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Lipid droplet formation in leprosy: Toll-like receptor-regulated organelles involved in eicosanoid formation and *Mycobacterium leprae* pathogenesis

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ABSTRACT

A hallmark of LL is the accumulation of Virchow's foamy macrophages. However, the origin and nature of these lipids, as well as their function and contribution to leprosy disease, remain unclear. We herein show that macrophages present in LL dermal lesions are highly positive for ADRP, suggesting that their foamy aspect is at least in part derived from LD (also known as lipid bodies) accumulation induced during ML infection. Indeed, the capacity of ML to induce LD formation was confirmed in vivo via an experimental model of mouse pleurisy and in in vitro studies with human peripheral monocytes and murine peritoneal macrophages. Furthermore, infected cells were shown to propagate LD induction to uninfected, neighboring cells by generating a paracrine signal, for which TLR2 and TLR6 were demonstrated to be essential. However, TLR2 and TLR6 deletions affected LD formation in bacterium-bearing cells only partially, suggesting the involvement of alternative receptors of the innate immune response besides TLR2/6 for ML recognition by macrophages. Finally, a direct correlation between LD formation and PGE_2 production was observed, indicating that ML-induced LDs constitute intracellular sites for eicosanoid synthesis and that foamy cells may be critical regulators in subverting the immune response in leprosy. *J. Leukoc. Biol.* 87: 371–384; 2010.

Abbreviations: ADRP=adipose differentiation-related protein, B6 mice=C57BL/6J mice, BCG=bacillus Calmette-Guerin, BP=Bodipy (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene), CHO=cholesterol, CHOE=CHO ester, CM=conditioned medium, COX-2=cyclooxygenase type 2, Cyt B=cytochalasin B, DAPI=4′,6-diamino-2-phenylindole, EIA=enzyme immunoassay, FL=fluorescence channel, HPTLC=high-performance thin-layer chromatography, i.pl.=intrapleurally, LD=lipid droplet, LL=lepromatous leprosy, MFI=mean fluorescence intensity, ML=*Mycobacterium leprae*, MOI=multiplicity of infection, NIH=National Institutes of Health, NS-398=N-(2-cyclohexyloxy-4-nitrophenyl) methane sulfonamide, PI=propidium iodide, $TLR^{-/-}$=TLR-signaling deficient, TT=tuberculoid leprosy, WT=wild-type

Introduction

Leprosy, a chronic, infectious disease caused by the obligate intracellular bacterium ML, is still a major source of morbidity in developing countries [1]. The disease principally affects the skin and the peripheral nervous system, in which the leprosy bacillus is found preferentially inside macrophages and Schwann cells [2–4].

Leprosy manifests as a spectrum of clinical forms in correlation with the nature and magnitude of the innate and adaptive immune response generated during ML infection [3, 5, 6]. At one extreme of the spectrum, individuals with TT have few lesions and manifest a contained and self-limited infection in which scarce bacilli are detected as a result of the generation of a strong cellular immune response against ML. At the other end, LL is a progressive and disseminated disease characterized by extensive bacterial multiplication within host cells and low cell-mediated immunity to the pathogen. Although major advances have been made, the mechanisms responsible for the permissive infection observed in individuals with LL remain only partially understood.

The first line of defense against microbial pathogens is composed of macrophages, so that their innate responses are critical in the early containment of infection. Their capacity to engulf and expose microbes to the acidic and hydrolytically active environment of the phagosome is in most cases sufficient to bring about the demise of these microbes [7, 8]. In addition, macrophages are equipped with a full

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range of TLRs and other pattern-recognition receptors capable of sensing the microbial presence [9–13]. The stimulation of TLR signals triggers inflammation and induces macrophage transition to immune effector cells, which by antigen presentation, costimulatory activity, and production of cytokines and chemokines, will dictate the nature of the adaptive immune response to the pathogen. Macrophages are also essential players in mycobacterial infection [11, 13–16]. However, mycobacteria may persist and replicate in macrophages, whether in part by modulating the phagosomal compartment [16–18] or by inducing macrophage deactivation [16, 19].

The clinico-pathological bipolarity observed in leprosy may stem from the dual responses of monocytes and macrophages to ML in LL and TT patients [20]. Indeed, a histopathological hallmark of LL not seen in TT lesions is the presence of large collections of highly infected macrophages, in which bacilli are allowed to multiply and form *globis*. In 1863, Virchow [21] described these macrophages originally, also referred to as Virchow or Lepra cells and often characterized as foamy or lipid-laden cells. These cells accumulate large amounts of lipids, but the mechanisms that regulate this lipid accumulation, as well as whether they are bacterial and/or cell host products remain nebulous [22]. These lipids, including phospholipids and fatty acids, were thought initially to be derived from ML [22]. Very recently, however, a more detailed analysis of the lipid metabolism in LL lesions indicated the accumulation of host-derived, oxidized phospholipids in these cells [23]. Moreover, the same study showed that these lipids are able to down-regulate the innate immune response, suggesting that their accumulation in infected cells may favor bacterial growth and persistence in the host.

In parallel with this information, studies have shown recently that leukocytes are able to accumulate lipids in response to infection and inflammation and that these lipids are organized in nonmembrane-bound cytoplasmic organelles known as LDs or lipid bodies [24, 25]. Consistent with the role of leukocytes in inflammation, the LDs formed by these cells constitute production sites of inflammatory mediators, enriched by arachidonate esterified in neutral lipids, phospholipids, and eicoisanoid-forming enzymes [25–27].

In terms of mycobacterial infections, we have shown recently that *Mycobacterium bovis* BCG, but not *Mycobacterium smegmatis*, a saprophytic bacterium, induced a time- and dose-dependent increase in LD formation and that this induction was mediated through BCG recognition by TLR2 [24, 28]. Moreover, we have also demonstrated that the newly formed LDs were the predominant PGE_2 production sites in BCG-activated macrophages [28]. These data provide evidence that leukocyte LDs may play a critical role in immunity and inflammation.

LD structure consists of a neutral core composed of triacylglycerol, CHOE, and diacylglycerol surrounded by a half-unit membrane of a complex variety of phospholipids [29, 30]. One of the major proteins found on the LD surface is the ADRP (adipophilin), which has been implicated in LD biogenesis and assembling [25, 31]. Besides ADRP and eicosanoid-forming enzymes, other proteins present in LDs include enzymes involved in the biosynthesis and degradation of other lipid molecules, caveolin, proteins of the Rab family [32, 33], protein kinases [34, 35], and cytokines [28, 36], all of which are essentially pointing to a multifunctional role for these organelles in several cellular processes.

In the present study, we detected LDs inside macrophages present in the dermal lesions of LL patients and investigated the mechanisms involved in ML-induced LD formation. The data presented show that LD formation is induced during ML infection and suggest the involvement of these organelles in PGE_2 biosynthesis and leprosy pathogenesis.

MATERIALS AND METHODS

ML

James Krahenbuhl (National Hansen's Disease Program, Laboratory Research Branch, Louisiana State University, Baton Rouge, LA, USA), through National Institute of Allergy and Infectious Diseases/NIH (Bethesda, MD, USA), Contract No. 155262, kindly provided ML, prepared from footpads of athymic *nu/nu* mice. Part of the ML suspension was killed by γ-irradiation [37]. Prior to interactive assays with macrophages, dead bacteria were stained with PI (Sigma Chemical Co., St. Louis, MO, USA) and live bacteria with PKH26 Red Fluorescence cell linker kit (Sigma Chemical Co.). Equal volumes of dead bacterial suspension (1×10^9 ml^{-1} in 10 mM PBS, pH 7.2) and PI solution (100 μg ml^{-1} in PBS) were mixed by rotation for 15 min at room temperature. The PI-labeled bacteria were washed three times in PBS and finally suspended in RPMI 1640 at 1×10^9 bacilli ml^{-1} [38]. Alternatively, live ML was prelabeled with a PKH26, according to the manufacturer's instructions. Aliquots were stored at −20°C and thawed immediately before use. The quality of labeling was checked by fluorescence microscope.

Animals

B6 mice were obtained from the Oswaldo Cruz Foundation Breeding Unit (Rio de Janeiro, RJ, Brazil). Shizuo Akira (Osaka University, Osaka, Japan) donated TLR2 and TLR6 knockout mice in a homogeneous B6 background. Animals were caged with free access to food and fresh water in a room at 22–24°C and a 12-h light-dark cycle. Animal protocols were in agreement with the animal care guidelines of the NIH and were approved by the Animal Welfare Committee of the Oswaldo Cruz Foundation.

Isolation and treatment of mononuclear phagocytes with ML

Mouse resident peritoneal macrophages were recovered from unstimulated mice after peritoneal washings with RPMI medium as described previously [39]. Cells were plated at a density of 1×10^6 cells in 1 ml medium (RPMI 1640 supplemented with 2% heat-inactivated FBS and 1% penicillin-streptomycin; Invitrogen, Eugene, OR, USA)/well in a 24-well tissue-culture plate. For microscope visualization, cells were plated in tissue-culture dishes containing glass coverslips. Peritoneal macrophages were allowed to adhere for 2 h at 37°C in a 5% CO_2 atmosphere and subsequently washed with PBS to remove nonadherent cells. ML was added to the culture at a MOI of five bacilli/cell (5:1) for 1 h. In some experiments, cells infected with BCG (MOI=5) or *M. smegmatis* (MOI=5) or stimulated with LPS (500 ng ml^{-1}) were included as controls. Cultures were then washed three times with PBS and incubated for 24, 48, and 72 h at 37°C in 5% CO_2. Alternatively, mice were i.pl.-injected with ML (5×10^6 bacilli/cavity) in 100 μl sterile saline. Control animals received the same volume of sterile saline only. After 24 and 48 h, the animals were killed by CO_2 inhalation, and their thoracic cavities were washed with 1 ml heparinized PBS (10 UI ml^{-1}).

Human PBMCs were isolated from fresh heparinized blood withdrawn from healthy volunteers and prepared by way of Ficoll-Hypaque density gradient centrifugation (Sigma Chemical Co.). Monocytes were isolated from PBMC by exploiting their ability to adhere to plastic surfaces. The cells were allowed to adhere for 2 h in serum-free medium

and then washed to remove the nonadherent cells. Monocytes were treated with ML as described above.

In the inhibitory studies, cells were pretreated with Cyt B (20 μM, Sigma Chemical Co.) or the nonesteroidal drug NS-398 (1 μM, Biomol, Plymouth Meeting, PA, USA) for 30 min at 37°C. As mentioned previously, after two washings with RPMI medium, cells were treated with ML. The supernatants were collected after 48 h, centrifuged for 5 min at 3000 *g*, filtered (0.2 μm), and then frozen until testing for biological effects.

LD evaluation by microscopy

Cells adhering to coverslips were fixed in 3.5% paraformaldehyde in Ca^{2+}/Mg^{2+}-free HBSS, pH 7.4, for 10 min and stained by osmium tetroxide or BP [40, 41]. Briefly, slides were rinsed in 0.1 M cacodylate buffer (pH 7.4), stained in 1.5% osmium tetroxide (30 min), rinsed in water, immersed in 1.0% thiocarbohydrazide (5 min), rinsed in water, rinsed in 0.1 M cacodylate buffer, restained in 1.5% osmium tetroxide (3 min), rinsed in water, dried, and mounted. For fluorescent LD-labeling, cells were incubated with BP 493/503 dye (Molecular Probes, Eugene, OR, USA) at a final concentration of 1 μM for 45 min at 37°C in PBS, pH 7.8. The slides were stained with 2 μM DAPI (Invitrogen) at room temperature for 5 min. The coverslips were then mounted in 20% glycerol and 1% n-propyl gallate in PBS. Preparations were examined using an Olympus BX-FLA microscope equipped with a Plan Apo 100× 1.4 Ph3 objective (Olympus Optical, Japan) and CoolSNAP-Pro CF digital camera in conjunction with Image-Pro Plus Version 4.5.1.3 software (Media Cybernetics, Bethesda, MD, USA). The images were edited using Adobe Photoshop 5.5 software (Adobe Systems, McLean, VA, USA). The morphology of the fixed cells was observed, and LDs were enumerated at 100× in 50 consecutively scanned cells.

Analysis of LDs using FACS

The adherent cells were detached by trypsin-EDTA treatment (Invitrogen). Cells were washed in PBS, fixed with 4% paraformaldehyde, and incubated with 1 μM BP for 15 min at 37°C, and LD induction was measured at FL1 channel and was expressed as MFI. Bacterial association to cells was measured at FL2 or FL3 by PKH26- or PI-labeled bacteria, respectively. The index of bacterial association (percent) is expressed as percent of cells taking up PI- or PKH26-ML. Two-color flow cytometric acquisition and analysis were performed on a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany), and at least 10,000 cells were analyzed/sample. Quantitative data analysis was performed using BD CellQuest Pro software (BD Biosciences) and WinMDI analysis software.

Immunohistological analysis of ADRP expression

Lesional skin biopsies were obtained from a total of four LL patients diagnosed according to the Ridley-Jopling classification in attendance at the Leprosy Out-Patient Unit (Oswaldo Cruz Foundation). The Ethics Committee of the Oswaldo Cruz Foundation approved the procedures described in this study. Archived, snap-frozen in liquid N_2, tissue sections from leprosum lesions were subjected to immunohistochemical staining as described. Cryostat sections (5 μm-thick) were thawed on sylane-precoated slides and submitted to staining and immunostaining protocols. Standard staining was done with H&E for morphological analysis and Wade-Fite staining to identify the mycobacteria [42]. Immunostaining was performed to detect the ADRP protein and CD68 staining to highlight macrophage cells. For immunodetection of ADRP, biopsies were washed with PBS and blocked and permeabilized with blocking buffer [5% newborn calf serum (Invitrogen) and 0.01% Triton (Sigma Chemical Co.) in PBS] for 1 h at room temperature. Sections were incubated with mouse monoclonal anti-ADRP (AP125; Research Diagnostics Inc., Flanders, NJ, USA), diluted in blocking buffer at 1:25 for 1 h at room temperature. The streptavidin-biotin peroxidase immunostaining duet kit (StreptABComplex/HRP Duet, Dako, Hamburg, Germany) was used according to the manufacturer's protocol. A mouse IgG1 (BD Biosciences) was used as control. Sections were counterstained with hematoxylin. The slides were mounted in 20% glycerol and 1% n-propyl gallate in PBS (pH 7.8) containing 2 μM DAPI (Invitrogen). For immunofluorescence microscopy, biopsies were incubated with primary antibody against ADRP (1:25) or CD68 (1:100; Clone KP1, Dako), diluted in blocking buffer for 2 h at room temperature. Cells were washed extensively with PBS and then incubated with IgG goat anti-mouse secondary antibody conjugated to Alexa488 or Alexa568 (Molecular Probes; 1:250) for 1 h at room temperature. Cells were also labeled with the nuclear stain TO-PRO-3 (1:100; Molecular Probes) for 1 h. After washing with PBS, cells were lastly rinsed with water before mounting on slides. Negative controls were performed under each experimental condition by incubating tissue with secondary but not primary antibodies. Immunodetection was carried out using confocal microscopy. Fluorescence images were acquired with a LSM 510 Zeiss confocal microscope (Carl Zeiss Inc., Thornwood, NY, USA). Images were acquired, colored, and merged via LSM 510 Zeiss software.

Measurement of PGE_2

PGE_2 concentration was measured in cell-free supernatants via an EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA), using a plate reader (Labsystems, Helsinki, Finland). The assays were conducted according to the manufacturer's protocol.

Lipid extraction and analysis

Skin biopsies from LL patients and healthy volunteers were taken with a 6-mm punch. After mechanic disruption of tissues, lipids were extracted with chloroform, methanol, and water (1:2:0.8 by vol) [43] and then partitioned with chloroform and methanol (2:1 by vol), according to the standard procedure of Folch et al. [44]. Neutral lipids were analyzed by one-dimensional HPTLC on Silica gel 60 plates (Merck, Darmstadt, Germany). Plates were first developed in hexane-ethyl ether-acetic acid (60:40:1 by vol) until the solvent front reached the middle of the plate and then in hexane-chloroform-acetic acid (80:20:1 by vol). HPTLC plates were stained by spraying with a charring solution consisting of 10% $CuSO_4$, 8% H_3PO_4, and heated to 180°C for 5–10 min [45]. The charred TLC plate was then subjected to densitometric analysis using a photodensitometer with automatic peak integration (Camag TLC Scanner II). The percentage of CHO and CHOE was calculated from the total amount of lipid (set as 100%) isolated in each skin biopsy.

Cytokine analysis

IL-4, IFN-γ, IL-6, IL-17, TNF-α, IL-12, IL-1β, IL-10, MCP, and keratinocyte-derived chemokine were analyzed simultaneously using luminex technology in supernatants from ML-infected macrophages. A mouse multiplex cytokine kit (Upstate Biotechnology, Lake Placid, NY, USA) was obtained, and the assay was performed according to the manufacturer's instructions using the Bio-Plex system (Bio-Rad, Hercules, CA, USA). Data analysis was performed with the Bio-Plex Manager software.

Statistical analysis

Data analysis was performed using the GraphPad InStat program (GraphPad Software, Inc., San Diego, CA, USA), and the statistical significance ($P<0.05$) was determined by the Student's *t*-test.

RESULTS

Detection of LDs in skin biopsies of LL patients

As described above, a highly characteristic feature seen in dermal lesions of LL patients is the accumulation of infected foam macrophages, also referred to as Virchow cells [21]. Given the fact that other mycobacterial species such as *M. bovis* BCG are good inducers of LD formation in macrophages [28], we hypothesized that Virchow cells are the result of ML-induced LD accumulation during the course of leprosy.

To investigate this hypothesis, we immune-stained tissue sections of LL lesions with specific antibodies that recognize ADRP, a LD marker, and CD68, a macrophage marker. The LL lesion was characterized by many parasitized foam cells containing large multibacillary vacuoles (**Fig. 1A**). The lesion exhibited strong staining for ADRP-labeled LDs (Fig. 1B). Moreover, immunofluorescence staining for CD68 and ADRP demonstrated that the ADRP-reactive cells were CD68+, confirming they were macrophages (Fig. 1C is isotype control; Fig. 1D is ADRP; Fig. 1E is CD68+; Fig. 1F is merged). These data suggest that the foamy aspect of ML-infected macrophages in LL lesions is derived from the accumulation of lipids stored in cellular organelles identified as LDs.

As LDs are known to be enriched in free CHO and CHOE [46–48], the relative content of these lipids was then examined by HPTLC comparing skin biopsies from LL versus healthy controls. This analysis revealed an increase of

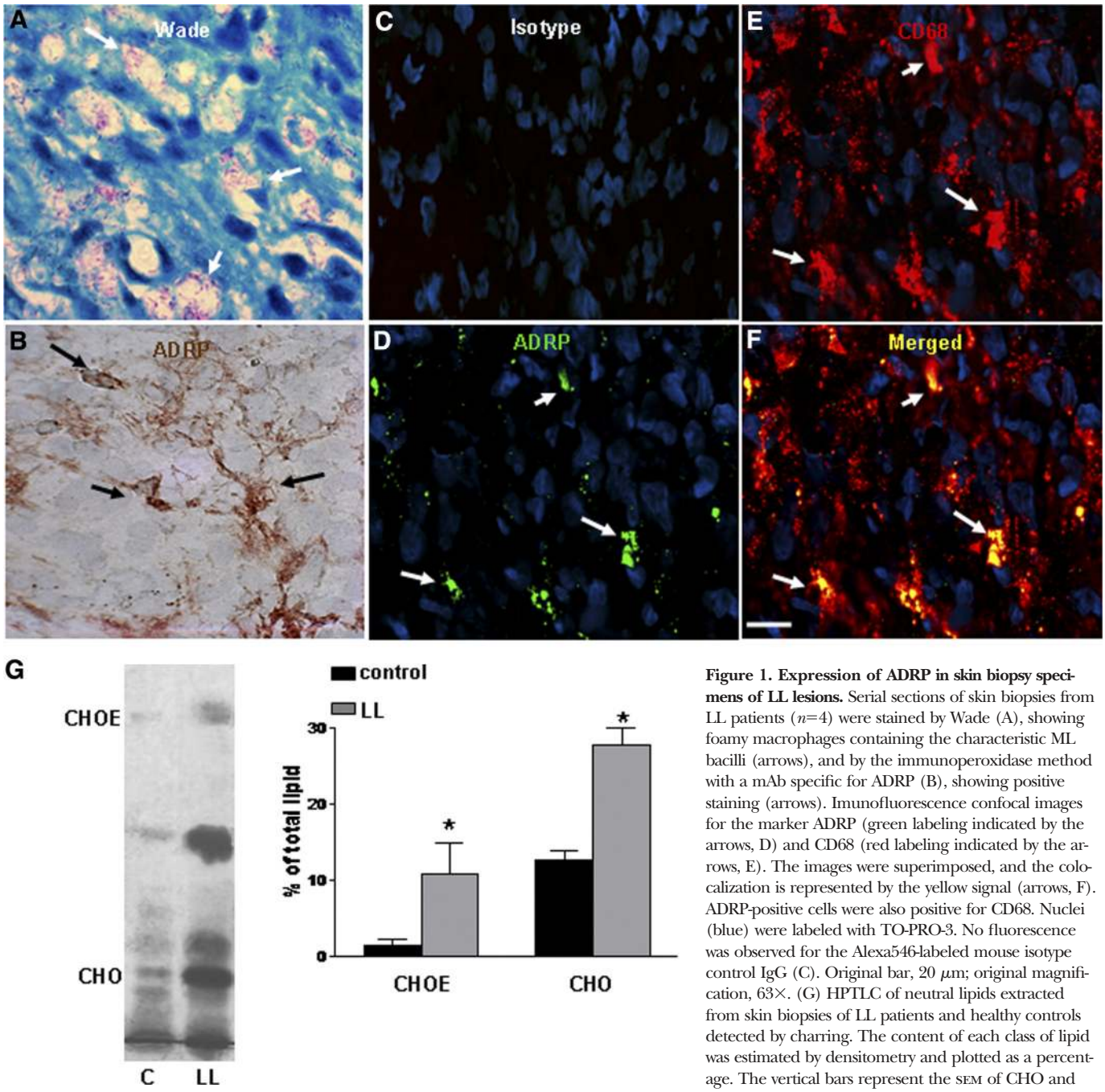

Figure 1. Expression of ADRP in skin biopsy specimens of LL lesions. Serial sections of skin biopsies from LL patients (n=4) were stained by Wade (A), showing foamy macrophages containing the characteristic ML bacilli (arrows), and by the immunoperoxidase method with a mAb specific for ADRP (B), showing positive staining (arrows). Imunofluorescence confocal images for the marker ADRP (green labeling indicated by the arrows, D) and CD68 (red labeling indicated by the arrows, E). The images were superimposed, and the colocalization is represented by the yellow signal (arrows, F). ADRP-positive cells were also positive for CD68. Nuclei (blue) were labeled with TO-PRO-3. No fluorescence was observed for the Alexa546-labeled mouse isotype control IgG (C). Original bar, 20 μm; original magnification, 63×. (G) HPTLC of neutral lipids extracted from skin biopsies of LL patients and healthy controls detected by charring. The content of each class of lipid was estimated by densitometry and plotted as a percentage. The vertical bars represent the SEM of CHO and CHOE levels of three LL patients and three controls performed in duplicates. Bands were identified using pure chemical standards. *, Statistically significant differences (P<0.05) when campared to control groups.

CHO and CHOE in LL as compared with healthy skin (Fig. 1G), suggesting that CHO homeostasis is modulated in response to ML infection, leading to its intracellular accumulation as LDs.

In vitro induction of LD formation by ML

To confirm the capacity of ML to induce the formation of LDs, murine peritoneal macrophages and human mononuclear phagocytes were stimulated in vitro with live and irradiated, killed bacteria. LD formation was determined by microscopic quantification and fluorescence changes measured by flow cytometry. For light microscopy analysis, cells were fixed appropriately, and LDs were enumerated after staining with osmium tetroxide (**Fig. 2A**). Alternatively, the cells were incubated with BP, a lipophilic fluorescent dye, and the LD formation was analyzed by fluorescence microscopy (Fig. 2, B and D) and flow cytometry (Fig. 2, C and E).

Murine and human ML-infected mononuclear phagocytes showed an increase of LD numbers and in the MFI of the BP probe in comparison with untreated cells (Fig. 2, C and E). Moreover, as shown in Figure 2D, the capacity to induce LD formation was independent of bacterial viability. Murine macrophages treated with live or dead ML resulted in similar bacterial associations (data not shown) and induction of LD formation (Fig. 2F). Therefore, as a result of difficulties in obtaining live bacteria, subsequent experiments used irradiated, killed mycobacteria. Cells were also stimulated with other mycobacterial species, corroborating the capacity of BCG (see Fig. 4A) but not *M. smegmatis* (data not shown) to induce LDs in macrophages. Finally, Figure 2G shows that ML-induced LD biogenesis was time-dependent. Murine macrophages were stimulated in vitro with bacteria for periods of time ranging from 24 h to 72 h, and LD formation reached maximum levels at 48 h after treatment (Fig. 2G).

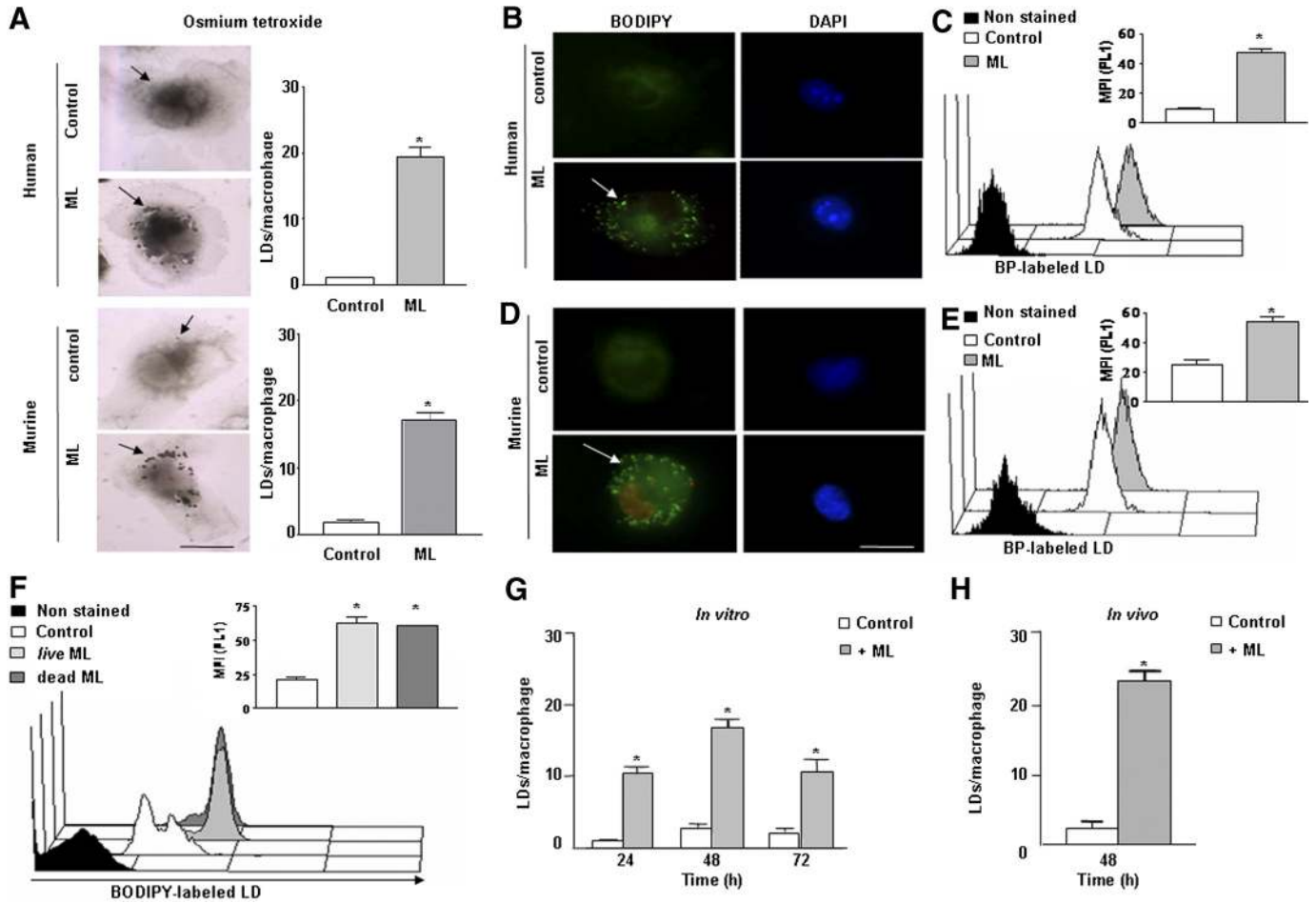

Figure 2. ML induces LD formation in human and murine mononuclear phagocytic cells. (A) LDs were visualized as black cytoplasmatic, punctuate inclusions (arrows) after osmium staining, and the quantification of these organelles was expressed in bar graphs as number of LD/macrophage. (B and D) BP-labeled LD at 48 h subsequent to ML treatment was visualized as green inclusions (arrows). Nuclei were labeled with DAPI. The LD level was monitored by flow cytometry using the BP staining method in human (C) and murine (E) cells. LD biogenesis was independent of bacterial viability (F). Inset graph shows MFI values of BP. Quantification of LD was determined by enumeration of these organelles after osmium staining in the time course of in vitro experiments (G) and in vivo assays (H). All experiments were performed using a MOI of 5, and LDs were determined after 48 h, except for the time-course experiments. *, Statistically significant differences ($P<0.05$) when compared with control groups. Original bars, 20 μm. Data are representative of four independent experiments and are expressed as the mean ± SD of triplicates.

Finally, the capacity of ML to induce LD formation in vivo was confirmed via an experimental model of mouse pleurisy induced upon bacterial i.pl. injection. Cells were recovered 24 h and 48 h after injection, and LDs were then stained with osmium tetroxide and quantified under a light microscope. Figure 2H shows that cells recovered 48 h after ML injection have increased formation of LDs markedly when compared with cells recovered from uninfected animals (from 2.5±0.5 LDs/macrophage in controls to 23.67±1.5 LDs/macrophage in ML-treated animals; P=0.0001). The same results were obtained when cells were analyzed at 24 h postinjection, regardless of whether the bacteria were alive or dead (data not shown). The in vivo and in vitro data obtained so far suggest that LD formation is a common response of macrophages during interaction with ML and that LD is induced notwithstanding mycobacterial viability.

ML-induced LD formation is observed in bacterium-associated cells and in cells with no bacterium

We next investigated whether the effect of ML on LD formation described herein was restricted to cells bearing bacteria. To this end, human peripheral monocytes were isolated and exposed to fluorescent-labeled ML. Cells untreated or exposed to PI-labeled ML were cultured for 48 h. Cells were then stained with BP, and ML-induced LDs were analyzed by flow cytometry and fluorescence microscopy. **Figure 3A** shows that in ML-treated cultures, the LDs were induced significantly in human macrophages bearing fluorescent-labeled bacterium (ML-positive cells, BP^+/PI^+) and in cells with no bacterium (ML-negative cells, BP^+/PI^-) in comparison with untreated cells. However, all bacterium-bearing cells were BP^+, and LD formation was significantly higher in these cells in comparison with those without bacterium (from a MFI value of 15.22±1.4 in ML-negative cells to 43.68±3.0 in ML-positive cells; P=0.0004). An identical result was obtained when murine macrophages were ML-stimulated in vitro (from a MFI value of 27.2±4.4 in ML-negative cells to 62.8±2.6 in ML-positive cells; P=0.002; Fig. 3B) and in vivo (from a MFI value of 132.7±23.1 in ML-negative cells to 633.3±7.0 in ML-positive cells; P=0.002; Fig. 3C). Moreover, immunofluorescence images obtained from murine macrophages treated with PI-labeled ML showed increased numbers of BP-staining LDs in bacterium-bearing cells and cells without bacterium (Fig. 3D).

Taken together, these results suggest that ML induces the secretion of soluble factors in bacterium-associated cells and that these soluble factors may act in a paracrine-signaling circuit to induce LD formation in uninfected cells.

TLR2, TLR6, and cytoskeleton are involved in ML induction of LD formation

The predominant receptor responsible for mycobacterium recognition on macrophages is TLR2 [49]. We have also shown previously that LD formation induced by BCG is mediated by TLR2 [28]. To understand how bacterial association modulates LD formation, the consequences resulting from the absence of TLR2 signaling in ML-stimulated LD formation were analyzed.

Peritoneal macrophages from WT and $TLR2^{-/-}$ mice were stimulated in vitro for 48 h with ML, and LD formation was estimated by flow cytometry analysis and quantified by microscopic analysis. As shown in **Figure 4A**, ML-induced LD formation was partially impaired in TLR2-knockout macrophages (Fig. 4A), although comparable levels of bacterial association were observed in mutant and WT macrophages (Fig. 4A). As expected, no effect of $TLR2^{-/-}$ on LD formation induced by LPS, a classical TLR4 ligand [50], was observed (Fig. 4A). In contrast, BCG, used as a positive control, behaved similarly to ML (Fig. 4A). These results show that although the induction of LD formation in response to ML appeared to be at least partially TLR2-dependent, it is dispensable for bacterial association, indicating that TLR2 does not mediate phagocytic recognition of ML in murine macrophages.

It has been demonstrated previously that TLR2 forms heterodimers with TLR1 or TLR6 in response to different ligands [12, 51–53]. In turn, we investigated whether the TLR2/TLR6 heterodimer is necessary for LD biogenesis during ML association by using $TLR6^{-/-}$ macrophages. LD formation was evaluated by FACS and microscopic analysis. As shown in Figure 4B, LD formation induced by ML was partially inhibited in the absence of TLR6. Moreover, as observed for TLR2, the absence of TLR6 did not affect bacterial association with murine macrophages (Fig. 4B). Thus, these results indicate that TLR6 plays a significant role in LD formation in response to ML, suggesting that the TLR2/6 heterodimer constitutes a ML receptor signal for LD biogenesis in macrophages.

To investigate whether bacterial phagocytosis is required to elicit LD formation, mononuclear cells were pretreated with the actin polymerization inhibitor Cyt B before the addition of ML. The inhibitory effect of Cyt B on phagocytosis was monitored by flow cytometry using bacteria prestained with PI. Figure 4C shows a 50% reduction in the number of cells with associated bacteria in the presence of the drug, coincident with the same reduction in number of LD (Fig. 4C, left) and BP fluorescence (MFI, Fig. 4C). This suggests that ML internalization is involved in triggering the signaling pathways that will culminate in LD formation, although other cytoskeleton-dependent mechanisms besides phagocytosis of the mycobacterium might also participate in the mechanisms involved in LD biogenesis.

TLR2, TLR6, and cytoskeleton are essential for the induction of LD formation by ML-infected cells in uninfected, neighboring cells

To better understand the mechanisms involved in LD induction by ML, we re-evaluated the effect of TLR2 and TLR6 depletions by looking separately at cells bearing or not bearing bacterium. This was performed by means of a two-color fluorescence FACS analysis using PI-labeled bacteria and LDs stained with BP. Interestingly, LD formation was abrogated completely in cells with no bacteria (PI^-; **Fig. 5, A** and **B**). However, the TLR2 or TLR6 effect on LD biogenesis in bacterium-bearing (PI^+) cells was less drastic, partially decreasing the LD formation observed in WT

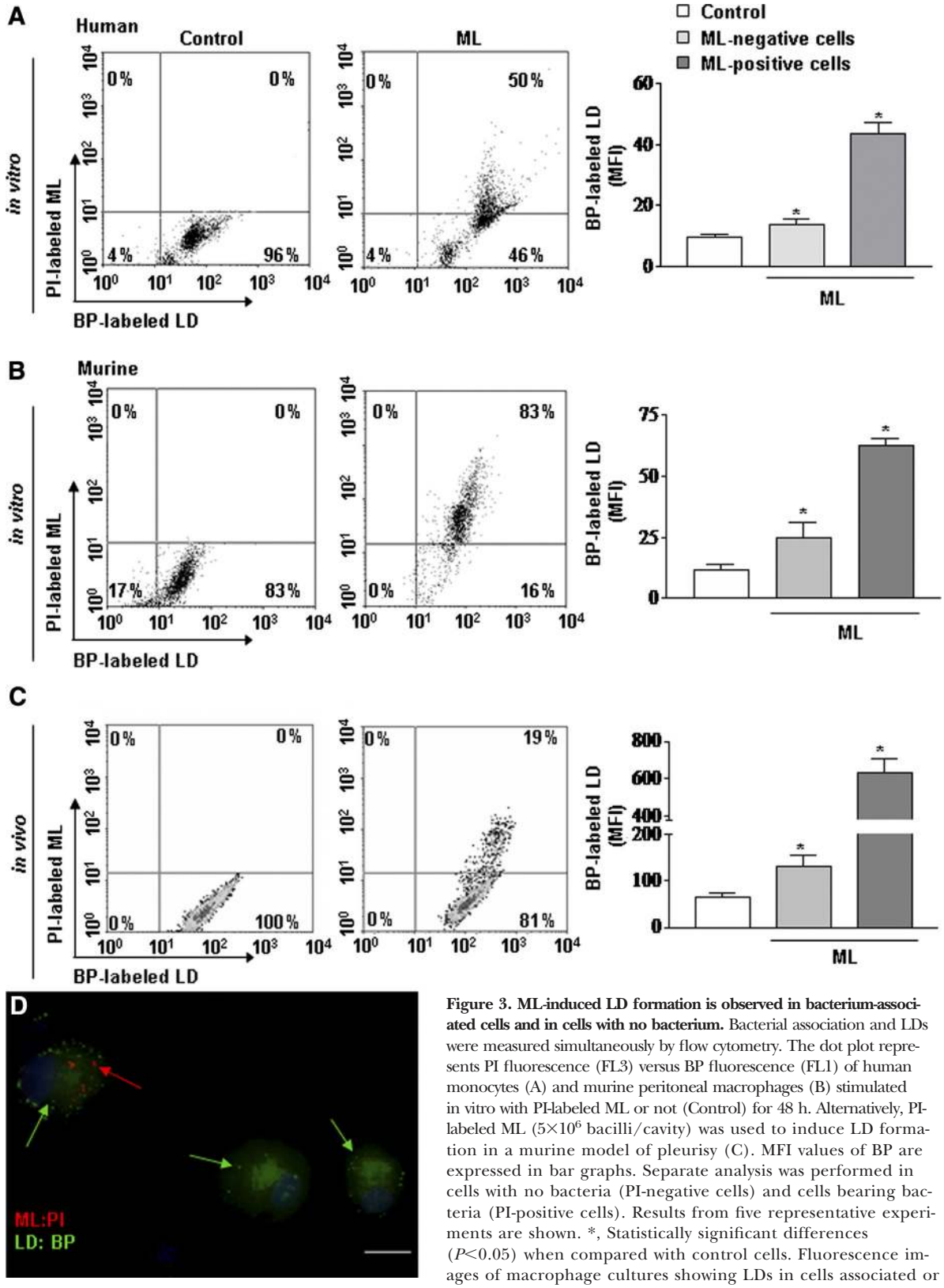

Figure 3. ML-induced LD formation is observed in bacterium-associated cells and in cells with no bacterium. Bacterial association and LDs were measured simultaneously by flow cytometry. The dot plot represents PI fluorescence (FL3) versus BP fluorescence (FL1) of human monocytes (A) and murine peritoneal macrophages (B) stimulated in vitro with PI-labeled ML or not (Control) for 48 h. Alternatively, PI-labeled ML (5×10^6 bacilli/cavity) was used to induce LD formation in a murine model of pleurisy (C). MFI values of BP are expressed in bar graphs. Separate analysis was performed in cells with no bacteria (PI-negative cells) and cells bearing bacteria (PI-positive cells). Results from five representative experiments are shown. *, Statistically significant differences ($P<0.05$) when compared with control cells. Fluorescence images of macrophage cultures showing LDs in cells associated or not with ML (D). Red arrow, PI-labeled ML; green arrows, BP-stained LDs. Original bar, 20 μm.

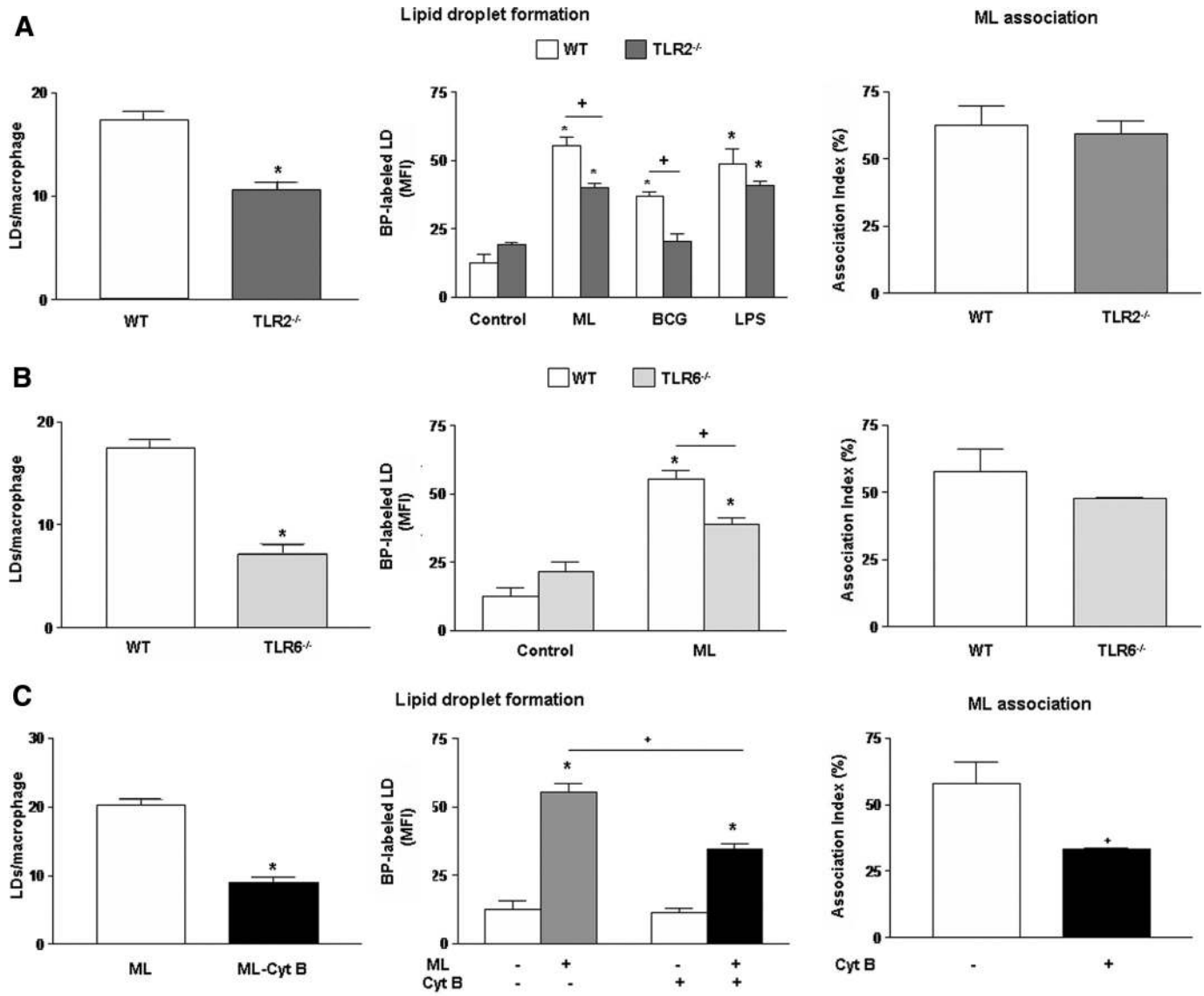

Figure 4. TLR2/TLR6 and cytoskeleton are involved in ML induction of LD formation. Peritoneal macrophages from WT, TLR2$^{-/-}$, or TLR6$^{-/-}$ B6 mice were treated with ML, and LD formation was determined by microscopic quantification (osmium staining) and flow cytometry as described in Figure 3. Reduced LD formation in response to ML was observed in TLR2 (A) and TLR6 (B) knockout macrophages, although the association index of ML with these cells was identical to that of WT macrophages. PI-labeled ML (PI-ML) was used to determine the percentage of cells bearing bacteria by flow cytometry. (A) Cells were also stimulated with BCG (MOI=5) or LPS (500 ng ml^{-1}) as positive and negative controls, respectively. (C) The addition of Cyt B reduced LD biogenesis and the bacterial association index significantly. Data are representative of five independent experiments. Data indicate mean ± SD of triplicates. *, Statistically significant differences (P<0.05) between ML-stimulated and control cells. +, Significant differences (P<0.05) between the different ML-treated cell groups.

cells. Identical results were obtained when the effect of Cyt B was analyzed (Fig. 5C), indicating that TLR2/TLR6 and the process of phagocytosis or other cytoskeleton-dependent mechanisms are required to complete induction of LD formation in bacterium-bearing cells. On the other hand, in infected cells, TLR2/TLR6 and phagocytosis are indispensable for the LD induction in uninfected, neighboring cells. Imunofluorescence images of the same cultures clearly show the exclusive presence of LDs in cells bearing ML in TLR2$^{-/-}$, TLR6$^{-/-}$, or Cyt B-treated macrophages (Fig. 5, A–C, right panel), in contrast to Cyt B-untreated WT cells, in which numerous LDs can be seen in infected and uninfected cells (Fig. 3D).

Media conditioned by ML-stimulated macrophages mimicked the LD induction activity of the bacteria

To test the hypothesis that ML induces macrophages to secrete soluble factors that regulate LD formation, the CM of ML-treated cells was tested for the capacity of CM to induce

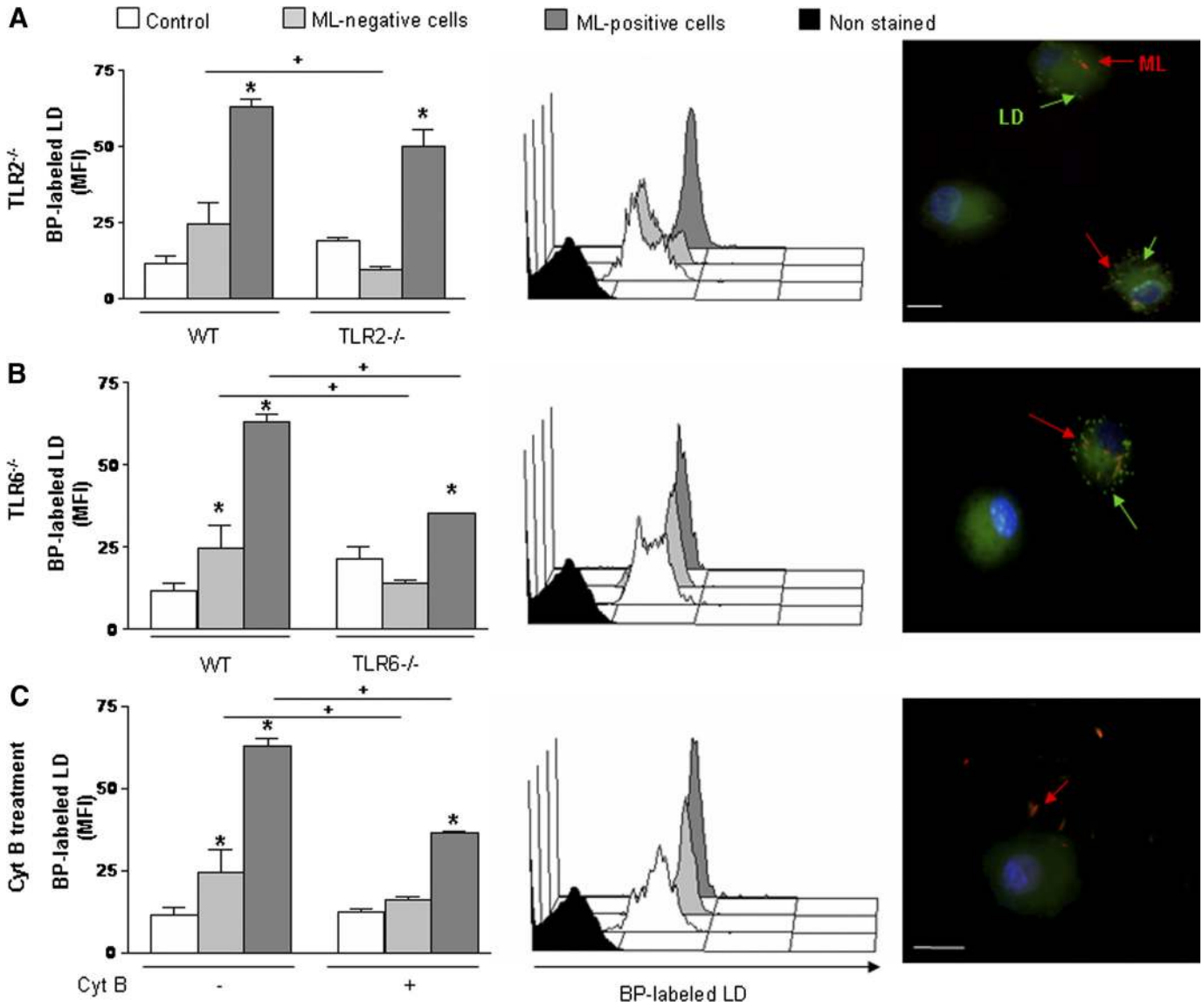

Figure 5. TLR2/TLR6 and cytoskeleton are essential for the induction of LD formation by ML-infected cells in neighboring, uninfected cells. Macrophages from WT, TLR2$^{-/-}$, and TLR6$^{-/-}$ B6 mice were stimulated with PI-labeled ML, and LD formation was monitored in cells bearing bacteria (PI^{+}) versus cells with no bacterium (PI^{-}; A and B). The same analysis was performed in WT macrophages pretreated or not with Cyt B (C). Histograms show LD fluorescence (FL1) in PI^{-} and PI^{+} ML-treated cells, in unstimulated macrophages, and unstained cells. (Right panels) Fluorescence images of BP-labeled LDs (green labeling, arrows) in macrophages after PI-labeled ML (red labeling, arrows) stimulation. Images correspond to TLR2$^{-/-}$ (A), TLR6$^{-/-}$ (B), or Cyt B-treated cells (C). Nucleated cells were marked with DAPI (blue). Original bars, 20 μm. Data are presented as the mean ± SD from three independent experiments. *, Statistically significant differences (P<0.05) between the macrophage groups treated with ML and control cells. +, Significant differences (P<0.05) between the WT and TLR macrophages and between macrophages pretreated or not with Cyt B.

LD formation. CMs were generated from peritoneal macrophages, treated or not with ML for 48 h, and then used to treat fresh cells for another 48 h. As shown in **Figure 6A**, incubation of macrophages with the CM from cells pretreated with ML resulted in significant LD induction relative to the CM from control cells (from a MFI value of 20.2±1.6 in control cells to 37.06±1.9 in cells treated with CM from ML-treated cells; P=0.002). This result confirms that soluble factors are secreted upon ML-macrophage interaction and that these factors are capable of inducing LD formation in uninfected cells.

Generation of the paracrine signal by ML-infected cells is dependent on cytoskeleton and TLR2

As cytoskeleton was shown previously to be essential in inducing LD formation in adjacent, uninfected cells, we next tested the capacity of the CM generated from ML-treated macrophages in the presence of Cyt B to induce LD forma-

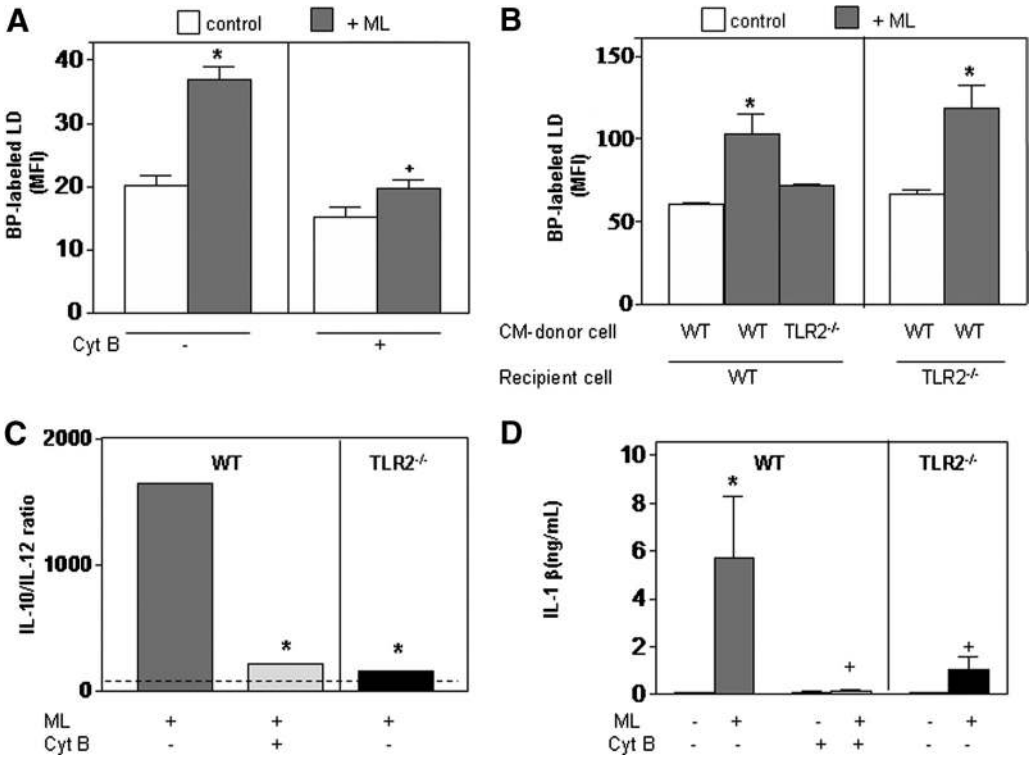

Figure 6. The generation of the paracrine signal in ML-infected cells is dependent on cytoskeleton and TLR2. (A) Murine macrophages were treated with CM from macrophages pretreated or not with ML, and LD formation was determined by flow cytometry. CM from ML-treated cells induced LD formation, in contrast to CM taken from control cells or cells pretreated with Cyt B prior to bacterial stimulation. (B) Effect of TLR on paracrine signaling induced by ML. WT or TLR2$^{-/-}$ macrophages were treated with CM from control WT cells, ML-treated WT cells (ML-WT), and ML-stimulated TLR2$^{-/-}$ cells (ML-TLR2$^{-/-}$). (C) Cytokine/chemokine levels were determined by multiplex assay in the CM following treatment with ML in macrophages pretreated with Cyt B or TLR2 depleted. IL-10/IL-12 ratios are expressed as percentage of the ratio observed in CM from control, ML-treated macrophages. (D) IL-1β production (ng mL^{-1}). Data are presented as the mean ± SE from three independent experiments. *, Statistically significant differences (P<0.05) between the macrophage groups treated with CM derived from ML-stimulated cells and cells treated with CM from control cells. +, Significant differences (P<0.05) between the macrophage groups treated with CM from ML-stimulated cells, pretreated or not with Cyt B.

tion. As expected, CM generated in the presence of the drug showed no effect on LD formation (Fig. 6A), suggesting that bacterial internalization is necessary to trigger cell-signaling events that would culminate in the release of LD-inducing molecules from ML-associated macrophages. As a whole, these results suggest that ML phagocytosis is essential to inducing the secretion of molecules that will act in a paracrine circuit to induce LD formation in adjacent cells.

The involvement of TLR2/TLR6 in the generation of this paracrine signaling was then investigated, as the absence of these receptors abrogated ML induction of LD formation completely in uninfected cells (Fig. 5, A and B). The TLR2/TLR6 heterodimer could be acting at the infected-cell level and participating in the signaling cascade necessary for the production of soluble factors, or the heretodimer may be acting as a pathogen-associated molecular pattern receptor on the membrane of uninfected cells in recognizing the bacterial molecules originating from cell-bearing bacteria. To discriminate between these two possibilities, we recovered CM from WT and TLR2$^{-/-}$ cells, pretreated or not with ML, and subsequently, tested the ability of CM to induce LDs in WT and TLR2$^{-/-}$ cells. When CM from WT ML-treated macrophages were used to stimulate TLR2$^{-/-}$ cells, a significant increase in LD formation was observed (from 60.3±0.6 LDs in control cells to 118.3±13.8 LDs in CM-treated cells; P=0.04), indicating that TLR2 is not required for macrophages to respond to LD-inducing molecules secreted by infected cells (Fig. 6B). However, CM obtained from ML-treated TLR2$^{-/-}$ cells failed to induce LD formation in cells expressing TLR2 (WT; from a MFI value of 66.8±2.2 in control cells to 71.4±1.3 in CM-treated cells; P=0.2), indicating that infected macrophages require TLR2 expression to generate the paracrine signal capable of inducing LD formation.

As reviewed extensively elsewhere, mycobacterial infection modulates the production by macrophages of a number of cytokines [54, 55]. In addition, it has been described previously that cytokines and chemokines may participate in the signaling that leads to LD formation [25, 47, 56, 57]. Thus, to investigate the potential involvement of cytokines/chemokines as LD-inducing molecules secreted by ML-infected macrophages, a multiplex cytokine analysis was performed comparing active (CM produced by ML-infected WT macrophages) with inactive (CM derived from ML-infected TLR2$^{-/-}$ and cells pretreated with Cyt B) LD-forming CM. The only cytokine that showed a significant decrease in CM Cyt B-treated and TLR2$^{-/-}$ ML-infected cells was derived from IL-1β (Fig. 6C). Interestingly, the IL-10/IL-12 ratio (taken as a reflection of an anti- vs. proinflammatory balance) followed a similar trend as IL-1β, with lower values in inactive CM, suggesting that this cytokine balance may favor LD formation in uninfected cells. In these assays, no correlating was observed with TNF-α and MCP-1 levels, corroborating previous results, in which these mediators were shown not to participate in LD induction promoted by BCG [28].

Correlation between LD formation and PGE_2 production in ML-treated mononuclear cells

We have demonstrated previously that LDs formed in response to BCG constitute sites for eicosanoid synthesis, leading to increased production of PGE_2 by infected macrophages [24, 28]. We then investigated whether regulation of LD numbers by ML also correlated with PG production.

Figure 7, A and **C**, shows that human and murine macrophages produced significantly increased levels of PGE_2 after 48 h of ML infection, coinciding with the time-point of the highest LD formation. Moreover, NS-398, a nonsteroidal, anti-inflammatory drug, shown previously to inhibit BCG-induced LD formation and PGE_2 production [28], was also able to abrogate ML-induced LD formation and PGE_2 production completely in murine macrophages (Fig. 7A). Lastly, treatment with Cyt B or the TLR2 knockout, conditions shown previously to reduce ML-induced LD formation, was also shown to inhibit PGE_2 production partially (Fig. 7, A and B). Thus, within the context of ML infection, these results indicate a correlation between LD formation and lipid mediator production.

DISCUSSION

Leprosy provides an excellent opportunity to investigate mechanisms of innate and adaptive immunity in humans. As LDs are emerging as key organelles involved in the innate immune response during bacterial infection [24, 28, 58], in the present study, we investigated the generation of these organelles during ML infection. The data herein presented show that LD formation is induced during ML infection, based on the following findings: the identification of LDs inside macrophages present in the dermal lesions of LL patients via immunodetection of ADRP; and the rapid accumulation of these organelles in ML-treated human monocytes and murine macrophages and subsequent to in vivo i.pl. infection.

Our results indicating the accumulation of LDs enriched in CHO and CHOE in LL lesions are consistent with previous reports demonstrating that several host genes involved in lipid metabolism, including ADRP and CD36, a selective lipid receptor, are up-regulated in LL lesions and that at least part of the lipids accumulated in macrophages are host-derived, oxidized phospholipids [23, 59, 60]. In further support of these data, another recent report has attested to the localization of ADRP in phagosomes containing ML in LL lesions and the capacity of ML to induce the expression of this protein in human phagocytes [61].

In vivo and in vitro assays showed that ML-induced LD formation was independent of bacterial viability, suggesting that bacteria may play a passive role in LD formation and that the host cell might be the active player in this process. This observation is consistent with our previous data showing that dead BCG (ref. [28] and K. A. Mattos, unpublished results) and mannose-capped lipoarabinomannan purified from BCG, a cell-wall constituent also present in ML, are able to induce LD in macrophages [28]. Another interesting observation was the capacity of ML to induce LD accumulation in bacterium-associated cells and in those cells with no bacterium. It was then possible to investigate the mechanisms of LD induction by ML

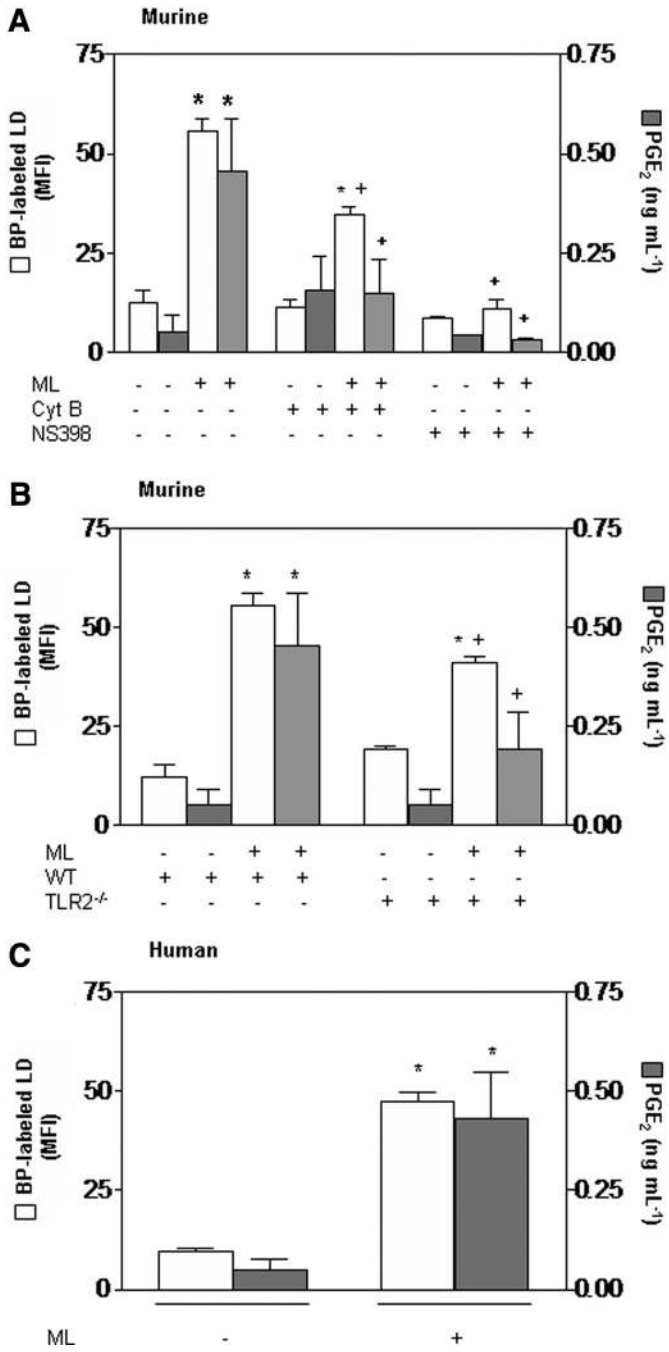

Figure 7. Correlation of PGE_2 production and LD formation in response to ML. The levels of LDs and PGE_2 were analyzed in murine peritoneal macrophages: (A) pretreated with Cyt B, NS-398 (1 μM), or an equal volume of vehicle or (B) WT peritoneal macrophages and $TLR2^{-/-}$ macrophages stimulated with ML or not. (C) LDs and PGE_2 production were analyzed in monocytes from human peripheral blood in response to ML. Induction of PGE_2 was measured by EIA in culture supernatants, and LD levels were determined by flow cytometry of BP-labeled cells. Data are representative of four experiments with SD. *, Statistically significant differences ($P<0.05$) between macrophage groups treated with ML and untreated, control cells. +, Significant differences ($P<0.05$) between different ML-treated cell groups.

in both cell populations. The TLR2 depletion did show a strong inhibitory effect on LD formation in cells with no bacteria, having less of an impact in infected cells. This preferential effect of the TLR2 absence on cells not bearing bacteria is explained by its effect on the generation of the paracine signal. This conclusion is supported by the inability of the CM from TLR2$^{-/-}$ cells, in contrast to the CM originating from WT cells, to induce LD formation in uninfected macrophages. Moreover, the generation of the paracrine signal was also abrogated by Cyt B, suggesting that TLR2 internalization is essential for this process to occur. This hypothesis is supported by a recent report showing that TLR2 is internalized and localized on the membrane of phagosomes that contain ML [62].

To define potential candidates involved in the paracrine signaling, we assessed the levels of some cytokines/chemokines in CM from ML-infected macrophages. Interestingly, a positive correlation of the IL-10/IL-12 ratio with the capacity of CM to induce LD formation in uninfected cells was found. This observation correlates with the characteristic pattern of IL-10 predominance in LL lesions [63]. Moreover, IL-10 has been implicated recently in foam cell formation in atherosclerosis disease, reinforcing the potential participation in vivo of the cytokine balance in the generation and maintenance of abundant LDs in LL lesions. Additionally, significantly higher levels of IL-1β were found in CM capable of inducing LDs, suggesting a potential contribution of this cytokine in promoting intracellular lipid storage, as has been pointed out recently [48]. A more detailed investigation of the soluble factors responsible for the paracrine LD-forming signaling produced by ML-infected macrophages is under course.

As a potential cofactor for LD induction in ML infection, the possible role of TLR6 was then studied, as it has been shown that the latter heterodimerizes with TLR2 [10, 64, 65]. The TLR6 deletion alone showed an even higher impact than the TLR2 deletion on ML-induced LD formation in infected macrophages (Fig. 5, A and B). These results are in agreement with the expression profile of TLRs in leprosy lesions reported recently. Krutzik et al. [66] showed that TLR2 was expressed more strongly in TT lesions as opposed to LL lesions. On the other hand, Bleharski et al. [67], although analyzing gene expression profiles in skin lesions of LL and TT leprosy patients, demonstrated that TLR6 was up-regulated significantly in LL lesions. It may, therefore, be speculated that these data are in correlation with the essential role of TLR6 in ML-induced LD formation, herein demonstrated along with the capacity of LL macrophages to accumulate LDs, in contrast to the reduced presence of these organelles in macrophages present in TT lesions [61].

Our data also indicate that ML-induced LD formation in infected macrophages depends on additional receptors associated with the innate immune response besides TLR2 and -6. Accordingly, TLR2-dependent signaling was shown to be essential, although not sufficient, to the LD biogenesis induced by BCG infection [28]. However, nonpathogenic *M. smegmatis*, which also triggers TLR2-dependent cytokine production, failed to induce LD formation, suggesting that cofactors may be involved in the mechanisms involved in LD formation in pathogenic mycobacteria [28, 68]. PGE_2 is a potent immune modulator that down-regulates Th1 responses and bactericidal activity toward intracellular organisms [69, 70], and LDs are intracellular sites for eicosanoid production. Our data suggest that ML-induced LDs also constitute intracellular sites for eicosanoid synthesis, which is sustained by the observation of a significant correlation between LD formation and PGE_2 generation by phagocytes treated with the bacteria. These data are in accordance with published reports, indicating the elevated production of PGE_2 by human monocytes from LL patients, and in the athymic mouse leprosy model [71–73]. Moreover, the expression of the inducible enzyme COX-2 was shown to be significantly higher in biopsies of LL than TT leprosy patients [19]. In reality, the high concentrations of PGE_2 produced by COX-2-positive macrophages in LL were able to inhibit Th1 cytokine production, most probably contributing to T cell anergy at this disease pole [19]. Thus, enhanced levels of macrophage-generated PGE_2 induced by ML could act as an endogenous-negative modulator of the immune response occurring in the microenvironment of the LL lesion.

In conclusion, it could be speculated that the lipid storage phenomenon observed in LL may play a critical role in leprosy pathogenesis by facilitating bacterial persistence in the host in at least two different ways. First, as described above, the Virchow cells, present abundantly in LL lesions, are probably catalytically active sites of PGE_2 synthesis, thereby favoring the inhibition of macrophage bactericidal activities and the down-regulation of the immune response. Second, it has been demonstrated recently that lipids constitute an important nutritional source for mycobacterial persistence in the host [74, 75]. Indeed, in a recent analysis of the ML proteome, we pointed out the presence of the fatty acid β-oxidation and glyoxylate cycle enzymes, reinforcing the idea that fatty acids, rather than carbohydrates, are more likely to be the dominant carbon substrate used by ML during infection [76].

AUTHORSHIP

K. A. M., H. D., P. T. B., and M. C. V. P. designed the study; K. A. M., H. D., L. S. R., and V. C. G. O. performed experiments; K. A. M., H. D., and L. S. R. collected and analyzed data; E. N. S. provided leprosy biopsies; H. D. and P. T. B. provided reagents and mice; K. A. M., P. T. B., and M. C. V. P. wrote the manuscript; G. M. P. performed flow cytometry analysis; G. C. A. performed lipid analysis; M. C. V. P., P. T. B., and E. N. S. gave technical support and conceptual advice. All authors discussed the results and implications and commented on the manuscript at all stages.

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