *L. major* (grey bars) or *L. amazonensis* (black bars) infection. After 1·5, 3, 6 or 24 h of infection, cells were fixed and stained with H&E. Nonspecific IgG-treated and infected cells were used as a negative control. The results are expressed as the percentage of infected cells, with the IgG-treated control cells considered as 100% of each infection (white bars). *L. major* infection varied from 13·4% to 61·5%, and *L. amazonensis* infection varied from 33·3% to 94·9% in nonspecific IgG-treated cells. Note that blockage of MARCO significantly reduced *L. major* infection (45·2%) as early as 1·5 h post-infection. This reduction decreased to 20% and stabilized at 6 h until 24 h post-infection ($P < 0{\cdot}0001$, one-way ANOVA). Bars represent an average ± SE of replicates from one to five experiments performed in triplicate (**$P < 0{\cdot}01$, Dunnett's Multiple Comparison Test).

antibody towards MARCO (mAb ED31) was added to the cell cultures 30 min before *Leishmania* infection. Interestingly, MARCO neutralization significantly reduced the percentage of *L. major* infection, but although apparently reduced *L. amazonensis* infection this reduction was not statistically significant (Figure 2). Blockage of MARCO by mAb ED31 reduced *L. major* infection by 45·2% as early as 1·5 h post-infection, and this reduction decreased to 20·0% and stabilized at 6 h until 24 h post-infection (Figure 2). As inhibition of *L. major* infection in CBA/J macrophages was observed as early as 1·5 h post-infection following MARCO neutralization by mAb ED31, we suggest that MARCO is involved in *L. major* uptake by these cells. As infection inhibition rates in anti-MARCO-treated cells occur as early as 1·5 h after infection and were not statistically different ($P > 0{\cdot}05$) over time, we conclude that the antibody had an effect on an early event after infection, but had no effect on parasite survival (Figure 2).

As neutralization was partial, we hypothesized that *L. major*–MARCO interaction is dependent on the contribution of other phagocytic receptors that are recognizing *Leishmania* simultaneously with MARCO. To determine the exclusive influence of MARCO in *Leishmania* recognition and uptake, co-localization studies were performed using *Leishmania* and nonphagocytic CHO cells transiently transfected with mouse MARCO cDNA (Figure S1a,b in Supporting Information). Cells were classified into two groups, associated with 1 or > 1 parasites per cell according to the number of parasites associated with MARCO-positive and MARCO-negative cells. Thus, the data shown in Tables S1–S6 (Supporting Information) represent three independent experiments organized in contingency tables. We observed that the proportion of MARCO-positive cells that interact with 1 and > 1 promastigotes was higher when compared to the proportion of MARCO-negative cells at both 4°C (Tables S1–S6 in Supporting Information) and 37°C (not shown). Indeed, chi-square analyses showed significant differences among groups ($P < 0{\cdot}0001$). In addition, it is important to note that nonphagocytic MARCO-positive CHO cells interact in the same proportion to *L. major* and *L. amazonensis* promastigotes.

Together, these data suggest that MARCO interacts similarly in a first step with both *L. major* and *L. amazonensis*. However, we state that this interaction has different consequences for the fate of these parasites inside cells. Our data suggest that MARCO only participates in downstream events of *L. major* infection. The evidence that supports this idea is that the percentage of MARCO-positive cells was only up-regulated in *L. major* infection, and that mAb ED31 only reduced *L. major* infection *in vitro*. As mAb ED31 did not modify *L. amazonensis* infection, MARCO is probably not essential for the establishment of this infection.

L. major-induced MARCO expression *in vivo*

Detection of MARCO by immunohistochemistry *in vivo* was conducted to extend the *in vitro* data showing that MARCO was more highly expressed in *L. major*-infected macrophages compared to *L. amazonensis*-infected cells. In Figure 3, we observe that MARCO expression in both spleens from uninfected (Figure 3a–c) and *L. amazonensis*-infected mice (Figure 3d–f) was less frequent and weaker than MARCO expression in spleens from *L. major*-infected mice (Figure 3g–i). A comparative observation of Figure 3 shows that MARCO-expressing cells were uniformly distributed in follicular marginal zones of the spleen (Figure 3a,d,g). In addition, MARCO-labelling in *L. major*-infected spleen sections (Figure 3g) was much more evident than in uninfected (Figure 3a) and *L. amazonensis*-infected mice (Figure 3d). Surprisingly, MARCO expression in

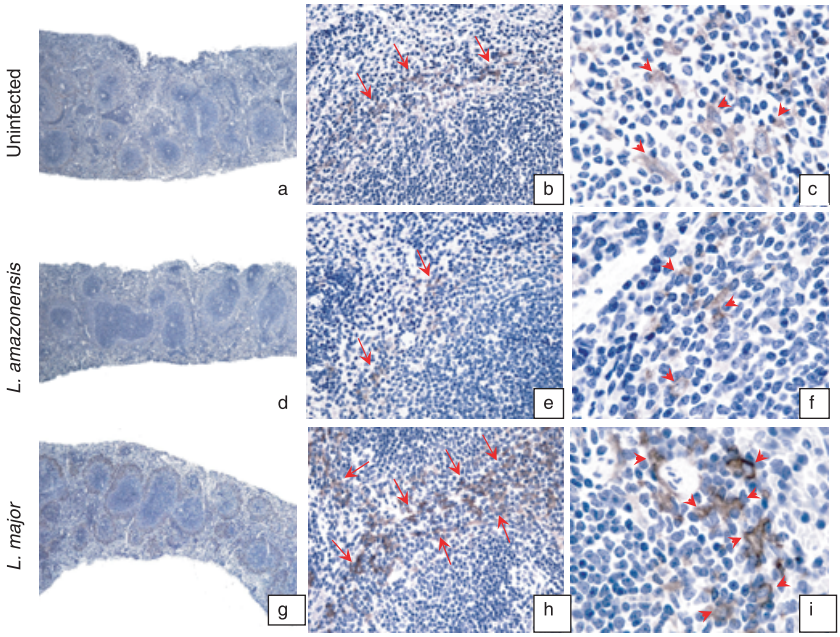

Figure 3 Induction of MARCO expression in spleens of *L. major*-infected mice *in vivo*. After 7 days of *L. major* or *L. amazonensis* infection, spleens were collected and analysed by immunohistochemistry with the specific anti-MARCO mAb ED31 as described in the Material and Methods. Saline-injected CBA/J mice were used as a negative control. The pictures clearly show MARCO-labelled spleen marginal zone macrophages localized in follicles from the white pulp of uninfected (a), *L. major* (d) and *L. amazonensis*-infected mice (g) (40×). MARCO-labelling of the spleen section of *L. major*-infected mice (d) is much more evident than that of uninfected (a) and *L. amazonensis*-infected mice (g) (40×). In *L. major*-infected spleen sections, MARCO expression is also more frequent (h) and intense (i) than in cells from uninfected (b and c) or *L. amazonensis*-infected mice (e and f) (200× and 400×, respectively). Pictures are representative of one experiment out of two using at least four mice per group.

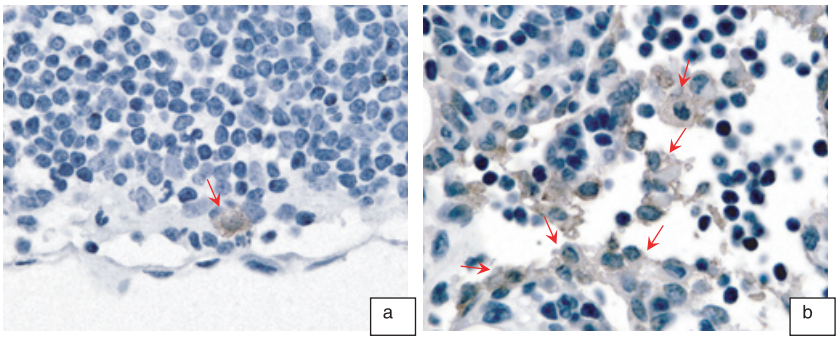

Figure 4 Induction of MARCO expression in lymph nodes of *L. major*-infected mice *in vivo*. After 7 days of *L. major* or *L. amazonensis* infection, lymph nodes were collected and analysed by immunohistochemistry with the specific anti-MARCO mAb ED31 as described above. Saline-injected CBA/J mice were used as a negative control. In draining lymph nodes, MARCO expression was only present in macrophages localized in the subcapsular sinus. MARCO expression was higher in lymph nodes from *L. major*-infected mice (arrowheads) (400×) (b) in comparison to those from *L. amazonensis*-infected mice (arrows) (400×) (a). Pictures are representative of one experiment out of two using at least four mice per group. MARCO-expressing cells are indicated in brown (arrows).

L. amazonensis-infected mice (Figure 3d–f) was even less evident than MARCO expression in spleens of saline-injected control mice (Figure 3a–c). At high power views (400×) of spleen sections, the differences in MARCO expression were more evident (Figure 3c,f,i). In the draining lymph nodes, MARCO expression was observed in many macrophages present in the subcapsular sinus of *L. major*-infected mice (Figure 4b). On the other hand, only very few MARCO-expressing macrophages were similarly seen in the lymph nodes from *L. amazonensis*-infected mice (Figure 4a) compared to uninfected mice (not shown). The differences in these collected data were only observed 7 days after *Leishmania* infection, although investigations were conducted at 1 and 3 days after infection (not shown).

MARCO-specific mAb ED31 reduces the *L. major*-induced inflammatory response in lymph nodes from CBA/J mice

To further confirm the contribution of the SR MARCO on *L. major* infection, the specific anti-MARCO mAb ED31 was intravenously injected, followed by subcutaneous injection of *L. major* promastigotes in the hind footpads of CBA/J mice. At 10 days after infection, the footpad

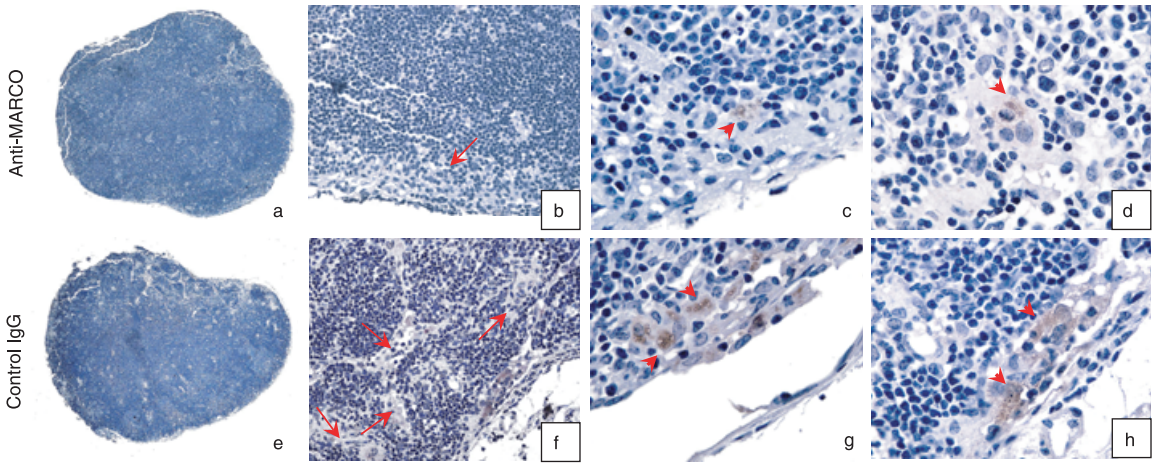

Figure 5 Effect of MARCO neutralization *in vivo* on immune-inflammatory responses of lymph nodes from *L. major*-infected mice. Mice previously treated with mAb ED31 (Figure 5a–d) or isotype control IgG (Figure 5e–h) were infected with *L. major* in the hind footpad as described in Material and Methods. Ten days later, the popliteal draining lymph nodes from these mice (n = 4 mice per group) were evaluated. Sections were subjected to immunolabelling using an antibody raised against *Leishmania*. Histological features of lymph node architecture are shown. Draining lymph nodes from anti-MARCO mAb-treated mice are homogeneous, with well-preserved lymphoid follicles and capsule (Figure 5a; 40×). Lymph nodes from isotype control IgG mice were slightly disorganized. There is a discrete swelling of the subcapsular sinus and discrete follicular rearrangement (Figure 5e; 40×). (Figure 5b–h) Details from (a) and (e) showing a comparison of histiocytes infiltration (arrows) in subcapsular cortical areas. The subcapsular cortical area has a larger collection of macrophages in lymph nodes from anti-MARCO mAb-treated mice (Figure 5b; 100×) compared to lymph nodes from control mice (Figure 5f; 100×). Macrophages containing an amastigote form of *Leishmania* and parasite antigen (arrow heads) are more frequent in lymph nodes from control mice (Figure 5g,h) than in lymph nodes from anti-MARCO mAb-treated mice (Figure 5c,d) (100× and 400×, respectively).

inflammatory infiltrates of CBA/J mice treated with IgG or anti-MARCO mAb ED31 followed by *L. major* infection, displayed no differences, and were characterized by an acute inflammatory response with moderate infiltration of granulocytes and mononuclear cells located at the dermis, as previously described (8). These inflammatory infiltrates were diffuse, and consisted predominantly of macrophages with a few neutrophils and lymphocytes (8). Macrophages with an epithelioid appearance formed small granulomas. Many macrophages and a few granulocytes were parasitized (not shown). Some epithelioid macrophages containing amastigote forms of immunolabelled *L. major* or parasite antigens were seen in small parasitophorous vacuoles. Occasionally, extensive areas of the dermis were occupied by parasitized macrophages and, rarely, lymphocytes were observed at the periphery of the injury (not shown).

The effect of MARCO neutralization was also evaluated in the draining lymph nodes of infected mice that received intravenous injections of anti-MARCO mAb ED31, and compared to control IgG-treated mice. The parameters analysed were the maintenance of lymph node architecture, presence of parasite, presence of parasite antigen, and histiocytosis, as described in Material and Methods. As depicted in Figure 5(a–d), mice that were injected with neutralizing anti-MARCO mAb ED31 showed fewer immunolabelled parasites in the draining lymph nodes compared to control infected mice (Table 1). In the draining lymph nodes of mice receiving soluble neutralizing anti-MARCO mAb ED31, there were small collections of macrophages in the cortical area near the subcapsular sinus, and a few small granulomas (Table 1, Figure 5a). Occasionally, isolated amastigote forms of *Leishmania* and parasite antigens were observed inside macrophages (Figure 5b–d). In comparison, mice infected with *L. major* that received control IgG showed larger collections of macrophages infected with many amastigote forms of *Leishmania* in the cortical zone

Table 1 Qualitative histological observations of lymph nodes from *L. major*-infected CBA/J mice treated with neutralizing specific anti-MARCO mAb ED31 or with control IgG

Features	Control IgG	Anti-MARCO
Parasitism	Intense	Discrete
Lymph node architecture	Discrete disorganization	Preserved
Histiocytosis	Intense	Discrete
Presence of parasite Ag	Intense	Discrete

Immunohistochemistry for *Leishmania* was performed on formalin-fixed and paraffin-embedded 3 µm-thick sections of lymph nodes from IgG-treated and anti-MARCO ED31 mAb-treated CBA/J mice as described in Material and Methods. Qualitative histological findings were observed in all mice from each group (n = 4).

of the lymph nodes (Figure 5g,h). A summary of the qualitative differences observed between draining lymph nodes from control and anti-MARCO-treated mice are depicted in Table 1.

DISCUSSION

The surfaces of phagocytes are adorned with many receptors known as PRR. These receptors are able to recognize and decode their ligands expressed on the surface of infectious agents and apoptotic cells, ultimately triggering engulfment or modulation of phagocyte signalling (22). Additionally, PRRs bind to modified proteins, lipids, and carbohydrates (23), leading to the coordination of gene expression with induction of effector functions (24). The molecules present on modified host cell and pathogen surfaces form a specific mosaic, known as a molecular pattern. The recently identified MARCO is a SR that possesses similarities to SR-AI/II (14). MARCO contains a triple-helical collagenous domain and a SR cysteine-rich (SRCR) domain at the C-terminus (14,23), the probable binding site of the specific mAb ED31 (17). Although possessing similarities to SR-AI/II in structure, distribution, and ligand binding repertoire, MARCO is unique. This receptor is able to bind Gram-positive and Gram-negative bacteria (13,15–17) and is constitutively expressed by subpopulations of macrophages, including spleen marginal zone macrophages and freshly harvested peritoneal macrophage populations (14,18).

As described by others (14), we observed a low constitutive expression of MARCO in inflammatory peritoneal macrophages that was more significantly enhanced by *L. major* than by *L. amazonensis* stimulation (Figure 1). The positive modulation of MARCO expression *in vitro* (Figure 1) and *in vivo* in response to *L. major* but not *L. amazonensis* infection (Figures 3 and 4), as well as the lower MARCO expression in spleens and lymph nodes of *L. amazonensis*-infected CBA/J mice compared to saline-injected controls, suggest that MARCO may participate in the host-parasite dynamic of *L. major* infection.

In co-localization studies using nonphagocytic MARCO-positive CHO cells, it was observed that a higher number of MARCO-positive than MARCO-negative cells interacts with parasites. These data indicate that ligands present on the *Leishmania* surface recruit MARCO receptors to participate in parasite recognition. Surprisingly, MARCO-positive cells interact in the same proportion to *L. major* and *L. amazonensis* promastigotes (Figure S1c, Tables S1–S3 in Supporting Information). These results suggest that MARCO interacts similarly in a first step with both *L. major* and *L. amazonensis*. However, this interaction has different consequences for the fate of the parasites inside cells. Indeed, a significant reduction in *L. major* but not *L. amazonensis* infection was observed in CBA/J macrophages upon MARCO neutralization *in vitro* (Figure 2). This reduction was detected as early as 1·5 h after infection (Figure 2), suggesting that MARCO actually participates in downstream events of only *L. major* infection. It has been described that the binding sites for bacteria and mAb ED31 are in close proximity but are not the same (25). However, studies have shown that this antibody is capable of blocking the binding of the bacteria to the MARCO receptor (17). It has been proposed that the inhibitory effects of mAb ED31 on bacterial interaction with macrophages could be mediated indirectly by steric hindrance of adjacent ligand-binding sites (17). We suggest that the inhibitory effect of mAb ED31 in *L. major* infection may be mediated by a similar mechanism. To explain the actual role of MARCO in *L. major* infection, we hypothesize that this receptor directly influences *L. major* phagocytosis. The *L. major*–MARCO interaction, being not mutually exclusive, could also be mediated by receptor-induced modifications in cell signalling during parasite internalization, which somehow influences the outcome of the parasite infection. It has been previously demonstrated that TLRs, a very well-known class of PRRs, are involved in cell signalling, but not in particle phagocytosis (23,26). It is important to note that the downstream events that interfere with *L. major* infection are initiated very early (mAb ED31 was able to reduce *L. major* infection as early as 1·5 h after infection). It is plausible that MARCO also plays a role later in *L. major* infection, based on *in vivo* studies. Although tested after 1 and 3 days of infection (data not shown), MARCO up-regulation was only detected at 7 days after infection *in vivo*. It has been previously shown that MARCO functions as a back-up system through which the organism can quickly arm more cells with highly efficient phagocytic receptors (27).

Although MARCO is present in the initial *L. amazonensis*–cell interaction, it is probably not essential for the establishment of infection, as mAb ED31 did not modify *L. amazonensis* infection. This evidence reinforces the idea that binding does not necessarily reflect phagocytosis (22). There are several lines of evidence that *Leishmania* interacts with a particular set of macrophage receptors, which differ depending on parasite species (5,28,29). It is clear from these data that there is a difference between *L. major* and *L. amazonensis* interaction with MARCO which results in a more extend uptake of *L. major* by macrophages.

Alterations in macrophage function induced by mAb ED31 can be one explanation for the reduction of *L. major* infection caused by this antibody *in vitro* as well as *in vivo*. It has been very well described that antibodies added to macrophages ligate to specific receptors on the macrophage surface by the Fc-γ region (30). This is probably not the case

in our study, as such an effect in *L. major* infection of mice induces an alternative activation of antigen presenting cells in the culture, resulting in expression of the modulatory cytokine IL-10, and enhancement of parasite infection (31,32). As we observed that soluble mAb ED31 reduced *L. major* infection *in vitro* and *in vivo*, it is not likely that the antibody effect is dependent on modulation of the macrophage response by Fcγ receptor ligation. In contrast to our observations, it has been described that immobilized anti-MARCO mAb ED31 at 22 h after IFN-γ stimulation of macrophage cultures enhances nitrite accumulation *in vitro* (33). It is not plausible to link the anti-MARCO effect in *L. major* infection observed in our experiments (Figure 2) with the previously described antibody effect on the activation of NO macrophage killing mechanism (33). In this previous work, the authors used immobilized antibodies which are known to stimulate macrophages by inducing receptor cross-linking on the cell surface (34). Additionally, we showed in a previous study that NO and iNOS mRNA expression was only detected in IFN-γ-treated thioglycolate-elicited CBA/J macrophages at a later time point after infection (9).

It is noteworthy that the distribution of MARCO in the spleens and lymph nodes from *Leishmania*-infected mice (Figures 4 and 5) was similar to that described in other pathogen models (14,18). To our knowledge, this is the first report implicating up-regulation of MARCO in a protozoan parasite infection *in vivo*. Interestingly, it seems that *L. major*-induced enhancement of MARCO expression *in vivo* was much greater compared to that observed in *in vitro* experiments. It is possible that, as previously suggested for LPS stimulation (17), the *in vivo* up-regulation of MARCO is mediated through positive feedback by autocrine activation of cytokines produced by activated macrophages (35). We have previously described that CBA/J macrophages stimulated with IFN-γ were able to significantly enhance TNF-α mRNA in response to *L. major* infection when compared to *L. amazonensis* infection (9). An autocrine regulation of SR mediated by stimulation of TNF-α has been described for LPS-induced down-regulation of SR-A I/II in monocytes (35). More recently, using peritoneal elicited macrophages from wild type and MARCO-deficient mice, it was shown that ligation of MARCO to immobilized mAb induced enhancement of IL-12 and IFN-γ-stimulated NO production by wild type, but not MARCO-null macrophages (33). In a previous observation, we showed that CBA/J mice respond to *L. major* with the production of IFN-γ in draining lymph nodes (8). In summary, these data suggest that up-regulation of MARCO in response to *L. major* at 7 days (Figures 3 and 4) after *L. major* infection is an event related to the adaptive immune response. However, we cannot disregard the possibility that, at 1 and 3 days after *L. major* infection, MARCO was expressed at low levels that were not detected using the immunohistochemistry method with a low sensitivity.

MARCO neutralization by the specific anti-MARCO *in vivo* induced no alterations at the site of parasite inoculation (not shown). When we analysed lymph nodes from mice treated with anti-MARCO, we observed a reduction in the inflammatory response and in the number of immunolabelled parasites and parasite antigens. This apparent discrepancy can be explained by the possibility that inflammatory macrophages recruited to the site of infection probably express low levels of MARCO, similar to the low expression previously described for thioglycolate inflammatory macrophages (14). In summary, based on our results, we present evidence for the first time that MARCO has a role in *L. major* infection *in vitro* as well as *in vivo*. Further experiments need to be performed to determine the actual role of MARCO in the outcome of *L. major* infection *in vivo*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 *Leishmania* interaction with MARCO-transfected CHO cells. CHO cells (5×10^4 per mL) were cultured for 24 h and transfected with recombinant pcDNA3·0 containing full length MARCO DNA, kindly provided by Dr Timo Pikkarainen, or pcDNA3·0 empty control vector using Lipofectamine™ (Invitrogen). After 24 h, MARCO-transfected CHO cells were allowed to interact with *L. major* (a) or *L. amazonensis* (b) promastigotes in stationary-phase of growth. After binding at 4°C for 30 min, cells were washed to remove unbound parasites and then fixed in paraformaldehyde. CHO cells were re-incubated for additional 90 min at 37°C as described. At the end of the incubation time, CHO cell cultures were fixed in 4% paraformaldehyde. MARCO and *Leishmania* were then double-labelled with

specific antibodies anti-MARCO and anti-*Leishmania* similarly as described for macrophage in item 2·4. The pictures show a higher number of parasites bounded to MARCO-transfected CHO cells (arrows) than to nontransfected cells (arrow heads). Cells successfully transfected and expressing MARCO contain as much *L. amazonensis* (a) as *L. major*-associated to them (b) (100×). At least 1000 cells were quantified at 8–10 fields using Image Pro-Express® 6·0.

Tables S1–S6 Number of *Leishmania* bounded to MARCO-transfected CHO cells and nontransfected cells. CHO cells were transfected with recombinant pcDNA3·0 containing full length MARCO DNA using LipofectamineTM (Invitrogen). After 24 h, MARCO-transfected CHO cells were allowed to interact with *L. major* or *L. amazonensis* promastigotes in stationary-phase. After binding at 4°C for 30 min, cells were washed to remove unbound parasites and then fixed in paraformaldehyde. MARCO and *Leishmania* were then double-labelled with specific antibodies as described above. Cells were classified into two groups, associated with 1 or > 1 parasites per cell according to the number of parasites associated with MARCO-positive and MARCO-negative cells. The data represent three independent experiments organized in contingency tables. The proportion of MARCO-positive cells that interact with 1 and > 1 promastigotes was higher when compared with MARCO-negative cells at 4°C. Chi-square analyses was performed and showed significant differences among groups ($P < 0{\cdot}0001$). In addition, MARCO-positive cells interact in the same proportion to *L. major* and *L. amazonensis* promastigotes.

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