Biochimica et Biophysica Acta 1838 (2014) 2011–2018

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Integrity of the Actin Cytoskeleton of Host Macrophages is Essential for *Leishmania donovani* Infection

Saptarshi Roy [a,1], G. Aditya Kumar [b,1], Md. Jafurulla [b,1], Chitra Mandal [a,*], Amitabha Chattopadhyay [b,**]

[a] CSIR-Indian Institute of Chemical Biology, Raja S.C. Mullick Road, Kolkata 700 032, India
[b] CSIR-Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

ARTICLE INFO

Article history:
Received 16 November 2013
Received in revised form 19 March 2014
Accepted 18 April 2014
Available online 26 April 2014

Keywords:
Leishmania
actin cytoskeleton
promastigotes
F-actin quantitation
cytochalasin D
FACS

ABSTRACT

Visceral leishmaniasis is a vector-borne disease caused by an obligate intracellular protozoan parasite *Leishmania donovani*. The molecular mechanism involved in internalization of *Leishmania* is poorly understood. The entry of *Leishmania* involves interaction with the plasma membrane of host cells. We have previously demonstrated the requirement of host membrane cholesterol in the binding and internalization of *L. donovani* into macrophages. In the present work, we explored the role of the host actin cytoskeleton in leishmanial infection. We observed a dose-dependent reduction in the attachment of *Leishmania* promastigotes to host macrophages upon destabilization of the actin cytoskeleton by cytochalasin D. This is accompanied by a concomitant reduction in the intracellular amastigote load. We utilized a recently developed high resolution microscopy-based method to quantitate cellular F-actin content upon treatment with cytochalasin D. A striking feature of our results is that binding of *Leishmania* promastigotes and intracellular amastigote load show close correlation with cellular F-actin level. Importantly, the binding of *Escherichia coli* remained invariant upon actin destabilization of host cells, thereby implying specific involvement of the actin cytoskeleton in *Leishmania* infection. To the best of our knowledge, these novel results constitute the first comprehensive demonstration on the specific role of the host actin cytoskeleton in *Leishmania* infection. Our results could be significant in developing future therapeutic strategies to tackle leishmaniasis.

1. Introduction

Leishmaniasis is a vector-borne disease, caused by various species of the genus *Leishmania*, which are obligate intracellular protozoan parasites. Leishmaniasis causes substantial public health problems, especially in tropics, subtropics and the Mediterranean basin, and is usually fatal if left untreated [1–4]. Leishmaniasis threatens about 350 million men, women and children in 98 countries around the world. As many as 12 million people are believed to be currently infected, with about 1–2 million estimated new cases occurring every year [5,6]. In socioeconomic terms, leishmaniasis is often associated with poverty [7] and is believed to be one of the most neglected diseases due to limited funding available for diagnosis, treatment and control [8]. According to available estimates, leishmaniasis is considered to be second in mortality and fourth in morbidity among all tropical diseases [9]. Based on clinical syndromes, leishmaniasis is classified into four major types: cutaneous, muco-cutaneous, visceral (also known as *kala-azar*) and post-*kala-azar* dermal leishmaniasis. Among these, visceral leishmaniasis (VL) is fatal in the absence of treatment [3]. The current worldwide increase in leishmaniasis to epidemic proportions, and the emergence of VL as an important opportunistic infection among people infected with HIV-1 [10] have given rise to an urgency to provide treatment for leishmaniasis.

Leishmaniasis is transmitted by the bite of the infected female sandfly (*Phlebotomus* spp.) while taking a bloodmeal from a host [11]. The lifecycle of *Leishmania* has two distinct forms: an extracellular promastigote flagellar form found in the mid-gut of sandflies, and an intracellular amastigote form that resides in phagolysosomes of host macrophages. After entering the bloodstream, promastigotes are internalized by dendritic cells and macrophages, and subsequently transform into amastigotes by losing their flagella [3,12]. The entry of promastigotes into host macrophages involves multiple host–parasite interactions such as recognition of specific ligands on the parasite cell surface by receptors on the macrophage cell surface [4]. Studies aimed at understanding the molecular mechanisms of entry of *Leishmania* into host cells have led to the identification of a number of candidate receptors facilitating multiple routes of entry, thereby highlighting the redundancy in the entry process [2,13,14]. These include membrane

Abbreviations: CD, cytochalasin D; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PE, phycoerythrin; VL, visceral leishmaniasis

* Corresponding author. Tel.: +91 33 2429 8861; fax: +91 33 2473 5197.
** Corresponding author. Tel.: +91 40 2719 2578; fax: +91 40 2716 0311.
E-mail addresses: cmandal@iicb.res.in (C. Mandal), amit@ccmb.res.in (A. Chattopadhyay).

[1] Equal contribution.

http://dx.doi.org/10.1016/j.bbamem.2014.04.017
0005-2736/

receptors on the host macrophage cell surface such as the mannose-fucose receptor, receptor for advanced glycosylation end products, the fibronectin receptor, the Fc receptor and complement receptors such as CR1 and CR3. Due to the large variety of receptors responsible for parasite entry into host macrophages, no panacea is available for the treatment of leishmaniasis.

The entry of intracellular parasites such as *Leishmania* involves interaction of the parasite with the plasma membrane of host cells. In this context, we were the first to demonstrate the requirement of host membrane cholesterol in the binding and internalization of *Leishmania donovani* into macrophages using complementary approaches [12,15–20]. Membrane cholesterol has also been shown to be necessary for the entry of *Leishmania chagasi* into host macrophages [21]. Interestingly, depletion of plasma membrane cholesterol has recently been reported to result in possible reorganization of the cortical actin cytoskeleton [22–27]. With the overall goal of delineating plasma membrane components of host macrophages responsible for the entry of *Leishmania* and arrive at a comprehensive molecular mechanism of parasite entry, in this work, we have explored the role of the actin cytoskeleton in parasite entry. Our results show that destabilization of the actin cytoskeleton of host macrophages results in a dose-dependent reduction in the attachment of *Leishmania* promastigotes, along with a concomitant reduction in the intracellular amastigote load. Importantly, we demonstrate here that *Leishmania* infection is strongly correlated with cellular F-actin level. To the best of our knowledge, these novel results constitute the first comprehensive demonstration on the specific role of the host actin cytoskeleton in *Leishmania* infection.

2. Materials and methods

2.1. Materials

$MgCl_2$, $CaCl_2$, cytochalasin D (CD), antibiotic antimycotic solution, gentamicin sulfate, IMDM (Iscove's Modified Dulbecco's Medium), M-199 (Medium-199), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), FITC (Fluorescein isothiocyanate) and Giemsa stain were obtained from Sigma (St. Louis, MO). Fetal calf serum (FCS) was purchased from Gibco/Life Technologies (Grand Island, NY), PE (phycoerythrin) rat anti-mouse CD14 antibody was obtained from BD Biosciences (Franklin Lakes, NJ) and Alexa Fluor 546 phalloidin was obtained from Molecular Probes/Invitrogen (Eugene, OR). All other chemicals used were of the highest available purity. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

2.2. Methods

2.2.1. Cell culture

Murine macrophage cell line J774A.1 (American Type Culture Collection) was maintained as described previously [15,28] with some modifications. Cells were maintained in IMDM medium supplemented with 2.4 g/l sodium bicarbonate, 10% heat-inactivated FCS, and antibiotic antimycotic (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) solution in a humidified atmosphere with 5% CO_2 at 37 °C.

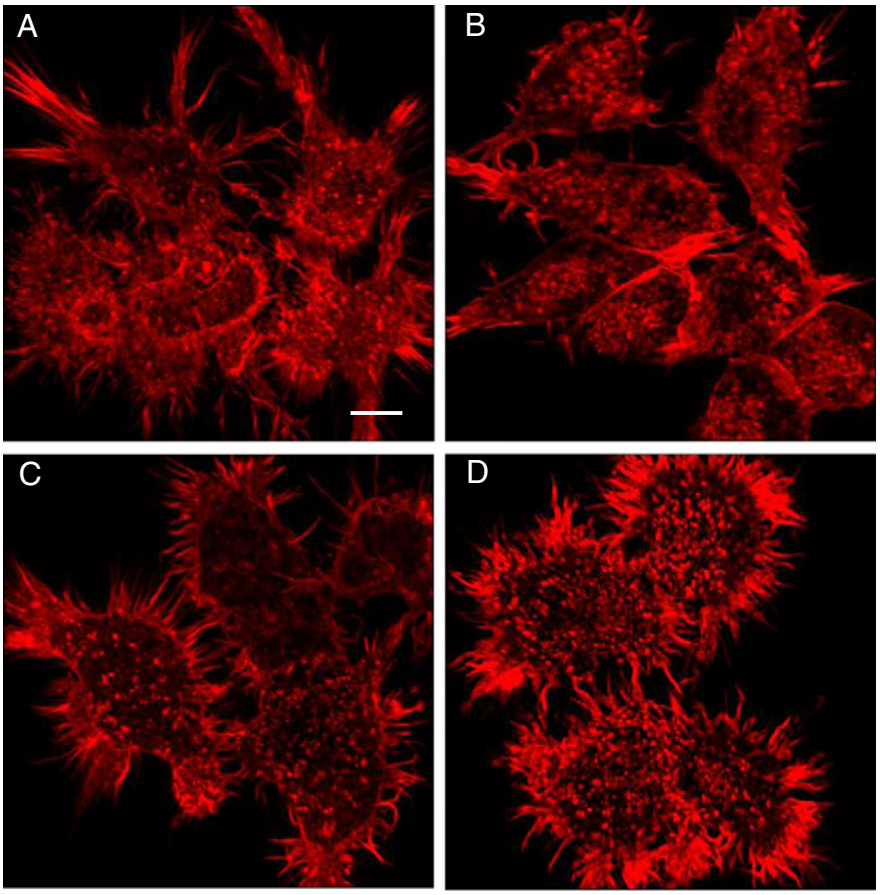

Fig. 1. Organization of the actin cytoskeleton in J774A.1 macrophages treated with increasing concentrations of cytochalasin D (CD). The actin cytoskeleton was stained with Alexa Fluor 546 phalloidin. Projections of 11 sections from the base of the coverslip (~3.5 μm from the base into the cell) are shown. Panel A shows representative projected image for control macrophages, and panels B–D show corresponding images for macrophages treated with 2.5, 5 and 10 μM CD, respectively. Loss of F-actin filaments and formation of F-actin aggregates can be observed upon treatment with increasing concentrations of CD. The scale bar represents 10 μm. See Materials and methods for other details.

2.2.2. Parasite culture

Leishmania donovani strain AG83 (MHOM/IN/1983/AG83) promastigotes were maintained as described previously [29]. Promastigotes were maintained in M-199 medium, supplemented with 200 μg/ml gentamicin sulfate and 10% heat-inactivated FCS at 22 °C. To ensure that only virulent promastigotes were used, we routinely checked their capacity to infect hamsters.

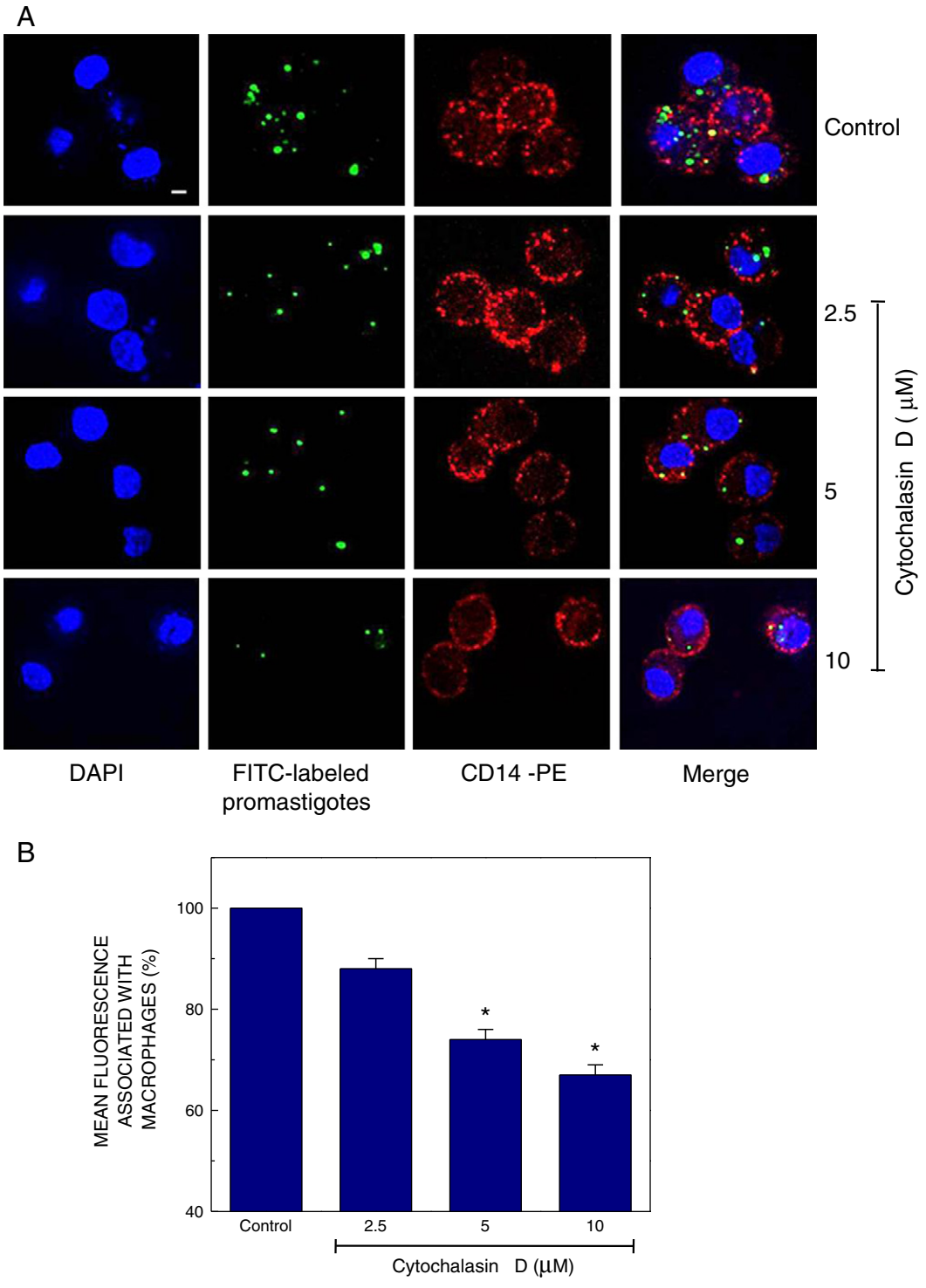

Fig. 2. Effect of destabilization of the actin cytoskeleton of J774A.1 macrophages on binding of FITC-labeled *Leishmania donovani* promastigotes. Actin cytoskeleton of macrophages was destabilized using increasing concentrations of CD. Promastigotes were added onto macrophages at a multiplicity of infection of 10:1 (parasite to macrophage). Panel A shows representative confocal microscopic images of promastigotes bound to untreated (control) and CD-treated macrophages. Macrophage nucleus was stained with DAPI (blue), promastigotes were labeled with FITC (green) and CD14 (macrophage surface marker) was labeled with PE rat anti-mouse CD14 antibody (red). The scale bar represents 10 μm. Panel B shows quantitative estimates of FITC-labeled promastigotes bound to untreated (control) and CD-treated macrophages, as monitored by flow cytometry. Values are normalized to the fluorescence associated with untreated (control) macrophages. Data represent means ± S.E. of duplicate points from four independent experiments (* corresponds to significant ($p < 0.05$) difference in mean fluorescence associated with macrophages treated with CD relative to control macrophages). See Materials and methods for other details.

2.2.3. Cytochalasin D treatment of cells

Destabilization of macrophage actin cytoskeleton was carried out using CD as described earlier [30,31] with some modifications. Briefly, a stock solution of 2 mM CD was prepared in DMSO, and further concentrations for treatments were prepared upon dilution of the stock in serum-free IMDM medium. J774A.1 macrophages were grown for 48 h followed by incubation in IMDM medium without serum and antibiotic antimycotic supplements for 3 h. Actin cytoskeleton was destabilized by treating macrophages with increasing concentrations of CD in serum-free IMDM medium for 30 min at 37 °C. Following the treatment, medium containing CD was removed and macrophages were washed twice with PBS (in order to remove excess CD) before infecting with *Leishmania* promastigotes. The amount of DMSO was always <0.5% (v/v). Treatment of control cells with similar amounts of DMSO did not show any change in cellular morphology.

2.2.4. MTT viability assay

MTT assay was carried out as described earlier [32,33] to assess the viability of J774A.1 macrophages treated with CD. Macrophages in the mid log phase were plated at a density of ~1×10^4 in 96-well plates and treatments were carried out as described in Section 2.2.3. MTT was dissolved in serum-free IMDM medium and added to macrophages at a final concentration of 0.5 mg/ml, followed by incubation at 37 °C for 3 h. Formazan crystals formed upon reduction of MTT salt by mitochondrial enzymes in live cells [32] are insoluble in aqueous medium. Cells were centrifuged in 96-well plates and the formazan crystals formed were subsequently dissolved in DMSO after discarding the medium. The color obtained was measured by absorbance at 492 nm in a Multiskan Ex microplate reader (Thermo Scientific, Hudson, NH).

2.2.5. F-actin labeling of macrophages

Actin labeling in J774A.1 macrophages was carried out as described previously [31]. Briefly, macrophages were plated at a density of ~1×10^4 on glass coverslips and grown in IMDM medium for 48 h followed by incubation in serum-free IMDM medium for 3 h and subsequent treatment with CD. Macrophages were then washed with buffer A (PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$), and fixed with 3.5% (v/v) formaldehyde for 10 min. Permeabilization of cells was carried out in buffer A with 0.5% Triton X-100 (v/v) for 6 min. Cells were washed, stained with Alexa Fluor 546 phalloidin for 1 h, and mounted.

2.2.6. Fluorescence microscopy and F-actin quantitation

All images were acquired on an inverted Zeiss LSM 510 Meta confocal microscope (Jena, Germany). F-actin was imaged by exciting Alexa Fluor 546 phalloidin at 561 nm and collecting emission from 575 to 630 nm. F-actin quantitation was carried out using a technique recently developed by us [31]. Briefly, images of z-sections were acquired with a 63×/1.4 NA oil immersion objective under 1 airy condition, with a fixed step size of 0.32 μm. Projections of 11 sections (~3.5 μm from the base into the cell) were generated, and area of the projected images was determined manually using the software provided with Zeiss LSM 510 Meta confocal microscope. Iso-surfaces (defined as contours made upon joining voxels of equal fluorescence intensity) were generated from z-sections corresponding to each projected image using Imaris 6.0.0 software (Bitplane AG, Zurich, Switzerland). Iso-surfaces were obtained upon fluorescence intensity thresholding of z-sections followed by applying a Gaussian filter. The estimated volumes enclosed by iso-surfaces were normalized to the projected area of cells for a given field.

2.2.7. FITC labeling and binding analysis of L. donovani promastigotes

L. donovani promastigotes were labeled with FITC as described previously [15,34] with some modifications. Briefly, parasites grown to mid log phase were washed with PBS and labeled with 0.1% FITC in buffer B (50 mM carbonate buffer, pH 8.0) for 1 h at 22 °C. Parasites were extensively washed in PBS and suspended in IMDM medium supplemented with 2% heat-inactivated FCS for further experiments. Uniform labeling of promastigotes with FITC was confirmed using flow cytometry. Macrophages treated with CD were washed twice with PBS to remove excess CD. FITC-labeled promastigotes were added onto macrophages at a multiplicity of infection of 10:1 (parasite to macrophage) and incubated for 4 h at 37 °C. Subsequently, macrophages were collected, washed and suspended in PBS. The fluorescence from FITC-labeled parasites associated with 10,000 macrophages was monitored using FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed using the in-built CellQuest Pro analysis software.

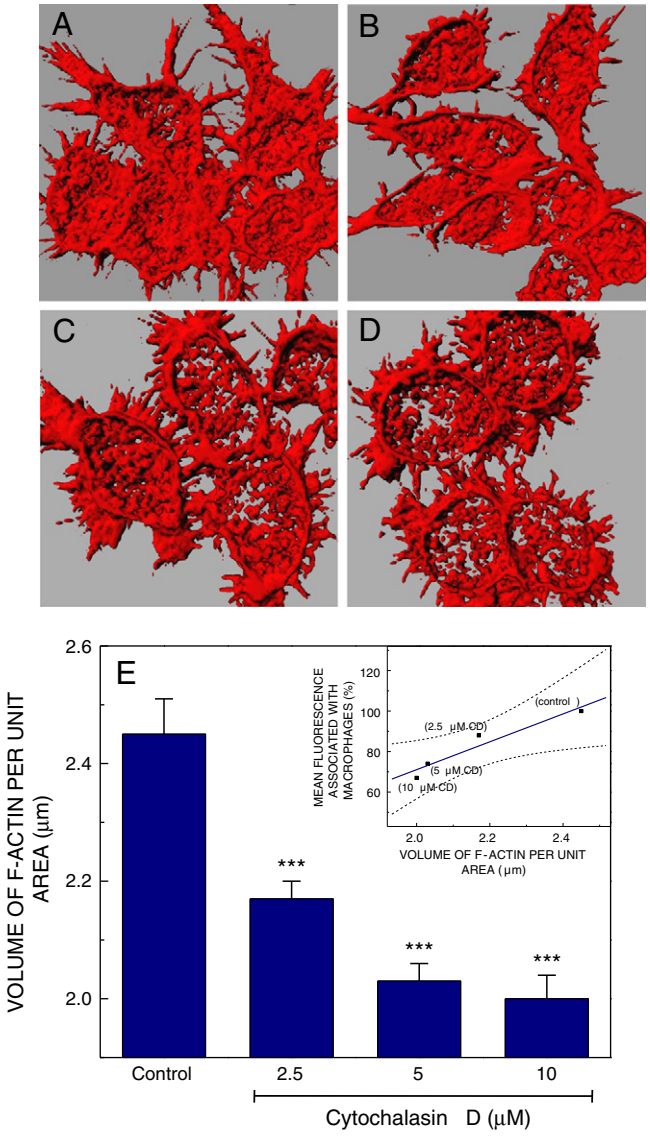

Fig. 3. Iso-surface generation and quantitation of F-actin in J774A.1 macrophages. Iso-surfaces of the sections corresponding to projections shown in Fig. 1 were generated using the iso-surface tool in Imaris. In order to quantitate F-actin, the volume enclosed by the iso-surface was normalized to the projected area of cells obtained using the software provided with LSM 510 Meta confocal microscope. Panel A shows iso-surface for control macrophages, and panels B–D show iso-surfaces corresponding to macrophages treated with 2.5, 5 and 10 μM CD, respectively. Values obtained upon quantitation of F-actin in control and CD-treated cells are shown in panel E. Data represent means ± S.E. of at least 11 independent measurements (*** corresponds to significant ($p < 0.001$) difference in F-actin content in macrophages treated with CD relative to control macrophages). The inset shows correlation of binding of *L. donovani* promastigote and F-actin content of macrophages with increasing concentrations of CD. Data plotted are taken from Figs. 2 and 3E. Linear regression analysis yielded a correlation coefficient (r) ~0.96. The significance of the correlation is apparent from the 95% confidence band (plotted as dashed lines). See Materials and methods for other details.

2.2.8. Microscopic analysis of intracellular amastigote counts

Microscopic analysis of infected macrophages was carried out as described earlier [15,19] with some modifications. *L. donovani* promastigotes were added onto macrophage monolayers grown on coverslips at a multiplicity of infection of 10:1 (parasite to macrophage) and infection was allowed to proceed for 24 h at 37 °C. Macrophages were washed to remove non-phagocytozed parasites and fixed with methanol before staining with Giemsa. The number of amastigotes in

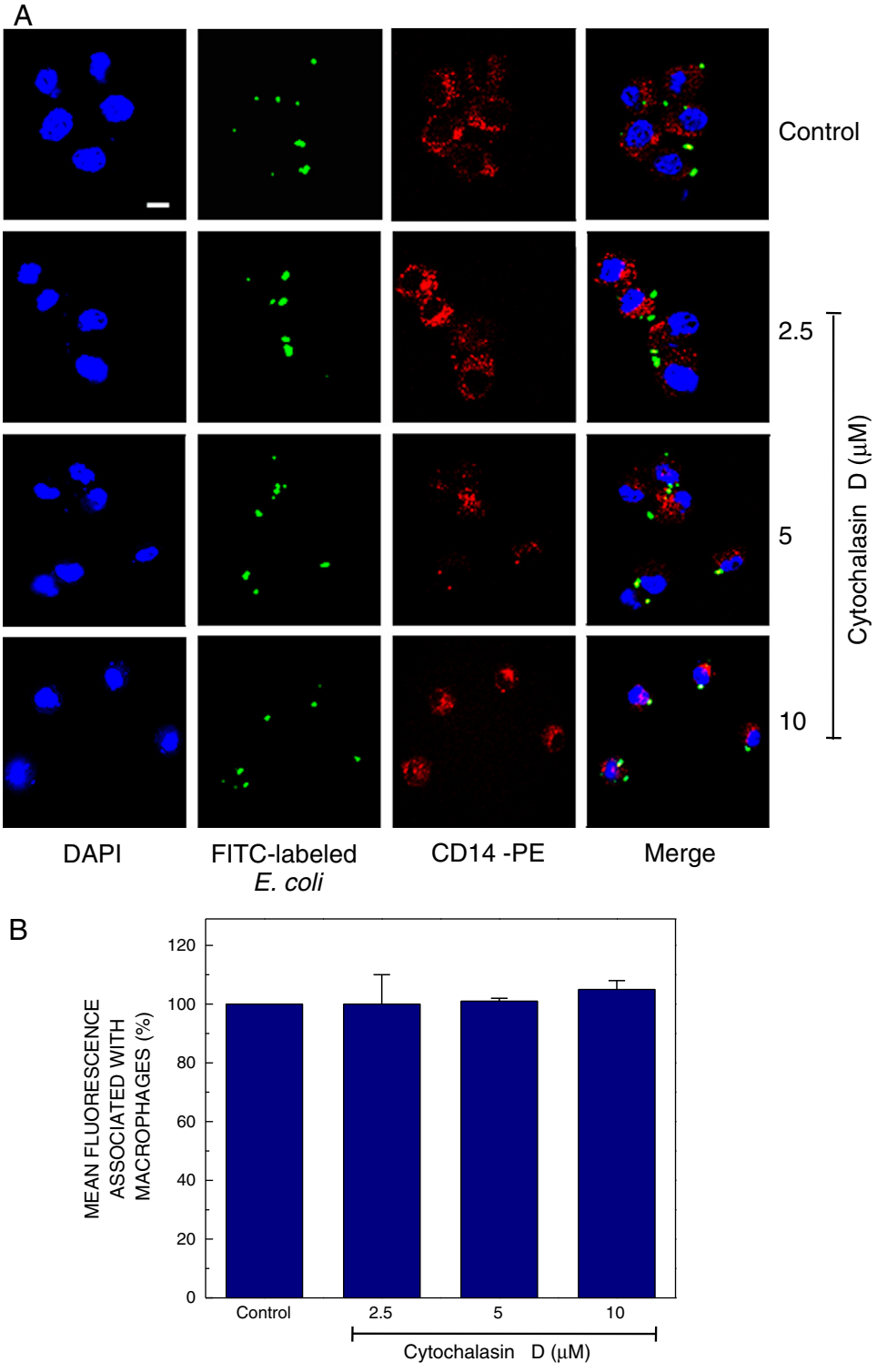

Fig. 4. Effect of destabilization of the actin cytoskeleton of J774A.1 macrophages on binding of FITC-labeled *E. coli* DH5α cells. *E. coli* were added to macrophages, treated with increasing concentrations of CD, at a ratio of 100:1 (bacteria to macrophage). Panel A shows representative confocal microscopic images of *E. coli* bound to untreated (control) and CD-treated macrophages. The macrophage nucleus was stained with DAPI (blue), bacteria were labeled with FITC (green) and CD14 (macrophage surface marker) was labeled with PE rat anti-mouse CD14 antibody (red). The scale bar represents 10 μm. Panel B shows quantitative estimates of FITC-labeled *E. coli* bound to untreated (control) and CD-treated macrophages, as monitored by flow cytometry. Values are normalized to the fluorescence associated with control macrophages. Data represent means ± S.E. of duplicate points from three independent experiments. See Materials and methods for other details.

macrophages was visually scored using a Zeiss microscope with a 100× oil objective, and the amastigote count was normalized to 100 macrophages.

2.2.9. FITC labeling and binding analysis of E. coli

E. coli DH5α cells were labeled with FITC as described previously [15,34] with some modifications. Briefly, *E. coli* was grown overnight in Luria broth at 37 °C under shaking. Bacteria were labeled with 0.1% FITC in buffer B for 30 min at 37 °C while being shaken. Cells were pelleted down and washed extensively with PBS to remove unbound stain. FITC-labeled bacteria were added onto macrophages at a multiplicity of 100:1 (bacteria to macrophage) and incubated for 30 min at 37 °C. Cells were processed and analyzed using flow cytometry as described in Section 2.2.7.

2.2.10. Fluorescence imaging of L. donovani and E. coli binding to macrophages

Macrophages were plated at a density of 2×10^4 on glass coverslips and grown in IMDM medium for 48 h. Macrophages were then treated with CD and subsequently infected with FITC-labeled *L. donovani* promastigotes or *E. coli*, as described in Sections 2.2.7 and 2.2.9, respectively. Cells were washed with PBS to remove unbound promastigotes (or bacteria) and the CD14 macrophage surface marker was labeled with PE rat anti-mouse CD14 antibody for 30 min at 4 °C. Cells were washed, fixed with 3.5% (v/v) formaldehyde and mounted in media containing DAPI. Images were acquired on an Andor Spinning Disc Confocal microscope (Belfast, U.K.) with a 60×/1.42 NA oil immersion objective.

2.2.11. Statistical analysis

Significance levels were estimated using Student's two-tailed unpaired *t*-test using Graphpad Prism software version 4.0 (San Diego, CA). The correlation between promastigote binding and F-actin content of macrophages treated with increasing concentrations of CD was analyzed using the same software with 95% confidence interval. Plots were generated using Microcal Origin software, version 6.0 (OriginLab, Northampton, MA).

3. Results

3.1. Actin organization in host macrophages is altered upon treatment with cytochalasin D

Actin is one of the most abundant cytosolic proteins in eukaryotic cells and exists in both monomeric (globular or G-actin) and polymeric (filamentous or F-actin) forms. F-actin is maintained in cells in dynamic equilibrium with soluble G-actin. Cytochalasins act as potent inhibitors of actin polymerization. Earlier *in vitro* studies have indicated that CD severs polymerized actin by predominantly binding to the barbed (fast growing) end of the actin filament thereby shifting the equilibrium toward depolymerization [35]. However, the mechanism of action *in vivo* appears to be a combination of the above effect of the drug and a secondary cellular response, leading to intensive disruption of the actin cytoskeletal network [36]. In order to monitor the role of the actin cytoskeletal network on the extent of leishmanial infection, we treated host murine macrophage cell line J774A.1 with CD. Fig. 1 shows the effect of increasing concentrations of CD on the organization of the actin cytoskeleton in host macrophages. The figure shows confocal images of the actin cytoskeleton (stained with Alexa Fluor 546 phalloidin) of host macrophages treated with CD. Treatment of host macrophages with CD resulted in fragmentation of F-actin, and the consequent loss of F-actin filaments and formation of F-actin aggregates can be observed under these conditions (see Fig. 1). We found the action of CD on macrophages to be fast, and changes in cellular morphology were visible within minutes of treatment for higher concentrations of CD. We chose to use an optimal concentration range of CD in these experiments so that the cellular morphology would remain more or less intact. We also optimized the period of treatment such that cellular morphology is retained over the time of measurement for the maximal concentration of CD used. In order to assess the effect of CD on cell viability, J774A.1 cells were tested for viability using MTT viability assay following CD treatment. MTT assay is a cell proliferation assay and provides an estimate of the cell growth rate and viability of the cells. No appreciable cell death was observed over the range of concentrations of CD used (see Fig. S1).

3.2. Actin destabilization in host macrophages reduces binding of Leishmania promastigotes

We assessed the role of the actin cytoskeleton on the extent of leishmanial infection by studying (a) parasite interaction with the host cell surface by monitoring the binding of fluorescently (FITC)-labeled promastigotes, the extracellular form of the parasite, by flow cytometric analysis and confocal microscopy; and (b) the eventual presence of the intracellular amastigote form of the parasite inside host macrophages. Fluorescent derivatization of promastigotes with FITC is a convenient method to accurately monitor host-parasite interaction at the cell surface, since each cell is analyzed individually for its ability to bind FITC-labeled promastigotes [15,34,37]. Fig. 2A shows fluorescence microscopy images of *Leishmania donovani* promastigotes bound to macrophages upon destabilization of the host actin cytoskeleton. The figure shows reduction in promastigotes bound to macrophages upon actin destabilization. We quantitated the reduction in promastigote binding upon actin destabilization using flow cytometry (shown in Fig. 2B). Fig. 2B shows a dose-dependent reduction in the binding of promastigotes (normalized to control cells) to host macrophages upon treatment with increasing concentrations of CD. We observed a modest (~12%) reduction in promastigote binding upon mild actin destabilization (2.5 μM CD). With increasing actin destabilization using higher concentrations of CD, we observed progressive reduction in promastigote binding. For example, promastigote binding was reduced by ~33% when 10 μM CD was used (see Fig. 2B). These results show that actin destabilization affects the ability of *Leishmania* promastigotes to interact with the host macrophage cell surface.

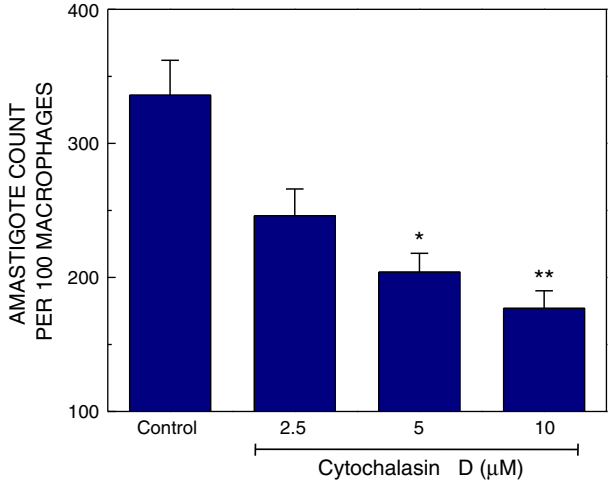

Fig. 5. Effect of destabilization of the actin cytoskeleton on internalization of the parasite assessed by amastigote count in infected J774A.1 macrophages. Macrophages treated with CD were exposed to parasites at a multiplicity of infection of 10:1 (parasite to macrophage) for 24 h. The number of intracellular amastigotes in macrophages was counted subsequent to Giemsa staining. Data represent means ± S.E. of duplicate points from three independent experiments (* and ** correspond to significant ($p < 0.05$ and $p < 0.01$) difference in amastigote count in macrophages treated with CD relative to control macrophages). See Materials and methods for other details.

3.3. F-actin content exhibits dose-dependent reduction upon treatment with cytochalasin D

In order to analyze the reduction in parasite binding in terms of actin reorganization, it is important to quantitatively estimate the extent of actin destabilization under these conditions. Unfortunately, intensity-based analysis is not suitable for quantitation of actin reorganization. This is because treatment with CD results in fragmentation of actin filaments into smaller F-actin aggregates, which appear brighter under a fluorescence microscope. In order to overcome this problem, we recently developed a high resolution microscopy-based approach to quantitatively assess the changes in actin organization. This method allows quantitation of F-actin by high magnification imaging followed by image reconstruction [31]. Fig. 3 (panels A–D) shows iso-surface images corresponding to projected images shown in Fig. 1. In order to quantitatively estimate F-actin, the volumes enclosed by the iso-surfaces were normalized in each case to the projected area of cells. The F-actin content upon CD treatment quantitated this way is shown in Fig. 3E. As can be seen from the figure, CD treatment resulted in a dose-dependent reduction in cellular F-actin level.

3.4. Reduction in promastigote binding to host macrophages strongly correlates with cellular F-actin content

In order to examine a possible correlation between the reduction in cellular F-actin level and the corresponding decrease in promastigote binding to host macrophages, we plotted F-actin levels (from Fig. 3E) *vs.* parasite attachment to host cells (from Fig. 2B). This plot is shown as an inset in Fig. 3E. A linear regression analysis between F-actin level and promastigote binding produced a positive correlation of ~0.96. The 95% confidence intervals contained all the data points, implying a significant relationship between the two parameters observed. Such a tight correlation between F-actin level and parasite binding implies a distinct molecular basis of promastigote binding with cellular actin.

3.5. Actin destabilization of host macrophages does not affect the binding of *E. coli*

To evaluate the specific role of the host actin cytoskeleton in *Leishmania* infection, as a control, we monitored the effect of actin destabilization of host macrophages on the binding of *E. coli* DH5α. Fig. 4A shows fluorescence microscopy images of *E. coli* bound to macrophages upon destabilization of the host actin cytoskeleton. We observed that the binding of *E. coli* remains invariant over the range of CD concentrations used, *i.e.*, is independent of cellular F-actin level. This observation was further confirmed by quantifying the number of *E. coli* bound to macrophages using flow cytometry (shown in Fig. 4B). These results are in sharp contrast to the dose-dependent reduction in promastigote binding (shown in Fig. 2B) upon progressive actin destabilization (Fig. 3E). These results therefore point to the specificity of actin-dependent interaction between *Leishmania* promastigotes and the macrophage cell surface.

3.6. Reduction in promastigote binding is associated with loss of intracellular load of amastigotes

The above results demonstrate that actin destabilization leads to a reduction in the ability of promastigotes to interact with and bind to host macrophages. For efficient infection to take place, binding of the parasite should be followed by internalization and survival. The reduced binding of the promastigotes should therefore manifest as a reduction in the number of amastigotes, the intracellular form of the parasite. The number of amastigotes was scored visually after staining the infected macrophages with Giemsa. Fig. 5 shows that treatment of macrophages with increasing concentrations of CD resulted in a concomitant reduction in the number of amastigotes present (relative to control cells) in the macrophages. The reduction in amastigote count was ~27% when 2.5 μM CD was used (see Fig. 5). With increasing concentrations of CD, further reduction in amastigote load was observed, with ~47% reduction in amastigote count upon treatment with 10 μM CD. These results comprehensively demonstrate that actin destabilization affects the intracellular load of amastigotes. A linear regression analysis between F-actin level and intracellular amastigote load gave a positive correlation of ~0.99 (data not shown).

4. Discussion

The actin cytoskeleton is involved in a variety of cellular responses besides providing structural support. The extent of actin polymerization and depolymerization is orchestrated by a number of actin binding proteins in response to diverse stimuli [38]. This offers a mechanism such that dynamic changes in the actin cytoskeleton act as a transducer in communicating signaling transients. While the role of the actin cytoskeleton in cellular processes such as trafficking and motility has been extensively studied [39], the role of the actin cytoskeleton in the entry of intracellular pathogens has been addressed only rarely.

A number of studies have indicated the crucial requirement of membrane cholesterol in host-pathogen interaction (recently reviewed in [12]). In this overall scenario, we previously demonstrated the requirement of membrane cholesterol in *Leishmania donovani* infection [15,16,19]. Several studies have suggested a possible relationship between membrane domains and the actin cytoskeleton [24]. As mentioned above, depletion of plasma membrane cholesterol has earlier been reported to result in possible reorganization of the cortical actin cytoskeleton [22–27]. For example, it has been reported that depletion of plasma membrane cholesterol could induce changes in the underlying actin cytoskeleton by loss or redistribution of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] molecules in the membrane [22]. In addition, membrane cholesterol depletion has been reported to cause inhibition of neutrophil motility and reduction in actin-dependent protrusions in human neutrophils in response to chemoattractants [40]. With this background, our present results showing reduction in leishmanial infection upon actin reorganization assume significance. Although there have been some earlier reports suggesting a possible role of the actin cytoskeleton in the entry of *Leishmania* species into host cells [41–44], our results represent the first comprehensive demonstration on the specific role of the host actin cytoskeleton in *Leishmania* infection. More importantly, we show here that the host F-actin level and parasite binding are strongly correlated (shown as inset in Fig. 3E). Interestingly, it has been speculated that cytoskeletal reorganization is a requisite for pathogen entry into host cells [45]. To this end, we utilized the above mentioned high resolution imaging approach to quantitate possible changes in host F-actin content upon infection. Our results show that *L. donovani* infection itself does not result in a significant change in F-actin content of host macrophages (see Fig. S2). These observations allow speculation about possible mechanism underlying the interaction of the intracellular pathogen with the host cell surface.

A number of membrane receptors have been recognized as possible targets for facilitating the entry of *Leishmania* into host cells [2,13,14], and metabolic pathways required by the parasite for its survival and virulence have been identified [46]. Cellular signaling and dynamics of membrane receptors have been reported to be regulated by the actin cytoskeleton [27,30,47]. In addition, activation of membrane receptors (particularly, G protein-coupled receptors) induces reorganization of the actin cytoskeleton [31]. A number of studies have shown that membrane domains (sometimes termed 'lipid rafts') are implicated in the entry of pathogens into host cells [45,48,49]. Several key molecules involved in tethering membrane domains to the actin cytoskeleton have been identified [50]. In addition, a feedback mechanism has been proposed between membrane domains and the actin cytoskeleton during cell migration and activation [51]. In this overall context, our present

results on the novel role of the actin cytoskeleton in leishmanial infection in host macrophages assume relevance.

Taken together, our present results show actin as a major cell surface component (besides cholesterol) responsible for parasite entry into host cells. A close correlation between F-actin level and parasite binding and amastigote load reinforces the molecular significance of the role of the actin cytoskeleton in leishmanial infection. We envision that these results could be significant in developing future therapeutic strategies to tackle leishmaniasis in particular, and diseases caused by other intracellular pathogens in general.

Acknowledgments

This work was supported by the Council of Scientific and Industrial Research (Govt. of India) Network project HOPE (BSC0114) and the Indian Council of Medical Research (Govt. of India). S.R. and G.A.K. thank the Council of Scientific and Industrial Research for the award of Research Fellowships. A.C. is an Adjunct Professor at the Special Centre for Molecular Medicine of Jawaharlal Nehru University (New Delhi) and Indian Institute of Science Education and Research (Mohali), and Honorary Professor at the Jawaharlal Nehru Centre for Advanced Scientific Research (Bangalore). A.C. and C.M. gratefully acknowledge support from J.C. Bose Fellowship (Department of Science and Technology, Govt. of India). We thank Hirak Chakraborty and Nandini Rangaraj for helpful discussions, and members of A.C.'s laboratory for critically reading the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.04.017.

References

[1] B.L. Herwaldt, Leishmaniasis, Lancet 354 (1999) 1191–1199.
[2] J. Alexander, A.R. Satoskar, D.G. Russell, *Leishmania* species: models of intracellular parasitism, J. Cell Sci. 112 (1999) 2993–3002.
[3] F. Chappuis, S. Sundar, A. Hailu, H. Ghalib, S. Rijal, R.W. Peeling, J. Alvar, M. Boelaert, Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat. Rev. Microbiol. 5 (2007) 873–882.
[4] P. Kaye, P. Scott, Leishmaniasis: complexity at the host-pathogen interface, Nat. Rev. Microbiol. 9 (2011) 604–615.
[5] World Health Organization website, http://www.who.int/leishmaniasis/en/ .
[6] J. Alvar, I.D. Vélez, C. Bern, M. Herrero, P. Desjeux, J. Cano, J. Jannin, M. den Boer, WHO Leishmaniasis Control Team, leishmaniasis worldwide and global estimates of its incidence, PLoS One 7 (2012) e35671.
[7] J. Alvar, S. Yactayo, C. Bern, Leishmaniasis and poverty, Trends Parasitol. 22 (2006) 552–557.
[8] G. Yamey, E. Torreele, The world's most neglected diseases (editorial), Br. Med. J. 325 (2002) 176–177.
[9] C. Bern, J.H. Maguire, J. Alvar, Complexities of assessing the disease burden attributable to leishmaniasis, PLoS Negl. Trop. Dis. 2 (2008) e313.
[10] D. Wolday, N. Berhe, H. Akuffo, S. Britton, *Leishmania*–HIV interaction: immunopathogenic mechanisms, Parasitol. Today 15 (1999) 182–187.
[11] E. Handman, D.V.R. Bullen, Interaction of *Leishmania* with the host macrophage, Trends Parasitol. 18 (2002) 332–334.
[12] A. Chattopadhyay, M. Jafurulla, Role of membrane cholesterol in leishmanial infection, Adv. Exp. Med. Biol. 749 (2012) 201–213.
[13] M.G. Rittig, C. Bogdan, *Leishmania*-host–cell interaction: complexities and alternative views, Parasitol. Today 16 (2000) 292–297.
[14] D. Sacks, S. Kamhawi, Molecular aspects of parasite–vector and vector–host interactions in leishmaniasis, Annu. Rev. Microbiol. 55 (2001) 453–483.
[15] T.J. Pucadyil, P. Tewary, R. Madhubala, A. Chattopadhyay, Cholesterol is required for *Leishmania donovani* infection: implications in leishmaniasis, Mol. Biochem. Parasitol. 133 (2004) 145–152.
[16] P. Tewary, K. Veena, T.J. Pucadyil, A. Chattopadhyay, R. Madhubala, The sterol-binding antibiotic nystatin inhibits entry of non-opsonized *Leishmania donovani* into macrophages, Biochem. Biophys. Res. Commun. 339 (2006) 661–666.
[17] T.J. Pucadyil, A. Chattopadhyay, Cholesterol: a potential therapeutic target in *Leishmania* infection? Trends Parasitol. 23 (2007) 49–53.
[18] A. Chattopadhyay, R. Madhubala, Method of treating leishmaniasis using methyl-beta-cyclodextrin, U.S. patent # 7186702 (2007).
[19] Y.D. Paila, B. Saha, A. Chattopadhyay, Amphotericin B inhibits entry of *Leishmania donovani* into primary macrophages, Biochem. Biophys. Res. Commun. 399 (2010) 429–433.
[20] A. Chattopadhyay, M. Jafurulla, A novel mechanism for an old drug: amphotericin B in the treatment of visceral leishmaniasis, Biochem. Biophys. Res. Commun. 416 (2011) 7–12.
[21] N.E. Rodríguez, U. Gaur, M.E. Wilson, Role of caveolae in *Leishmania chagasi* phagocytosis and intracellular survival in macrophages, Cell. Microbiol. 8 (2006) 1106–1120.
[22] J. Kwik, S. Boyle, D. Fooksman, L. Margolis, M.P. Sheetz, M. Edidin, Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 13964–13969.
[23] F.J. Byfield, H. Aranda-Espinoza, V.G. Romanenko, G.H. Rothblat, I. Levitan, Cholesterol depletion increases membrane stiffness of aortic endothelial cells, Biophys. J. 87 (2004) 3336–3343.
[24] F.R. Maxfield, I. Tabas, Role of cholesterol and lipid organization in disease, Nature 438 (2005) 612–621.
[25] H.-I. Tsai, L.-H. Tsai, M.-Y. Chen, Y.-C. Chou, Cholesterol deficiency perturbs actin signaling and glutamate homeostasis in hippocampal astrocytes, Brain Res. 1104 (2006) 27–38.
[26] M. Sun, N. Northup, F. Marga, T. Huber, F.J. Byfield, I. Levitan, G. Forgacs, The effect of cellular cholesterol on membrane–cytoskeleton adhesion, J. Cell Sci. 120 (2007) 2223–2231.
[27] S. Ganguly, A. Chattopadhyay, Cholesterol depletion mimics the effect of cytoskeletal destabilization on membrane dynamics of the serotonin$_{1A}$ receptor: a zFCS study, Biophys. J. 99 (2010) 1397–1407.
[28] R. Jain, A. Ghoshal, C. Mandal, C. Shaha, *Leishmania* cell surface prohibitin: role in host–parasite interaction, Cell. Microbiol. 12 (2010) 432–452.
[29] S. Bandyopadhyay, M. Chatterjee, T. Das, S. Bandyopadhyay, S. Sundar, C. Mandal, Antibodies directed against *O*-acetylated sialoglycoconjugates accelerate complement activation in *Leishmania donovani* promasigotes, J. Infect. Dis. 190 (2004) 2010–2019.
[30] S. Ganguly, T.J. Pucadyil, A. Chattopadhyay, Actin cytoskeleton dependent dynamics of the serotonin$_{1A}$ receptor correlates with receptor signaling, Biophys. J. 95 (2008) 451–463.
[31] S. Ganguly, R. Saxena, A. Chattopadhyay, Reorganization of the actin cytoskeleton upon G-protein coupled receptor signaling, Biochim. Biophys. Acta 1808 (2011) 1921–1929.
[32] D.T. Vistica, P. Skehan, D. Scudiero, A. Monks, A. Pittman, M.R. Boyd, Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production, Cancer Res. 51 (1991) 2515–2520.
[33] P. Singh, Y.D. Paila, A. Chattopadhyay, Role of glycosphingolipids in the function of human serotonin$_{1A}$ receptors, J. Neurochem. 123 (2012) 716–724.
[34] B. Khatua, K. Bhattacharya, C. Mandal, Sialoglycoproteins adsorbed by *Pseudomonas aeruginosa* facilitate their survival by impeding neutrophil extracellular trap through siglec-9, J. Leukoc. Biol. 91 (2012) 641–655.
[35] P. Sampath, T.D. Pollard, Effects of cytochalasin, phalloidin, and pH on the elongation of actin filaments, Biochemistry 30 (1991) 1973–1980.
[36] M. Schliwa, Action of cytochalasin D on cytoskeletal networks, J. Cell Biol. 92 (1982) 79–91.
[37] B.A. Butcher, L.A. Sklar, L.C. Seamer, R.H. Glew, Heparin enhances the interaction of infective *Leishmania donovani* promastigotes with mouse peritoneal macrophages: a fluorescence flow cytometric analysis, J. Immunol. 148 (1992) 2879–2886.
[38] C.G. dos Remedios, D. Chhabra, M. Kekic, I.V. Dedova, M. Tsubakihara, D.A. Berry, N.J. Nosworthy, Actin binding proteins: regulation of cytoskeletal microfilaments, Physiol. Rev. 83 (2003) 433–473.
[39] P.A. Janmey, The cytoskeleton and cell signaling: component localization and mechanical coupling, Physiol. Rev. 78 (1998) 763–781.
[40] L.M. Pierini, R.J. Eddy, M. Fuortes, S. Seveau, C. Casulo, F.R. Maxfield, Membrane lipid organization is critical for human neutrophil polarization, J. Biol. Chem. 278 (2003) 10831–10841.
[41] D.J. Wyler, In vitro parasite–monocyte interactions in human leishmaniasis. Evidence for an active role of the parasite in attachment, J. Clin. Invest. 70 (1982) 82–88.
[42] D. Ghosh, P. Chakraborty, Involvement of protein tyrosine kinases and phosphatases in uptake and intracellular replication of virulent and avirulent *Leishmania donovani* promastigotes in mouse macrophage cells, Biosci. Rep. 22 (2002) 395–406.
[43] J. Morehead, I. Coppens, N.W. Andrews, Opsonization modulates Rac-1 activation during cell entry by *Leishmania amazonensis*, Infect. Immun. 70 (2002) 4571–4580.
[44] E. Azevedo, L.T. Oliveira, A.K. Castro Lima, R. Terra, P.M.L. Dutra, V.P. Salerno, Interactions between *Leishmania braziliensis* and macrophages are dependent on the cytoskeleton and myosin Va, J. Parasitol. Res. 2012 (2012) 275436.
[45] B.P. Head, H.H. Patel, P.A. Insel, Interaction of membrane/lipid rafts with the cytoskeleton: impact on signaling and function: membrane/lipid rafts, mediators of cytoskeletal arrangement and cell signaling, Biochim. Biophys. Acta 1838 (2014) 532–545.
[46] M.J. McConville, T. Naderer, Metabolic pathways required for the intracellular survival of *Leishmania*, Annu. Rev. Microbiol. 65 (2011) 543–561.
[47] D.A. Jans, R. Peters, P. Jans, F. Fahrenholz, Vasopressin V_2-receptor mobile fraction and ligand-dependent adenylate cyclase activity are directly correlated in LLC-PK1 renal epithelial cells, J. Cell Biol. 114 (1991) 53–60.
[48] J.-S. Shin, S.N. Abraham, Caveolae as portals of entry for microbes, Microbes Infect. 3 (2001) 755–761.
[49] J. Riethmüller, A. Riehle, H. Grassmé, E. Gulbins, Membrane rafts in host–pathogen interactions, Biochim. Biophys. Acta 1758 (2006) 2139–2147.
[50] G.R. Chichili, W. Rodgers, Cytoskeleton–membrane interactions in membrane raft structure, Cell. Mol. Life Sci. 66 (2009) 2319–2328.
[51] A. Viola, N. Gupta, Tether and trap: regulation of membrane-raft dynamics by actin-binding proteins, Nat. Rev. Immunol. 7 (2007) 889–896.