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Original article

Pleomorphic forms of *Borrelia burgdorferi* induce distinct immune responses

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Abstract

Borrelia burgdorferi is the causative agent of tick-borne Lyme disease. As a response to environmental stress *B. burgdorferi* can change its morphology to a round body form. The role of *B. burgdorferi* pleomorphic forms in Lyme disease pathogenesis has long been debated and unclear. Here, we demonstrated that round bodies were processed differently in differentiated macrophages, consequently inducing distinct immune responses compared to spirochetes *in vitro*. Colocalization analysis indicated that the F-actin participates in internalization of both forms. However, round bodies end up less in macrophage lysosomes than spirochetes suggesting that there are differences in processing of these forms in phagocytic cells. Furthermore, round bodies stimulated distinct cytokine and chemokine production in these cells. We confirmed that spirochetes and round bodies present different protein profiles and antigenicity. In a Western blot analysis Lyme disease patients had more intense responses to round bodies when compared to spirochetes. These results suggest that round bodies have a role in Lyme disease pathogenesis.

Keywords: *Borrelia burgdorferi*; Pleomorphism; Immune response; Colocalization

1. Introduction

Spirochete bacteria *Borrelia burgdorferi*, transmitted by *Ixodes* ticks, is the causative agent of Lyme disease [1]. Innate immune responses initiated by phagocytic macrophages and dendritic cells are important in the clearance of a *B. burgdorferi* infection. Activated macrophages stimulate the adaptive immune response by the production of cytokines and chemokines, and presentation of processed bacterial antigens to naive T cells [2]. *B. burgdorferi* is engulfed into macrophages either by Fcγ-receptor mediated phagocytosis, conventional phagocytosis, or coiling phagocytosis [3]. The Fcγ-receptor mediated phagocytosis requires opsonization of the bacteria before internalization. *B. burgdorferi* does not have lipopolysaccharides (LPS) [4] that results in the recognition by Toll-like receptors (TLRs), such as TLR2/TLR1 heterodimer, on host cell surface [2] with cooperation of intracellular TLRs 7/8 and 9 on the endosomes [5–7]. They recognize pathogen-associated molecular patterns (PAMPs) on the *B. burgdorferi* surface, and initiate signaling cascades that lead to the formation of a phagocytic cup and spirochete internalization.

TLR mediated signaling cascades induce production of several cytokines and chemokines that determine the early immune responses in the host. The phagocytosis and degradation of the bacteria in the phagolysosomes activate TLR signaling within phagolysosomes and further promote cytokine production [5]. *B. burgdorferi* induce expression of multiple cytokines and chemokines in mouse [8] and human [9,10] macrophages.

B. burgdorferi is pleomorphic, and can undergo the morphological transformation as a response to environmental factors [11–15]. Pleomorphic forms of *B.*

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burgdorferi have been observed in a handful of clinical samples [16–20]. In addition, the spinal fluid [21] and human serum [14] have shown to induce RBs that can revert back to motile spirochetes when placed into normal culture medium *in vitro*. Moreover, in preliminary studies RBs have displayed a difference in biochemical characteristics [14], protein profiles, and antigenicity [11,12,18], when compared to the spirochetes. It has been suggested that the pleomorphic forms of *B. burgdorferi* may explain the numerous Lyme disease symptoms and heterogeneous immune responses in individuals with Lyme disease [16]. Although the phenomenon and occurrence of pleomorphic bacteria are quite widely reported in many clinically important bacteria species such as *Escherichia coli* [22], their role in Lyme disease pathogenesis is poorly understood. There is an essential need for immune studies examining the immune response to *B. burgdorferi* pleomorphic forms.

Here, we compared immune responses initiated by spirochetes and RBs. Macrophages internalized more spirochetes per cell and demonstrated higher lysosomal processing compared to RBs. The coiling phagocytosis for RBs could not be clearly demonstrated although involvement of F-actin in internalization of both forms was confirmed. Spirochetes and RBs stimulated distinct cytokine patterns in macrophages. Furthermore, there were differences in protein expression and antigenic properties between the two forms. Interestingly, Lyme disease patients displayed stronger reactivity against RB antigens. We confirmed that pleomorphic forms indeed induce a distinct immune response compared to spirochetes, and they may have a role in Lyme disease pathogenesis.

2. Materials and methods

2.1. Bacterial strains and growth conditions

B. burgdorferi strain B31 was purchased from ATCC (ATCC 35210, Manassas, USA). The fluorescent infectious *B. burgdorferi* strain GCB726 with GFP was kindly provided by George Chaconas, University of Calgary, Canada. Cultures were grown, and RBs were induced as previously described [14].

2.2. Monocyte cell culturing and differentiation to macrophages

Human acute monocytic leukemia (THP-1) cell line was obtained from ATCC (ATCC TIB-202) and cultured in RPMI−1640 medium (Sigma) at 37 °C, 5% CO_2. The medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin (pen/strep), 1% L-glutamine, and 0.05 mM 2-mercaptoethanol (Bio-Rad, CA, USA). Monocytes were differentiated to macrophages using 200 nM phorbol 12-myristate 13-acetate (Sigma, MO) as previously described [23].

2.3. Phagocytosis and colocalization experiments

For analysis of phagocytosis index (PI) and colocalization of spirochetes and RBs with F-actin and lysosomes, coverslips with differentiated macrophages were transferred to medium without 1% pen/strep. In PI studies, *B. burgdorferi* B31 strain was used while colocalization experiments were performed with fluorescent *B. burgdorferi* GCB726 strain. Cells were kept on ice for 20 min prior addition of the bacteria to synchronize phagocytosis. Either 50 μl (10×10^6 bacteria) of *B. burgdorferi* spirochetes or 2 h induced RBs in RPMI medium without antibiotics with a multiplicity of infection (MOI) of 40 were added to cells. For PI analysis cells were incubated for 2 h at 37 °C. For colocalization studies, cells were incubated for 2 h, 8 h, or 24 h. After each time point, cells were washed twice with PBS and fixed with 4% paraformaldehyde/PBS for 20 min RT. Cells were immunolabeled using the previously published protocol [24]. For PI analysis cells were immunolabeled with monoclonal mouse anti-*B. burgdorferi* OspA antibody (Santa Cruz Biotechnology, Dallas, USA) and with the secondary goat anti-mouse antibody conjugated with Alexa 488 (Invitrogen, Massachusetts, USA). For colocalization analysis with Lamp2, cells were immunolabeled similarly except rabbit anti-Lamp2 primary antibody and Alexa 555 or 594 were used. To study colocalization with F-actin cells were stained with Phalloidin-Tetramethylrhodamine B isothiocyanate (Santa Cruz Biotechnology) 1:1000 for 15 min. To inhibit phagocytosis in the colocalization experiments, macrophages were stimulated with spirochetes and RBs in the presence of 10 mM cytochalasin D (Sigma) that was added to the cells 30 min prior the bacteria. Cytochalasin D inhibits phagocytosis by disrupting actin polymerization. To prevent phagosome-lysosome fusion, 30 μM nocodazole was used to inhibit lysosomal pathway for *B. burgdorferi* and Lamp2 colocalization analysis. To allow phagocytosis nocodazole was added to the cells 30 min after the bacteria addition because nocodazole might inhibit phagocytosis in some amount. In both inhibition experiments, cells were fixed after 2 h incubation and immunolabeled/stained as explained above. As a last step, coverslips were mounted using Prolong Gold Antifade Reagent with DAPI (Life Technologies).

2.4. Confocal microscopy and image analysis

For determination of PI image stacks with 0.2 μm intervals from whole cells were acquired using Olympus confocal IX81microscope, 60× objective, 405 and 488 laser, and DIC. The image stacks for colocalization analysis were obtained using Nikon A1R confocal microscope with resonant scanning, 60× objective, and 488 and 561 lasers. In both experiments altogether 40 and 30 cells, respectively, from three independent experiments, were randomly selected and optically sectioned. The brightness and contrast settings of all images, as well as quantification of *B. burgdorferi* engulfment by macrophages from animated multidimensional z-stacks, were applied using open source Fiji software (NIH, USA). The noise from green and red fluorescent images was suppressed

Table 1
Phagocytosis percentages and indexes of *B. burgdorferi* associated differentiated macrophages.

Total	n	Phagocytosed cells % (N)	PhI ± SD
Macrophages and spirochetes	40	75% (30)	3.0 ± 1.8[a]
Macrophages and RBs	40	62% (25)	2.3 ± 1.8

[a] $P < 0.05$ (Mann–Whitney U test). Comparison analysis of phagocytic index between macrophages exposed to spirochetes and 2 h H_2O round bodies (RBs).

using Gaussian blur filter with Sigma (radius) 0.8. The colocalization analysis was performed with open source BioImageXD software [25]. Three slices in total from the top, middle and bottom of each cell z-stack were chosen for quantification of colocalization with Manders coefficient. Thresholds were adjusted manually to eliminate background fluorescence signals. If necessary, a region of interest (ROI) was drawn to image to exclude other cells or bacteria outside the cell from the analysis. For each cell weighted arithmetic mean of coefficients from three slices were calculated. Statistical significances of colocalizations were calculated during analysis using Costes algorithm, and only coincidence probabilities of $P = 1.00$ was taken into account.

2.5. Cytokine and chemokine analysis

For cytokine and chemokine analysis, differentiated macrophages were exposed to spirochetes and RBs for 24 h. *E. coli* DH5α cells were used as a positive control for cytokine expression with the same MOI. The media was collected and centrifuged 16 000 g for 5 min. The analysis was performed using Proteome Profiler™ Human Cytokine Array Panel A Array kit (R&D Systems, Minneapolis, USA), according to the manufacturer's instructions. The kit simultaneously profiles by immunoblotting and chemiluminescence relative levels of 36 inflammation mediators: C5a, CD40 ligand, G-CSF, GM-CSF, CXCL1, CCL1, CD54, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, CXCL10, CXCL11, MCP-1, MIF, MIP-1α, MIP-1β, Serpin E1, RANTES, CXCL12, TNFα, and sTREM-1. Samples were run as duplicates and experiments were performed twice. Immunoblot membranes were imaged with ChemiDoc XRS (Bio-Rad) and intensities were analyzed using ImageJ with Dot Blot Analyzer tool. The data was normalized to the positive control blots on the each membrane. Only those cytokine and chemokine responses that showed relative intensity values >0.01 are reported in this study.

2.6. Two-dimensional gel electrophoresis

Protein profiles of spirochetes and RBs were analyzed with two-dimensional gel electrophoresis (2D PAGE). Approximately $1–2 \times 10^9$ cells were centrifuged 3200 g for 15 min, supernatants were completely removed, and cell pellets were stored at −20 °C until use. 2D PAGE was performed according to the ZOOM IPGRunner System manufacturing instructions (Invitrogen) with modifications. Cells were lysed by sonication for 15 s in a Lysis buffer [910 µl of 1× ZOOM 2D Protein solubilizer 2 (Invitrogen), 3 µl 1 M Tris base (Sigma), 10 µl 2 M DL-dithiothreitol (DTT, Sigma), and 10 µl 10× Protease inhibitor single-use cocktail (Thermo Scientific, Massachusetts, USA), pH 8.4]. DNA was removed by centrifugation 16 000 g for 1 min in QIAShredder microcentrifuge tubes (Qiagen, Hilden, Germany). Dilution into the Lysis buffer and centrifugation washed away all the residual media components that could have interfered with the analysis. The protein concentrations were determined using Nanodrop ND-1000 spectrophotometer (Thermo Scientific) and samples were stored at +4 °C. The immobilized pH gradient (IPG) strips [broad range pH 3–10 (Invitrogen)] were equilibrated for 1 h at RT with the lysed samples (36 µg protein) in rehydration buffer [143 µl 1 × ZOOM 2D Protein Solubilizer 2 (Invitrogen), 0.7 µl 2 M DTT, 0.8 µl Carrier ampholytes pH 3–10 (Invitrogen), and 0.5 µl 0.2% bromophenol blue in EtOH]. Isoelectric focusing was performed using a stepwise program 175 V for 15 min, 1500 V for 45 min, and 2000 V for 45 min. Samples were run on NuPAGE Novex 4–12% Bis-Tris ZOOM gels (Invitrogen) during SDS-PAGE. The strips were restrained on the gel with 0.5% agarose in Tris buffer (124 mM Tris, 960 mM glycine, 17.3 mM SDS in H_2O) and resolved in 1× NuPAGE MES SDS Running buffer (Invitrogen) 200 V for 45 min, with 0.5 ml NuPAGE Antioxidant (Invitrogen) in the upper chamber. Mark12 unstained standard (Invitrogen) was used as the molecular weight standard. Gels were stained with SilverQuest SilverStaining kit (Invitrogen) according to the manufacturer's instructions, except the sensitizing and staining times were doubled (20 and 30 min, respectively). Gels were fixed for 40 min or o/n at RT and imaged with QuantityOne Chemidoc XRS (Bio-Rad). Experiments were performed three times. The intensities of the protein spots were measured with open source software Flicker (Open2DProt). The corresponding protein intensities of spirochetes and RBs were normalized with the molecular standard and mean intensities were compared to examine whether protein expressions were decreased or increased with respect to RBs.

2.7. Western blot

Western blots of whole lysates from spirochetes and RBs were probed with Lyme patient sera to compare the antigenicity of these two forms. Doxycycline treated bacteria (200 µg/ml, 48 h) were used as a control for cell damage. Spirochetes and RBs were collected (2400 g, 15 min), resuspended in 2× SDS reducing loading buffer (0.5 M Tris-HCL, pH 6.8, 10% SDS, 17% β-mercaptoethanol, 0.2% bromophenol blue) and heated 95 °C for 15 min. Protein concentrations were determined with Nanodrop ND-1000 spectrophotometer (Thermo-Scientific), and 20 µg of samples were resolved on 12% SDS-PAGE gels. High and low SDS Laemmli PAGE system molecular weight standards (Sigma) were used. Proteins were then transferred to nitrocellulose membranes (Whatmann, Sigma), blocked with 5% non-fat milk (1% TBS/

0.2% Tween20), and incubated with 1:100 diluted Lyme or Lyme negative sera for 1 h RT. Seven pre-screened Lyme positive patients with sustained manifestations and three negative sera samples were used. Diagnosis was conducted through routine tests used in Borreliosis Centrum Augsburg. Informed consents from the donors were not collected because the data were analyzed anonymously. The German Federal Institute for Drugs and Medical Devices (http://www.bfarm.de) approved the usage of these samples (ethical approval number 95.10-5661-7066). After a series of washes with 1% TBS/0.2% Tween20, membranes were incubated with 1:500 secondary anti-human polyclonal IgG Fc-AP conjugate antibody (Novus Biologicals, Abingdon, UK) for 1 h RT. Developed membranes were imaged with Chemidoc XRS and protein band intensities were analyzed with ImageJ software. The intensities between spirochetes and RBs were compared. The intensity was considered higher in spirochetes or RBs if the difference was $\geq$25%.

2.8. Statistical analyses

All the statistical analyzes were performed using IBM SPSS Statistics. The comparison of phagocytosis between groups was tested with Pearsson's χ^2-test. Non-parametric Mann–Whitney test was used for comparison of phagocytosis indexes. Colocalization and cytokine/chemokine data was analyzed with two-tailed unpaired t-test or with Mann–Whitney U-test. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Differentiated macrophages phagocytosed more spirochetes than RBs

After 2 h stimulation 75% and 62% of the differentiated macrophages associated with *B. burgdorferi* spirochetes or RBs, respectively, had phagocytosed bacteria (Table 1). Furthermore, the number of bacteria inside the cells (mean ± SD) at 2 h time point was quantified. Results demonstrated that macrophages stimulated with spirochetes engulfed significantly more bacteria per cell than cells associated with RBs ($P = 0.045$) (Table 1). This data suggests that RBs are less internalized in macrophages than spirochetes.

3.2. F-actin is involved in the phagocytic uptake of both B. burgdorferi forms

The internalization of spirochetes and RBs into macrophages was analyzed from confocal z-stacks and micrographs. Furthermore, colocalization of *B. burgdorferi* spirochetes and RBs with F-actin in differentiated macrophages at different time points was examined. The goal was to determine if the same mechanisms are involved in the internalization of these two bacteria forms (Fig. 1). F-actin is enriched in the macrophage pseudopodia used for coiling phagocytosis, the predominant uptake mechanism of spirochetes in macrophages [26–28]. As shown in Fig. 1A–F, F-actin was observed to accumulate in the area where bacteria were engulfed indicating the importance of F-actin in internalization of both *B. burgdorferi* forms. During phagocytosis, both spirochetes and RBs were surrounded by the F-actin (Fig. 1A, B, D) and colocalized with it as seen as yellow in Fig. 1A–F. Furthermore, F-actin was occasionally observed to wrap around the spirochetes that is characteristic for coiling phagocytosis. However, the similar long pseudopodia and wrapping of pseudopodia around the RBs was not observed although RBs were enclosed by F-actin and some coiling around them was detected (Fig. 1B, D). At 2 h time point, the colocalization percentage of spirochetes and RBs with F-actin was at its highest without statistical difference between these two forms. There was a minor decrease in the colocalization percentage at 8 h and 24 h time point, however; cells still internalized bacteria (Fig. 1G). Cytochalasin D was used as an inhibitor of phagocytosis for 2 h time point. Cytochalasin D inhibits actin polymerization that is essential for phagocytosis. Inhibition did not stop the uptake of the bacteria completely; nevertheless, it significantly decreased colocalization of both bacteria forms with F-actin ($P = 0.002$ for spirochetes and $P = 0.005$ for RBs) (Fig. 1G).

3.3. Spirochetes colocalize more with lysosomes than RBs

To investigate and compare processing of spirochetes and RBs in phagocytic cells, immunofluorescent colocalization analysis of these bacteria forms with lysosome-associated membrane protein 2 (Lamp2) at 2 h, 8 h, and 24 h time points was performed (Fig. 2A–F). At 2 h and 8 h time points there were no apparent difference in colocalization percentage between spirochetes and RBs, however; at 24 h post-stimulation the difference was significant ($P = 0.028$) demonstrating the more substantial colocalization of spirochetes with lysosomes (Fig. 2G). Nocodazole was used to inhibit the microtubules and autophagosome-lysosome fusion and stop bacteria cargo to lysosomes. Nocodazole treatment significantly decreased colocalization of spirochetes ($P = 0.005$) and RBs ($P = 0.000$) with lysosomes suggesting that lysosomal pathway is important in *B. burgdorferi* processing (Fig. 2G). However, lower colocalization of RBs with lysosomes indicated that RBs might be processed additionally via some other than lysosomal pathway in macrophages.

3.4. RBs induce distinct cytokine response compared to spirochetes

The immune responses of differentiated macrophages stimulated with *B. burgdorferi* spirochetes or RBs were studied using a multiplex analysis of relative levels of secreted cytokines and chemokines. Expression levels of all cytokines induced by these two forms are presented in Fig. 3A–R. Out of 36 examined immune-modulating mediators, *B. burgdorferi*

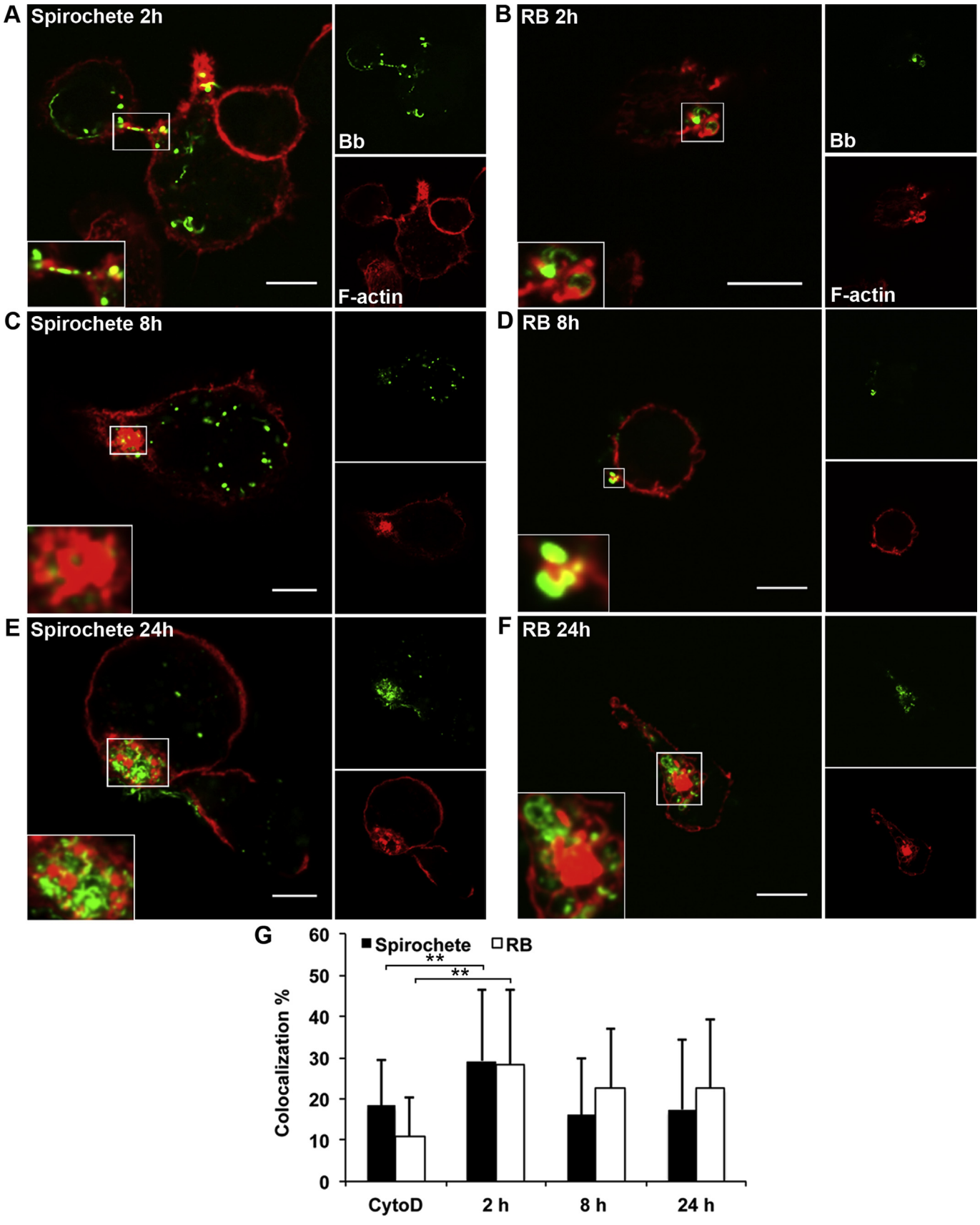


Fig. 1. Differentiated macrophages utilize coiling phagocytosis with F-actin rich pseudopodia to engulf both *B. burgdorferi* spirochetes and round bodies (RB). Confocal micrographs of differentiated THP-1 macrophages stimulated with green fluorescent *B. burgdorferi* (Bb) A) spirochetes for 2 h, B) 2 h H_2O RB for 2 h, C) spirochetes for 8 h, D) RBs for 8 h, E) spirochetes for 24 h, and F) RBs for 24 h with MOI 40. Cells were stained with phalloidin to indicate F-actin on macrophages (red). White squares depict the zoomed area on the left corner of the merged images. All image slices are from the center of the cell. Scale bars 10 µm. G) Mean colocalization percentages ± SD (n = 30) of F-actin with spirochetes or RBs after 2 h, 8 h, and 24 h. Cytochalasin D (CytoD) was used as inhibitor for phagocytosis. Inhibited colocalization was analyzed after 2 h stimulation. **P < 0.01.

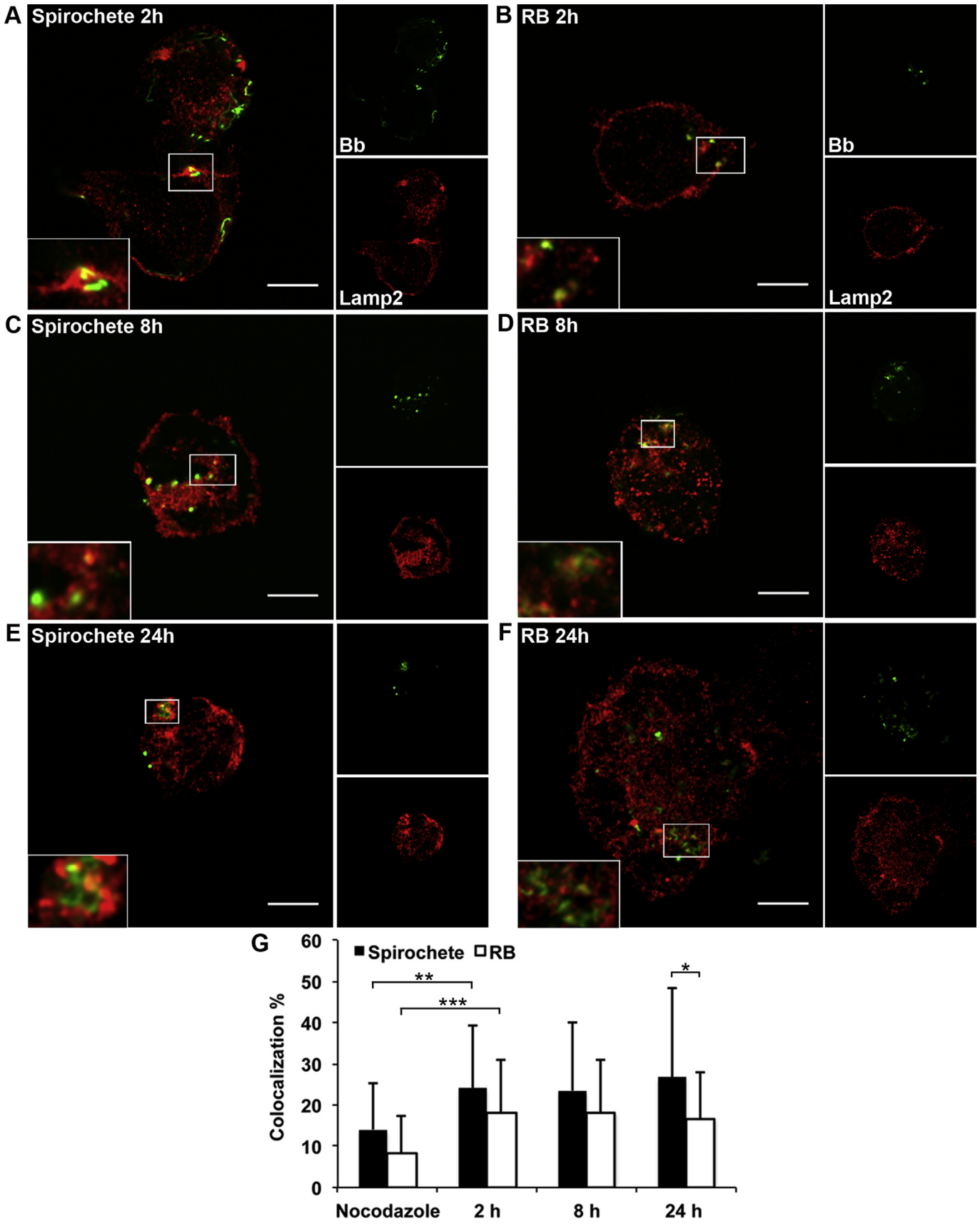
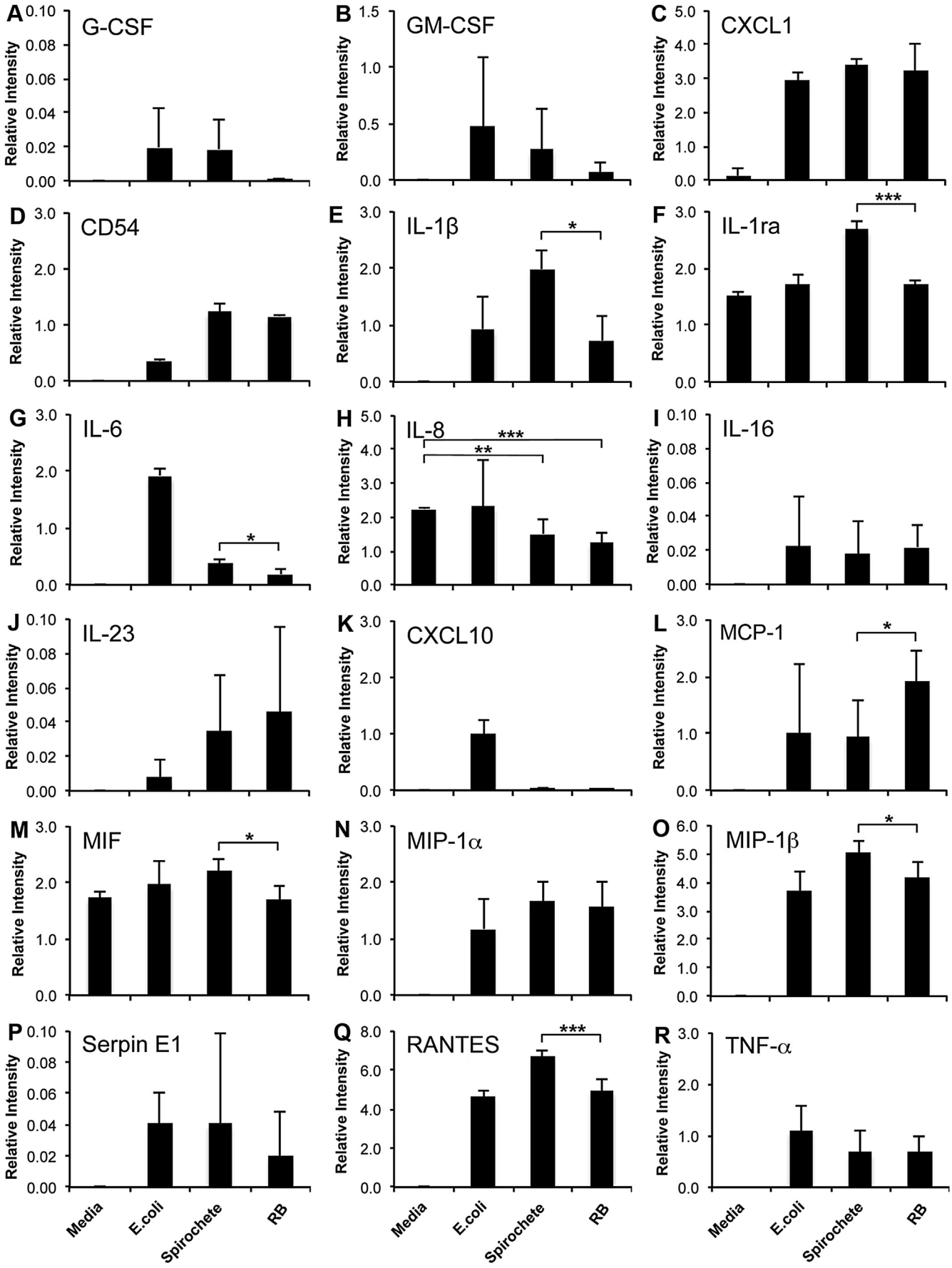


Fig. 2. *B. burgdorferi* spirochetes colocalize more with lysosomes than round bodies (RBs). Confocal micrographs of differentiated THP-1 macrophages stimulated with green fluorescent *B. burgdorferi* (Bb) A) spirochetes for 2 h, B) 2 h H_2O RB for 2 h, C) spirochetes for 8 h, D) RBs for 8 h, E) spirochetes for 24 h, and F) RBs for 24 h with MOI 40. Cells were immunolabeled with Lamp2 antibody and Alexa 555 or 594 (red) for confocal imaging. White squares depict the zoomed area on the left corner of the merged images. All image slices are from the center of the cell. Scale bars 10 μm. G) Mean colocalization percentages ± SD (n = 30) of spirochetes and RBs with Lamp2 at timepoints 2 h, 8 h, and 24 h. Nocodazole was used to inhibit phagosome-lysosome fusion at timepoint 2 h. *P < 0.05, **P < 0.01, ***P < 0.001.


A
G-CSF
B
GM-CSF
C
CXCL1
D
CD54
E
IL-1β
F
IL-1ra
G
IL-6
H
IL-8
I
IL-16
J
IL-23
K
CXCL10
L
MCP-1
M
MIF
N
MIP-1α
O
MIP-1β
P
Serpin E1
Q
RANTES
R
TNF-α
Relative Intensity
Media
E.coli
Spirochete
RB

induced clear expression of 18 distinct compounds. There were 14 cytokines that displayed weak signal on the threshold level of the detection, and they were omitted from the analysis. Chemokines MIP-1β and RANTES demonstrated relatively the highest expression of all studied mediators for both forms (Fig. 3O and Q, respectively). Spirochetes and RBs stimulated production of similar types of cytokines. However, there were significant differences in expression levels of seven specific ones. Spirochetes stimulated significantly higher secretion of IL-1β ($P = 0.012$), IL-1ra ($P = 0.000$), IL-6 ($P = 0.022$), MIF ($P = 0.015$), MIP-1β ($P = 0.028$) and RANTES ($P = 0.001$) compared to RBs (Fig. 3E–G, 3M, 3O, 3Q, respectively). Remarkably, RBs induced a significantly elevated level of MCP-1 ($P = 0.032$) (Fig. 3L) by the macrophages. Exposure to spirochetes and RBs decreased the IL-8 expression of the cells significantly compared to media control (Fig. 3H). Exposure of cells to *E. coli* did not have a similar decrease in expression (Fig. 3H).

3.5. Spirochetes and RBs have differences in protein profiles and antigenicity

Protein profiles of spirochetes and RBs were compared using two-dimensional gel electrophoresis. In total 77 different protein spots were distinguished from the gels with molecular weights ranging between 3 and 200 kDa (Fig. S1A). Corresponding proteins of spirochetes and RBs that displayed higher than 25% difference in mean relative intensity values ($n = 27$) are presented in Fig. S1A and Table 2. Fig. S1A presents 2D gels from all three experiments to address and demonstrate the reproducibility of the gels. Out of these 27 proteins, the expression of 15 proteins was increased in RBs, whereas, the expression decreased in 12 proteins when compared to spirochetes. The 15 proteins that displayed higher relative intensity in RBs were in the molecular range of 15–40 kDa. Proteins that expressed higher intensity in spirochetes were mostly larger, ranging from about 97 kDa to ≥200 kDa. There were two proteins with molecular weights of 4 and 21 kDa that were expressed less in spirochetes (spots 27 and 17, respectively, Fig. S1A, Table 2).

The antigenicity of spirochetes and RBs were compared using western blots probed with seven Lyme patients' sera (Fig. S1B). The intensities of bands on each membrane were compared between spirochetes and RBs (Table 3, Table S1). All the tested positive sera were reactive against both spirochetes and RBs; however, there were differences in reactivity against some antigens. For instance, only five and two patients out of seven had IgG antibodies against 58 kDa and 60 kDa antigens, respectively (Table 3, Table S1). In general, patients with sustained manifestations of Lyme disease reacted stronger against RBs compared to spirochetes. Interestingly, there were four antigens, 21, 39, 60, and 66 kDa, which were predominantly more immunoreactive compared to spirochetes (Table 3, Table S1). Patients reacted against these particular RB antigens more often than the similar ones in spirochetes suggesting that patients indeed react differently against spirochetes and RBs, and RBs have different antigenic properties compared to spirochetes.

4. Discussion

Macrophages are critical to fighting off *B. burgdorferi* infections in mice and humans [29–31,28]. It has been implied that the pleomorphic forms of this bacterium may help in the evasion from the immune system [32]. Our study demonstrated that the induced pleomorphic forms are recognized and engulfed by differentiated macrophages *in vitro*, however; macrophages engulfed significantly more spirochetes than RBs per cell (Table 1). This result indicates a difference in the uptake of these two forms.

Macrophages form F-actin rich pseudopodia when they interact and phagocytose *B. burgdorferi* spirochetes [26,27]. Actin polymerization required for phagocytosis is mediated by CR3 ($\alpha M\beta_2$) and Fcγ receptors [26]. However, it is reported that internalization of unopsonized *B. burgdorferi* is independent of the Fc receptor [33]. Furthermore, many studies have demonstrated *B. burgdorferi* internalization without opsonization [5,34,8,35,36]. Here, we demonstrated that F-actin participates in the uptake of *B. burgdorferi* both spirochetes and RBs without opsonization suggesting that F-actin is important also in the Fcγ independent phagocytosis (Fig. 1). In addition, the cytochalasin D significantly diminished the colocalization of F-actin with both bacteria forms supporting this observation (Fig. 1G). Although the cell were synchronized in the beginning of the experiments, the standard deviations (SD) for colocalization were rather high due to the different rate and timing of phagocytosis between the cells.

Coiling phagocytosis is employed by approximately 60–70% of the macrophage cells for engulfment of *B. burgdorferi* spirochetes [28]. Our results support the previous findings, where coiling phagocytosis is demonstrated for the spirochete uptake (Fig. 1A). However, such an extensive coiling and wrapping was not seen in RBs (Fig. 1B, D). F-actin accumulated in the phagocytosis site of macrophages, surrounded RBs and colocalized with them, nevertheless, within this study it remained unclear whether actual coiling phagocytosis occurred with RBs. RBs are nonmotile and they have completely different morphology compared to spirochetes that might explain why macrophages did not grow such a long and thin pseudopods and coil so profusely. There is also a possibility that spirochetes and RBs are internalized

Fig. 3. Immune mediator expression of differentiated macrophages exposed to *B. burgdorferi* spirochetes (S) and round bodies (RB). *B. burgdorferi* spirochetes, 2 h H_2O RB and *E. coli* DH5α cells with MOI of 50 were incubated with differentiated THP-1 cells for 24 h. Untreated immune cells incubated only with culture media (M) were included as a negative control, while *E. coli* DH5α cells (Ec) with MOI of 50 were used as a positive control for macrophage cytokine and chemokine expression. Mean cytokine production of 18 excreted cytokines (A–R) ± SD ($n = 2$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 2
Protein profile differences of *B. burgdorferi* spirochetes (S) and round bodies (RB).

Spot	~Mw (kDa)	S	RB	Difference%	Spot	~Mw (kDa)	S	RB	Difference%
1	>200	+	−	31.5	15	33	−	+	41.5
2	>200	+	−	45.2	16	31	−	+	27.7
3	>200	+	−	49.3	17	21	+	−	35.7
4	>200	+	−	58.8	18	21	−	+	39.9
5	200	+	−	47.5	19	21	−	+	34.0
6	200	+	−	26.2	20	27	−	+	33.9
7	200	+	−	37.6	21	27	−	+	49.9
8	120	+	−	27.5	22	27	−	+	42.2
9	160	+	−	34.9	23	18	−	+	38.4
10	97	+	−	33.4	24	18	−	+	35.4
11	40	−	+	36.5	25	15	−	+	29.7
12	33	−	+	32.6	26	15	−	+	33.7
13	33	−	+	26.6	27	4	+	−	28.7
14	33	−	+	51.6					

Table 3
Comparison of spirochete (S) and round body (RB) Western blots probed with Lyme patient's serum.

Mw (kDa)	Reactive sera ($n = 7$)	Higher intensity (>25%) against S	Higher intensity (>25%) against RB	No difference between S and RB
93	6	0	4	2
66	5	0	5	0
60	2	0	2	0
58	5	0	3	2
45	7	0	4	3
41	7	2	3	2
39	6	1	5	0
36	6	0	4	2
34	7	1	3	3
31	6	1	3	2
30	6	1	2	3
28	5	1	2	2
23	7	0	2	5
21	1	0	1	0
18	6	1	2	3

differently in macrophages: while spirochete uptake occurs via coiling phagocytosis, RBs internalization might rather utilize conventional phagocytosis.

After internalization with phagocytosis, bacteria are usually transported via the endocytic pathway to lysosomes and finally processed antigens are presented to T-cells by MHC II class molecules. The colocalization of *B. burgdorferi* spirochetes with immunolabeled lysosomal proteins [20,35,31] as well as LysoTracker stained acidic compartments [36] has been previously reported. In those studies, colocalization was observed from early 10 min time points up to 5 h of stimulation. Nevertheless, the quantification of actual colocalization was performed in only one study [31], where 45% of the spirochetes colocalized with lysosomal endopeptidase enzyme cathepsin L at 10 min time point and 57% after 5 h of incubation. Here, to compare lysosomal processing of spirochetes and RBs in macrophages, colocalization analysis was executed at three different time points. The colocalization of spirochetes and RBs with Lamp2 remained at the same level at all three time points 2 h, 8 h, and 24 h (Fig. 2G). In this study, the colocalization percentage of spirochetes with lysosomes was found smaller than in the previous study [31]. This difference may be due to the different analysis methods: in the previous study colocalization was analyzed using kinetic compartmental analysis, while in this study modern image analysis combined with Costes algorithm for statistical significances was used. Interestingly, RBs colocalized less with lysosomes compared to spirochetes at all time points, and the difference was significant after 24 h post-stimulation (Fig. 2G). This result indicates that spirochetes and RBs are processed differently in macrophages. It is suggested that coiling phagocytosis could lead to a presentation of antigens via MCH I molecules [37]. If spirochetes and RBs are phagocytosed differently, that could explain their different processing in macrophages.

Cytokines are thought to have an important role in Lyme disease pathogenesis. The examination of cytokine profiles induced by *B. burgdorferi* spirochete and RBs indicated the production of immune-modulating mediators consistent with the cytokine analysis of C3H and C57 mouse bone marrow-derived macrophages stimulated with *B. burgdorferi* [8]. Cytokines and chemokines expressed in our analysis (Fig. 3) as well as in previous mouse study were G-CSF, GM-CSF, IL-1β, IL-6, CXCL1, CXCL10, MCP-1, MIP-1α, MIP-1β, RANTES and TNF-α. Furthermore, the phagocytosis of spirochetes by human macrophages is reported to increase production of IL-1β, IL-6, IL-10, IFNγ, MCP-1 cytokines and MIP-1α, MIP-1β, TNFα, and CXCL10 chemokines [10]. Anti-inflammatory IL-10 has an important role as a regulator of inflammatory responses in Lyme disease [38] and IFNγ correlates positively with the Lyme disease severity in humans [39]. However, in this study neither spirochetes nor RBs induced IL-10 or IFNγ expression at a level that could be detected. In addition, we highlighted six cytokines with immunological relevance that has not been fully evaluated in Lyme disease (Fig. 3). The expression of CD54 (Fig. 3D), IL-16 (Fig. 3I), MIF (Fig. 3M) and Serpin E1 (Fig. 3P) indicated that there is an enhancement of adhesion, attraction and activation of immune cells. With respect to IL-8, significant decrease in production compared to media control for both spirochete and RB stimulated macrophages (Fig. 3H) could imply a cell specific response.

Furthermore, all of the expressed cytokines, except the IL-23 and MCP-1, were produced in higher levels when cells were stimulated with spirochetes. MCP-1 regulates the migration and infiltration of monocytes, T-cells, and NK-cells, and it potentially has a role in polarization of naïve T cells to Th2 type [40]. MCP-1 expression is required for the development of experimental Lyme arthritis in mice [41], and it is thought to be associated with other autoimmune diseases as well [42]. The higher expression levels of MCP-1, stimulated by *B. burgdorferi* RBs, could propose an involvement of the RBs in Lyme arthritis. In this study, RBs demonstrated decreased macrophage phagocytosis, differences in

phagocytosis mechanisms and lysosomal processing compared to spirochetes. These differences in the entry and processing could correspond to the lower cytokine and chemokine production in general as well as to different cytokine profiles of spirochetes and RBs. The lower expression of these cytokines by RBs could suggest suppressive immune response against these forms, or immune control dysfunction.

The protein profiling of spirochetes and RBs have performed earlier with RPMI serum starved [12] and 1 d H_2O induced RBs [11]. These studies reported protein spots with molecular weights less than 97 kDa. Here, we observed protein spots ranging from 3.5 kDa to higher than 200 kDa from both spirochetes and RBs (Fig. S1A, Table 2). Our results correspond to the genome data of *B. burgdorferi* B31 that the bacterium has proteins of molecular weights from 3.3 kDa to 254.2 kDa [43]. However, the 15 proteins detected with increased intensities in RBs were between the molecular weights of 15–40 kDa. Three proteins with higher protein expression in RBs (spots 11, 18 and 19 in Table 2) were determined corresponding to previously reported [12]. In another study [11] 6–16 kDa proteins were examined with mass spectrometry and the differences between spirochetes and 1 d H_2O induced RBs were not found. In contrast to their results, we observed two 15 kDa proteins in RBs (spots 24 and 26 in Table 2) with elevated expression when compared to spirochetes. This difference may be due to the different exposure times to H_2O, because the longer exposure time used may have decreased the protein expression.

In the previous Western blot analysis [12], 41 kDa (flagellar protein FlaB) and 46 kDa proteins exhibited less reactivity in 48 h serum starved RBs when probed with sera from two infected monkeys and one Lyme patient. In our study, all tested sera reacted against 41 kDa and 45 kDa antigen. These sizes correspond to very immunogenic antigens flagellar protein (FlaB) and VlsE, respectively, as previously reviewed [44]. However, two patients reacted more against 41 kDa flagella on spirochetes, three against RBs, and three had equal response to both (Table 3). Furthermore, four patients reacted more against RB VlsE. These discrepancies to previous findings may be due to the variability of patients' reactivity against this specific antigen, or in the distinct induction method of RBs. In addition, the number of tested sera was higher in our study.

Interestingly, the bands with a molecular weight of 39, 60, and 66 kDa demonstrated predominantly higher intensity in RBs compared to spirochetes. This supports the 2D PAGE results where smaller intensity spots displayed higher intensity in RBs. The 39, 60, and 66 kDa bands correspond to a laminin binding protein BmpA [45], heat shock protein GroEL [46], and p66 [47], respectively. These bands have been previously reported for *B. burgdorferi* B31 western blots probed with patients' sera [48]. Nonetheless, their reactivity against RBs has not been demonstrated before. The role of pleomorphic forms in Lyme disease has been previously criticized [49]. Nonetheless, there are *in vivo* studies that connect RB's to pathogenesis of Lyme disease [16,18,32]. Obviously, more studies are needed to demonstrate mechanisms how RB's are associated with Lyme disease. Overall, this data implies that patients react differently against *B. burgdorferi* spirochetes and RBs suggesting that RBs may have a role in Lyme pathogenesis. Different antigenicity between spirochetes and RBs could add value for diagnostic purposes.

The experiment design with *in vitro* cultured spirochetes and H_2O induced RB's may not be fully equivalent to the conditions *in vivo*. However, these cultured bacteria have similarities when compared to the forms found *in vivo*. For example, the fluorescent *B. burgdorferi* strain, used in these experiments, has shown similar movement *in situ* studies when compared to the spirochetes *in vitro* [50]. In this previous study [50] bacteria were adapted to mouse environment in 1% mouse blood before the experiment. Furthermore, this strain contains all the plasmids required for infectivity. H_2O RB's present similar morphology in these studies when compared to RBs seen in the histopathological samples of the dogs with myocarditis [51], and from chicken primary neuronal, and astrocytic cells [32]. In addition, RB's have been demonstrated to have specific staining properties with wheat germ agglutinin both *in vitro* [14] and in Langerhans cells *in vivo* [18].

Conclusively, these results imply that *B. burgdorferi* RBs have access to immune cells and have the ability to stimulate an immune response. However, there are differences in phagocytosis and processing of these two pleomorphic *B. burgdorferi* forms in macrophages. In addition, the immune response differs from spirochetes especially with lower expression of IL-1β, IL-1ra, MIF, MIP-1β and RANTES. Conversely, RBs stimulate a significantly higher expression amount of MCP-1. Spirochetes and RBs have differences in protein expression and they have different antigenic properties as seen in patients IgG responses. These results indicate that RBs may have the ability to induce distinct immune response compared to spirochetes and that they could be associated with different clinical symptoms seen in patients. We suggest that pleomorphic RBs should be included in the Lyme disease diagnostic tools. The recognition and detection of pleomorphic forms are detrimental to the proper diagnosis and treatment of *B. burgdorferi* infections.

Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micinf.2016.04.002.

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