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TLR6-Driven Lipid Droplets in Mycobacterium leprae-Infected Schwann Cells: Immunoinflammatory Platforms Associated with Bacterial Persistence

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The mechanisms responsible for nerve injury in leprosy need further elucidation. We recently demonstrated that the foamy phenotype of Mycobacterium leprae-infected Schwann cells (SCs) observed in nerves of multibacillary patients results from the capacity of M. leprae to induce and recruit lipid droplets (LDs; also known as lipid bodies) to bacterial-containing phagosomes. In this study, we analyzed the parameters that govern LD biogenesis by M. leprae in SCs and how this contributes to the innate immune response elicited by M. leprae. Our observations indicated that LD formation requires the uptake of live bacteria and depends on host cell cytoskeleton rearrangement and vesicular trafficking. TLR6 deletion, but not TLR2, completely abolished the induction of LDs by M. leprae, as well as inhibited the bacterial uptake in SCs. M. leprae-induced LD biogenesis correlated with increased PGE2 and IL-10 secretion, as well as reduced IL-12 and NO production in M. leprae-infected SCs. Analysis of nerves from lepromatous leprosy patients showed colocalization of M. leprae, LDs, and cyclooxygenase-2 in SCs, indicating that LDs are sites for PGE2 synthesis in vivo. LD biogenesis inhibition by the fatty acid synthase inhibitor C-75 abolished the effect of M. leprae on SC production of immunoinflammatory mediators and enhanced the mycobacterial-killing ability of SCs. Altogether, our data indicated a critical role for TLR6-dependent signaling in M. leprae–SC interactions, favoring phagocytosis and subsequent signaling for induction of LD biogenesis in infected cells. Moreover, our observations reinforced the role of LDs favoring mycobacterial survival and persistence in the nerve. These findings give further support to a critical role for LDs in M. leprae pathogenesis in the nerve. The Journal of Immunology, 2011, 187: 2548–2558.
biological processes. Contemporary evidence points to LDs as inflammatory organelles involved in the synthesis and secretion of inflammatory mediators (10). Our group recently demonstrated that the LDs formed in response to bacillus Calmette-Guérin (BCG) and M. leprae constitute sites for eicosanoid synthesis, ultimately leading to increased production of PGE2 by infected macrophages (9, 11, 12). PGE2 is a potent immune modulator that downregulates Th1 responses and bactericidal activity toward intracellular organisms (12–14). Our previous data also indicated that Mycobacterium-induced LD formation in infected macrophages depends on bacterial recognition via TLRs. Accordingly, we showed recently that TLR2 and TLR6 pathways are preferentially activated during LD biogenesis triggered by M. bovis BCG and M. leprae infection in macrophages (9, 11, 12, 15).

SCs are capable of secreting a vast array of cytokines and inflammatory mediators (i.e., IL-1, IL-6, IL-8, TNF-α, IL-10, IL-12, PGs, TGF-β, and NO) and actively participate in the immunoinflammatory responses in neurtic processes (16–21). We showed recently that M. leprae induces LD biogenesis and accumulation in bacterial-containing phagosomes in SCs and is responsible, at least in part, for originating foamy degeneration of M. leprae-infected SCs in NL nerves (22). Accordingly, as was proposed for macrophages in the context of dermal lesions (9, 23), it is reasonable to speculate that the lipid-storage phenomenon observed in M. leprae-infected SCs is an important contributor to the immunoinflammatory function of these cells in NL nerve lesions. In the current study, we investigated the involvement of TLRs in the induction of LDs by M. leprae in SCs. Moreover, we analyzed the capacity of M. leprae-infected SCs to secrete immunoinflammatory mediators, along with the manner in which this secretion is related to LD formation. The data indicated that host cell lipid metabolism and the innate immune response to M. leprae infection are intimately related in SCs, contributing to bacterial survival in the nerve and disease progression.

Materials and Methods

Patients and clinical specimens

LL patients were classified according to the criteria of Ridley (24). Nerve biopsy specimens (6-mm diameter) were obtained at the time of diagnosis. The specimens were snap-frozen in liquid nitrogen and stored at −70°C until sectioned. The procedures described in this work were approved by the Oswaldo Cruz Foundation Ethics Committee. Written informed consent was obtained from each patient.

Human SC cultures

Human primary SCs were isolated from peripheral nerve tissues and provided by Dr. Patrick Wood (University of Miami, through the Organ Procurement Organization, University of Miami, Miami, FL). The purity of these cultures was >95% by labeling with Ab to Ca2+-binding protein (S100; DakoCytoylation, Glostrup, Denmark). These cells were cultured as previously described (22). Alternatively, the ST88-14 tumor cell line was used for in vitro assays. ST88-14 was established from malignant schwannomas (neurofibrosarcomas) from patients with neurofibromatosis type 1 and was generously donated by Dr. J.A. Fletcher (Dana Farber Cancer Institute, Boston, MA). The cells were grown in RPMI 1640 medium (Invivogen) supplemented with 2% FBS and 20 mM L-glutamate.

Isolation of mouse primary SCs

C57BL/6 (B6) mice were obtained from the Oswaldo Cruz Foundation breeding unit. TL2R and TL6R knockout mice on a homogeneous B6 background were donated by Dr. S. Akira (Osaka University, Osaka, Japan). Mouse SCs were prepared from nerve explants from adult mice, as described previously (25). The purity of SCs was assessed by microscopic examination after immunostaining with anti-S100 Abs, which revealed >95% S100+ cells. These highly purified SCs were seeded as described above for the human SCs. The protocols were approved by the Animal Welfare Committee of the Oswaldo Cruz Foundation.

Mycobacteria strains and staining

M. leprae prepared from footpads of athymic nu/nu mice was kindly provided by Dr. J. Krahenbuhl (National Hansen’s Disease Program, Laboratory Research Branch, Louisiana State University, Baton Rouge, LA) through American Leprosy Missions, the Order of St. Lazarus, and the National Institute of Allergy and Infectious Diseases, Bethesda, MD; Contract No. 155262). Part of the M. leprae suspension was killed by gamma irradiation. Mycobacterium smegmatis (155; MSM) and Mycobacterium bovis BCG Pasteur (ATCC 35734) were grown at 37°C in Middlebrook 7H9 base ADC enrichment medium (Becton Dickinson, Franklin Lakes, NJ), supplemented with 0.2% glycerol, 0.05% Tween 80 (Sigma). Prior to interaction assays with SCs, bacteria were stained with a PKH26 Red Fluorescence Cell Linker Kit (Sigma), according to the manufacturer’s instructions.

Mycobacterium–SC interaction assays

SCs were suspended in culture medium without antibiotics and cultured at a density of 1 × 10^5 cells/well on 12-well plates precoated with laminin-1 for flow cytometric assays and at 7 × 10^3 cells/well on 24-well plates containing poly-L-lysin (Sigma)/laminin-coated glass coverslips for microscopy experiments. M. leprae was added to the culture at a multiplicity of infection (MOI) of 50 bacilli/cell (50:1) for 2, 24, and 48 h. In some experiments, cells were also infected with live BCG (MOI 50:1) or live MSM (MOI 50:1). Alternatively, SCs were stimulated with LPS (50 μg/ml), poly-9-cis-epoxycasparoside (LPS-9c) (3 μg/ml) or R848 (10 μg/ml), a synthetic double-stranded oligonucleotide containing unmethylated diacylated triphosphate oligodeoxynucleotide. After 48-h incubation, cell viability was determined by counting the living cells stained with 0.1% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The supernatants were collected, centrifuged, and frozen until use. All experiments were performed at 33°C in a 5% CO2 atmosphere.

LD staining and quantification

Cells adhering to coverslips were fixed in 4% paraformaldehyde in Ca2+/Mg2+-free HBSS (pH 7.4) for 10 min, and LDs were analyzed using different markers—osmium tetroxide, oil red O (ORO), or BODIPY 493/503 dye—as described (22). Coverslips were then mounted in 20% glycerol and 1% n-propyl gallate in PBS (pH 7.8). The morphology of the fixed cells was observed, and LDs were enumerated at 100× in 50 consecutively scanned cells. Alternatively, the area occupied by LDs per cell was estimated using flow cytometry. Corresponding mean fluorescent intensity (MFI) (green) values were obtained from the histograms. Biological association with cells was measured in the fluorescence channel 2 by PKH26-labeled bacteria. The percentage of eukaryotic cells with bacterial association was determined from the gated red cells. Index of bacterial association is expressed as the percentage of cells taking up PKH26-M. leprae.

Immunohistochemical analysis

Immunohistochemical procedures to detect LD organelles and M. leprae in SCs from LL and healthy nerve were performed, as described (22). Briefly, tissue sections were thawed on sylane-precleaned slides and submitted to immunostaining protocols and to acid fast bacilli with Wade staining, following an standard protocol (26). Immunostaining was performed by...
incubation with primary Abs anti-adipose differentiation-related protein (ADRP; Research Diagnostics, Concord, MA), anti-S100, and anti-M. leprae (anti-whole bacteria or anti-LAM) kindly provided by Dr. Patrick J. Brennan (Colorado State University, Fort Collins, CO; National Institutes of Health/National Institute of Allergy and Infectious Diseases Contract No. 1AI25469), as well as anti–COX-2 (C terminus; Oxford Biomedical Research, Oxford, MI). Two-color immunofluorescence staining was performed by serial incubation of sections with combinatorial Abs against S100 (1:20) and ADRP (1:25), M. leprae (1:25) and ADRP (1:25), COX-2 (1:20) and ADRP (1:25), and M. leprae (1:25) and COX-2 (1:20). The corresponding isotype Abs were used as negative controls.

Image acquisition in confocal microscopy

Preparations were examined using a Zeiss Axio Observer microscope equipped with an Plan Apo 40 or 100 objective (Carl Zeiss, Thornwood, NY) and a CoolSNAP-Pro CF digital camera in conjunction with Axion Vision Version 4.7.2 software (Carl Zeiss). The images were edited using AxioVision software. To determine the colocalization of fluorescent signals, images were acquired with an LSM 510 Zeiss confocal microscope. Images were acquired, colored, and merged using LSM 510 Zeiss software. Argon and neon-helium lasers (Mellets Griot) emitting at 488, 543, and 633 nm were used. Pictures of 20–40 confocal planes through the cell (z-stack), with a step size of 0.2–0.3 μm, were taken with a 63× objective every 30 s for 5–10 min using LSM image software. For some experiments, z-stacks of images were captured, processed, and rendered using the LSM imaging system (Carl Zeiss).

Measurement of PGE₃

PGE₃ concentration was measured in cell-free supernatants via an enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI), and the assays were conducted according to the manufacturer’s protocol.

Cytokine measurement

IL-12 p40 and IL-10 concentrations in culture supernatants were determined by ELISA using the specific Duo set kit (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions. The sensitivity of these assays was 2 pg/ml.

Nitrite determination

NO synthesis was assessed by measuring the accumulation of nitrite in cell supernatants, as detected by the Griess reagent (Sigma). Absorption was measured at 550 nm, and nitrite concentrations were determined by comparison with OD of the NaNO₂ standards.

M. leprae viability

A live/dead-staining protocol, based on the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen), was applied to determine the percentage of M. leprae viability in SCs treated with NS-398, C-75, or vehicle. In brief, SCs (2 × 10⁶ cells/well) were treated with drugs or vehicle for 30 min at 33˚C and then infected with M. leprae (50:1) for 72 h in RPMI 1640 cell culture medium. SC cultures were washed three times with PBS to remove any noninternalized bacteria and lysed with 0.1% saponin. Bacterial-containing suspensions were labeled with the LIVE/DEAD kit, and the percentages of live and dead bacteria were determined by flow cytometry, according to the manufacturer’s instructions. Flow cytometry measurements were performed on a FACScalibur and analyzed with CellQuest software (both from BD Bioscience).

Statistical analysis

Data analysis was performed using the GraphPad InStat program (GraphPad Software, San Diego, CA), and the statistical significance (p < 0.05) was determined by the Student t test.

Results

In vitro induction of LDs by M. leprae in SCs depends on bacterial viability

We showed previously that the foam phenotype in infected SCs, described as a characteristic feature seen in nerve lesions of LL patients, is derived from lipid accumulation in LD organelles, as observed in in vitro and in vivo studies (22). Double labeling of ADRP/S100 and ADRP/M. leprae showed that there is a predominant association of M. leprae with these lipid-containing vacuoles in foamy SCs, both in the nerves of LL patients (Fig.
M. leprae-induced LD biogenesis in SCs was shown to be dose and time dependent, reaching significant levels at an MOI of 50 after 24 and 48 h of incubation (Fig. 1C, 1D). Because M. leprae at an MOI of 50 for 48 h turned out to be an optimal condition for LD induction, this dose and incubation time were adopted in the remaining experiments. Given the fact that both live and dead M. leprae are found in affected nerves of leprosy patients, we next investigated whether the capacity to modulate LD formation was dependent on bacterial viability. Primary SCs were stimulated in vitro in either live or irradiated-killed bacteria, and LD formation was monitored by flow cytometry (Fig. 1E). Live M. leprae-infected SCs showed ~4- and 2-fold higher values of the MFI of BODIPY probe in comparison with dead M. leprae-treated cells and untreated cells, respectively (Fig. 1E). Alternatively, LDs were also enumerated after osmium tetroxide staining, and identical results were obtained (data not shown). Similar results were obtained with the ST88-14 schwannoma cell line (data not shown). Accordingly, the mycobacterial glycolipids mannosylated LAM, non-capped LAM, and PGL-1 were unable to induce LD biogenesis in SCs, as well as LPS, a potent inducer of LDs in macrophages (Fig. 1E). These data strongly reinforced that different from macrophages, M. leprae viability is necessary to actively upregulate the biogenesis of LDs in SCs.

Next, we investigated the capacity of other viable mycobacterial species, such as the pathogenic M. bovis BCG and the avirulent M. smegmatis (MSM), to induce LD in SCs, given the fact that M. bovis BCG is a good inducer of LD formation in macrophages (12). Interestingly, only M. leprae was able to induce LD formation, whereas BCG and MSM showed LD levels similar to those found in unstimulated cells (Fig. 1F). Moreover, SCs incubated with fluorescent latex beads showed only basal levels of LDs, despite the fact that they were readily internalized (22), indicating that phagocytosis itself is not sufficient to induce LD formation in SCs.

Only SCs bearing bacteria show increased numbers of LDs
In a previous study with macrophages, we showed that, in M. leprae-treated cultures, the LDs were significantly induced both in human macrophages bearing bacterium and in cells with no bacteria. We also showed that soluble factors are secreted upon M. leprae–macrophage interaction and that these factors are capable of inducing LD formation in uninfected cells (9). We then investigated whether the effect of M. leprae on SC LD biogenesis described in this article follows the characteristics previously seen in macrophages. To this end, SCs were exposed to fluorescent-labeled M. leprae. Untreated cells or those exposed to PKH-labeled M. leprae were cultured for 48 h; after staining with BODIPY, the induction of LDs was analyzed by flow cytometry and fluorescence microscopy.

Fluorescence images clearly showed increased numbers of LDs only in cells bearing bacteria (M. leprae+, PKH-labeled M. leprae).
and not in cells without bacterial internalization (M. leprae−), suggesting a close relationship between phagocytosis of M. leprae and the induction of LD formation (Fig. 2A). Similar results were obtained when LD biogenesis was analyzed by flow cytometry (Fig. 2B). A representative dot plot showed that M. leprae infection induced high levels of BODIPY fluorescence compared with control. Fig. 2C shows MFI values of BODIPY observed in cells with red fluorescence (cells with internalized M. leprae) and cells without red fluorescence (cells without internalized M. leprae). LDs were significantly induced only in SC populations bearing M. leprae in comparison with the population without bacterial association and untreated cells (MFI of 167.4 ± 10.84, 67.5 ± 6.3, and 59.1 ± 6.6, respectively). An identical result was obtained when ST88-14 SCs were stimulated with M. leprae in vitro (MFI of 109.2 ± 2.7 in untreated cells, 103.1 ± 0.5 in cells without internalized M. leprae, and 333.2 ± 22.6 in cells with internalized M. leprae).

To investigate whether bacterial phagocytosis is required to elicit LD formation, SCs were pretreated with the actin polymerization inhibitor (CytD) before M. leprae infection. The inhibitory effect of CytD on phagocytosis was monitored by flow cytometry using bacteria prestained with PKH26. Fig. 2D shows that CytD-treated cells had ~90% impaired phagocytic ability. Fig. 2E shows that M. leprae was unable to induce LDs in CytD-treated cells (MFI of 88.10 ± 4.47 in M. leprae-treated cells versus 95.6 ± 2.8 in uninfected CytD-treated cells). Moreover, conditioned medium derived from M. leprae-infected SCs was not able to induce the biogenesis of these organelles in cultures not infected with M. leprae, in contrast to our previous observation of M. leprae-stimulated macrophages (9) (Fig. 2G). These data suggested that M. leprae internalization is necessary for triggering the signaling pathways that will culminate in LD formation, although other cytoskeleton-dependent mechanisms, in addition to phagocytosis of Mycobacterium, might participate in the mechanisms involved in LD biogenesis.

**ER–Golgi transport and a functional cytoskeleton are essential for M. leprae-induced LD biogenesis**

Several lines of evidence suggest that LDs are derived from the ER–Golgi apparatus pathway and that their formation depends on the microtubule system of the cell (27, 28). To better understand the mechanisms behind the LD formation induced by M. leprae in SCs, a series of experiments was conducted using pharmacological inhibitors of pathways previously shown to participate in LD biogenesis in other cell types (9, 29, 30) (Table I). We inhibited ER–Golgi transport with brefeldin A and monensin and observed the significant reduction in formed LDs. In addition, we examined the effect of CytB, an inhibitor of actin filament polymerization at barbed ends. CytB-treated SCs showed a decrease in LD formation in cells treated with live M. leprae compared with untreated CytB cells at 48 h post-infection, replicating the inhibitory effects of the actin-disrupting drug CytD. In addition, the microtubule-binding drugs taxol and colchicin showed inhibitory effects on LD biogenesis. Finally, the role of PI3K signaling, known to be involved in cytoskeletal rearrangements in Mycobacterium-infected macrophages (31), was investigated. Treatment of SCs with LY294002, an inhibitor of PI3K, blocked LD biogenesis in M. leprae-infected SCs (Table I). This effect was confirmed using wortmannin, an alternative specific PI3K inhibitor (data not shown). Taken together, these results suggested that M. leprae-induced LD biogenesis in SCs is an ER–Golgi and actin- or microtubule-dependent event, confirming the important role of vesicular traffic in the nascent LD process.

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>Treatment</th>
<th>LD Formation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Vehicle (DMSO)</td>
<td>186.6 ± 35.57</td>
</tr>
<tr>
<td>M. leprae infection</td>
<td>Vehicle (DMSO)</td>
<td>625.2 ± 17.2**</td>
</tr>
<tr>
<td>Disruption of Golgi–ER</td>
<td>Brefeldin (2 μM)</td>
<td>307.1 ± 23.34*</td>
</tr>
<tr>
<td></td>
<td>Monensin (5 μM)</td>
<td>174.3 ± 3.49*</td>
</tr>
<tr>
<td>Actin filament destabilization</td>
<td>CytoD (20 μM)</td>
<td>275.6 ± 48.83*</td>
</tr>
<tr>
<td></td>
<td>CytoB (20 μM)</td>
<td>90.29 ± 3.96*</td>
</tr>
<tr>
<td>Microfilament destabilization</td>
<td>Taxol (1 μM)</td>
<td>350.0 ± 46.05*</td>
</tr>
<tr>
<td></td>
<td>Colchicin (1 μM)</td>
<td>376.5 ± 40.8*</td>
</tr>
<tr>
<td>PI3K inhibition</td>
<td>LY294002 (10 μM)</td>
<td>395.9 ± 40.9*</td>
</tr>
</tbody>
</table>

SCs were treated for 30 min before infection with M. leprae (MOI = 50), and LD formation was determined 48 h later by MFI of BODIPY by flow cytometric analysis. *Data are mean ± SD of five independent experiments. **p < 0.05 between the different M. leprae-treated cell groups.

**TLR6, but not TLR2, is essential for M. leprae-induced LD biogenesis in SCs**

As reported previously, the recognition of M. leprae by the TLR2/TLR6 heterodimer is an essential step leading to LD formation in infected macrophages (9). We investigated the functional role of these receptors on LD formation in the context of SCs. SCs isolated from mouse deleted of TLR2 (TLR2−/−) or TLR6 (TLR6−/−) were infected with M. leprae, and LD biogenesis was evaluated by flow cytometry and microscopic analysis. In marked contrast to macrophages, TLR2−/− SCs showed M. leprae-induced LD levels comparable to wild-type (WT) SCs (Fig. 3A–D). In contrast, LD formation was reduced to basal levels in TLR6−/− SCs (Fig. 3A–D). Moreover, in contrast to TLR2−/− SCs, M. leprae internalization was partially impaired in TLR6−/− SCs, suggesting a role for TLR6 as a bacterial phagocytic receptor in these cells (Fig. 3E). These data indicated a critical role for TLR6, independent of its association with TLR2, in M. leprae–SC interactions, favoring phagocytosis and subsequent signaling for induction of LD biogenesis in infected cells.

Interestingly, immunofluorescence images and flow cytometry analysis of the same cultures indicated the induction of LDs only in cells bearing M. leprae (Supplemental Fig. 1A, 1B), as observed before in human SCs (Fig. 2A). The profile of LD formation in TLR2−/− SCs, with or without bacteria, was identical to that observed in WT cells (Supplemental Fig. 1B). Of note, TLR6 deletion caused a significant decrease in LD formation, even in cells with internalized M. leprae (Supplemental Fig. 1B), reinforcing the involvement of TLR6, beyond phagocytosis, as a signaling platform necessary for M. leprae-induced LD formation in SCs. Together, this set of results corroborated the idea that the modulation of LD formation in response to M. leprae in SCs requires bacterial phagocytosis and that TLR6 plays a critical role as a phagocytic receptor and a trigger of downstream signals necessary for LD biogenesis.

**LDs are sites of eicosanoid generation in in vivo-infected foamy SCs**

To investigate whether LDs are involved in PGE2 production in SCs during the natural course of leprosy, cross-sections of nerve biopsies from three LL patients were immune stained with specific Abs that recognize the inducible cyclooxygenase, COX-2, the LD marker, ADRP, the SC marker S100, as well as M. leprae, using an Ab that recognizes LAM, a component of the mycobacterial...
envelope. Fig. 4A shows an infected nerve section stained for acid fast bacilli by the Wade procedure. This image shows SCs with a foamy appearance packed with fuchsin-stained mycobacterial globi. LL nerve specimens exhibited an intense immune reactivity to COX-2 and M. leprae, confirming the relationship between bacterial presence and COX-2 expression (Fig. 4B). Immunofluorescent staining for both COX-2 and ADRP demonstrated that COX-2/LD colocalization. Nuclei were stained with TO-PRO-3. Scale bar, 10 μm.

**FIGURE 4.** LD organelles are sites of eicosanoid production in SCs of LL nerve biopsy. Serial sections of nerve biopsies from LL patients (n = 4) were analyzed. In situ localization of COX-2 in M. leprae-infected SCs. A. Wade staining showing foamy SCs with multiple acid-fast bacilli. Scale bar, 20 μm. B. Double-immunofluorescent labeling with anti-LAM (red) and anti–COX-2 (green) reveals putative COX-2 production in cells with a strong bacterial presence. C. COX-2 (red) localization within LD organelles labeled with ADRP (green) within SCs. Inset: Magnified view of COX-2/LD colocalization. Nuclei were stained with TO-PRO-3. Scale bar, 10 μm.

M. leprae elicits an innate immune response in infected SCs that depends on LD formation and TLR6 signaling

Because LDs in leukocytes were shown to be involved in the production of inflammatory mediators, we investigated the relationship between LDs induced by M. leprae infection and immune response in the context of SCs. As shown in Fig. 5, both human and murine SCs produced significantly higher levels of PGE2 (Fig. 5A) and IL-10 (Fig. 5B) after 48 h of M. leprae infection compared with uninfected cells, coinciding with the time point of greatest LD formation. In contrast, M. leprae-infected SCs produced significantly lower levels of IL-12 (Fig. 5C) and NO (Fig. 5D) compared with the basal levels observed in uninfected cells. Moreover, M. leprae infection was able to attenuate SC production of IL-12 and NO induced by TNF-α, a potent proinflammatory cytokine (Fig. 5D, 5F). These changes in the production of immunoinflammatory mediators triggered by live M. leprae were not observed when cells were treated with dead bacteria (data not shown), coinciding with the inability of dead M. leprae to induce LD formation.

Because bacterial phagocytosis was previously shown to be essential for the induction of LD formation in SCs, we next tested the effect of CytD on the profile of immunomediators produced by SCs in response to M. leprae. As expected, CytD treatment completely inhibited M. leprae-induced PGE2 (Fig. 5A) and IL-10 (Fig. 5B) production. In addition, basal IL-12 and nitrite levels were not reduced, as observed in untreated cultures (Fig. 5C, 5E).

Confirming the results obtained with human SCs, M. leprae infection induced PGE2 (Fig. 5G) and IL-10 (Fig. 5H) production and inhibited basal levels of NO generation (Fig. 5F) in murine WT SCs. Moreover, a correlation was observed between the effects of TLR2 and TLR6 deletion on M. leprae-induced LD formation and inflammatory mediator secretion (Fig. 5G–I). Although TLR2−/− SCs showed an identical profile of mediator secretion to WT cells in response to M. leprae, it failed to modulate the profile of these mediators in TLR6−/− SCs. These data suggested a key role for LDs as inflammatory organelles in SCs during M. leprae infection, with the novel involvement of TLR6 signaling independently of TLR2.
To gain more insight into the role of LDs in the *M. leprae*-modulated innate immune response in SCs, the effect of the drugs NS-398 and C-75 on PGE$_2$, IL-10, IL-12, and NO production was investigated. NS-398, a nonsteroidal anti-inflammatory drug, and C-75, an inhibitor of FAS, were previously shown to inhibit *Mycobacterium*-induced LD formation in macrophages (9, 12, 32). As expected, these inhibitors drastically inhibited *M. leprae*-induced LD formation in SCs (Fig. 6A). Next, to confirm the relationship between LDs and the innate immune response elicited by *M. leprae*, and PGE$_2$ (G), IL-10 (H), and nitrite (I) were determined in culture supernatants. Data are presented as the mean ± SD from six independent experiments. *p < 0.05, *M. leprae*-stimulated versus control cells; +p < 0.05 between the different *M. leprae*-treated cell groups.

**FIGURE 5.** The innate immune response of SCs toward *M. leprae* (ML) depends on bacterial internalization and TLR6. Human SCs were pretreated or not with CytD prior to *M. leprae* infection. Supernatants were harvested 48 h later. A, PGE$_2$ production was measured by EIA. IL-10 (B) and IL-12 (C) production was measured by ELISA. E, Nitrite production. Infected SCs were stimulated with TNF-$\alpha$, and IL-12 (D) and nitrite (F) were measured. Murine WT, TLR2$^{-/-}$ and TLR6$^{-/-}$ SCs were infected with *M. leprae*, and PGE$_2$ (G), IL-10 (H), and nitrite (I) were determined in culture supernatants. Data are presented as the mean ± SD from six independent experiments. *p < 0.05, *M. leprae*-stimulated versus control cells; +p < 0.05 between the different *M. leprae*-treated cell groups.

**Discussion**

Pathogen-triggered dysregulation of host–cell lipid metabolism are emerging as a key feature in the response to mycobacterial infection. Accumulating evidence suggest that modulation of host lipid metabolism through *M. leprae*-induced LD formation is important in leprosy disease. However, the mechanisms that govern *M. leprae* infection-induced lipid synthesis and lipid accumulation in LDs and the role of LDs in bacterial persistence in the nerve are poorly understood. Our study offers novel evidence that *M. leprae* is able to increase SC lipid accumulation and PGE$_2$.
formation through specific and highly regulated mechanisms involving TLR6-signaling activation. Moreover, we demonstrated that increased LD biogenesis affects SCs’ production of inflammatory mediators and mycobacterial killing capacity, which adds support to the growing body of evidence that identifies LDs as a key component in immunity to infections.

We showed that the mechanisms of LD induction by *M. leprae* in SCs share similarities, but also exhibit some differences, with those previously observed in macrophages (9). LD biogenesis was induced in a time- and dose-dependent-response manner, with bacterial phagocytosis required for the induction. Moreover, by using selective drugs, we showed that LD biogenesis in *M. leprae*-infected SCs follows a classical route. LDs are known to originate in the ER–Golgi complex, and the ability of LDs to form and grow in size was described to be dependent on motor proteins and microtubule dynamics (28, 33). Induction of LDs in SCs by *M. leprae* was dependent on ER and Golgi apparatus, because it was inhibited by brefeldin and monesin drugs, known to disorganize the ER–Golgi complex. In addition, disturbance of microtubule and actin organization by specific drugs demonstrated that *M. leprae*-induced LD biogenesis also signals through a pathway dependent on cytoskeleton dynamics. Finally, the ability of *M. leprae* to induce LDs in SCs was abrogated using an inhibitor of PDE3K, an enzyme that participates in several signaling pathways, including cytoskeletal organization.

However, the induction of LDs in SCs displayed interesting unique features compared with macrophages. First, in contrast to macrophages, live *M. leprae*, but not dead bacterium or isolated *Mycobacterium* components, were able to induce LDs in in vitro-cultured SCs, suggesting that, in SCs, the bacteria may play an active role in LD formation. Second, in *M. leprae*-infected cultures, *M. leprae*-induced LD formation was observed in bacterium-associated cells but not in cells with no bacterium. In deep contrast to macrophages, media conditioned by *M. leprae*-stimulated SCs were unable to mimic the LD-induction activity of the bacteria, confirming the absence of soluble factors secreted by infected SCs able to promote LD biogenesis in neighboring, uninfected cells. A third important difference found between *M. leprae*-induced LD biogenesis in macrophages and SCs was related to the involvement of TLRs. We demonstrated previously that the formation of LDs in response to BCG and *M. leprae* is dependent on TLR2/TLR6 in infected macrophages (9, 12, 22, 32). Although SCs express TLR2 (34), signaling through this receptor was not essential to LD biogenesis, reinforcing our previous data indicating that *M. leprae*-induced LD biogenesis and recruitment to bacterial-containing phagosomes in SCs were independent of TLR2 bacterial sensing (22). Moreover, although TLR6 was essential in both cell types in sensing *M. leprae*, a potential additional role as a phagocytic receptor was observed in the case of SCs. Interestingly, a signaling that excludes the TLR2 pathway as a heterodimer with TLR6 has not been explored in inflammation in response to microbial infection, but it was recently described in sterile inflammation, such as atherosclerosis and Alzheimer’s disease (35). In this study, a new heterodimerization model of cellular receptors, consisting of TLR4/6, was identified. Whether TLR4 or other coreceptors participate in conjunction with TLR6 in the recognition and signaling of live *M. leprae* in SCs deserves future investigation. Additionally, the specific *M. leprae* chemical signatures recognized by TLR6 need characterization.

We investigated the functional role of TLRs in the immune response triggered by *M. leprae* in SCs. TLR6 deletion completely

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**FIGURE 6.** *M. leprae* elicits an innate immune response in infected SCs that depends on LD formation. Human SCs were pretreated or not with C-75 or NS-398 prior to *M. leprae* infection, and LD formation and inflammatory mediators were measured. A, LD formation was determined by MFI of BODIPY by flow cytometric analysis. B, PGE2 production was determined by EIA. IL-10 (C) and IL-12 (D) production was estimated by ELISA. E, NO production was estimated by measuring nitrite by the Griess reagent. F, Percentage of live and dead bacteria was evaluated using a LIVE/DEAD Baclight Bacterial Viability Kit in combination with flow cytometry after 72 h of drug treatment. Data are mean ± SD from six independent experiments. *p < 0.05, *M. leprae*-stimulated versus control cells; **p < 0.05 between the different *M. leprae*-treated cell groups.
abolished the *M. leprae*-induced immunomodulatory effect on SCs. In contrast, no involvement of bacterial sensing by TLR2 in the production of PGE₂, IL-10, NO, and IL-12 by SCs in response to *M. leprae* was observed. Because TLR6, but not TLR2, was essential for both *M. leprae*-induced LD formation and cytokine and PGE₂ production in SCs, these results corroborated the idea that lipid metabolism and immune response are closely related in infected SCs. TLR expression was found to correlate with the clinical presentation of leprosy (36). The crucial role of TLR6 in leprosy described in this article is in agreement with the reported expression profile of TLRs in leprosy lesions. Bieharski et al. (37), while analyzing gene-expression profiles in skin lesions of LL and TT patients, demonstrated that TLR6 was significantly upregulated in LL lesions. In contrast, Krutzik et al. (38) showed that TLR6 was more strongly expressed in TT lesions compared with LL lesions. These data are in agreement with the essential role of TLR6 in *M. leprae*-infected SC formation demonstrated in this article, as well as the capacity of LL cells to accumulate LDs.

Interestingly, *M. leprae*-infected SCs were less responsive to TNF-α, suggesting a deactivating phenotype, similar to the one described for *M. leprae*-infected macrophages (39). In this study, infection of mouse macrophages with *M. leprae* was shown to restrict IFN-γ–mediated activation, at least in part, by the induction of PGE₂. TNF-α receptors are constitutively expressed by SCs, and treatment of cells with TNF-α promoted activation of SCs (40). Furthermore, TNF-α alone or in synergism with TGF-β was shown to induce apoptosis in SCs (40, 41). Avoidance of host cell apoptosis is particularly essential for the successful infection of obligate intracellular pathogens, such as *M. leprae*, which rely on host cell integrity and metabolic activities to complete their replication cycle. Thus, the unresponsive phenotype of infected SCs to TNF signaling may delay or suppress host cell apoptosis and activation during infection and favor infection persistence in the nerve during the natural course of leprosy.

Because immunocompetence has been recognized as an important feature of SCs (42), the correlation between lipid metabolism and the innate immunity generated in response to *M. leprae* infection was investigated. We evaluated whether LDs constitute sites of eicosanoid production in *M. leprae*-infected SCs, in a similar manner to what was observed in the context of *M. leprae* and BGC infection in macrophages (9, 12). The colocalization between ADRP, COX-2, and *M. leprae* inside SCs of nerve biopsies from LL patients was a strong indicator that *M. leprae*-induced LDs constitute intracellular sites for eicosanoid synthesis in vivo. This was sustained by in vitro assays, in which a significant correlation between LD formation and PGE₂ generation was observed in SCs treated with *M. leprae*. We next evaluated the impact of the inhibition of LD formation on immunomodulation of host response and killing capacity of SCs infected by *M. leprae*. First, we tested the effect of NS-398 (COX-2 inhibitor) that, in addition to its ability to inhibit cyclooxygenase, has COX-independent inhibitory effects on LD biogenesis (43, 44). NS-398 significantly inhibited the LD formation and PGE₂ production in infected SCs. In different cell systems, LD biogenesis was shown to require new lipid synthesis in a mechanism tightly regulated by FAS (45–47). Accordingly, the FAS inhibitor C-75 significantly inhibited LD formation induced by BCG, *Trypanosoma cruzi*, and dengue virus infection (32, 46, 48). Although not a cyclooxygenase inhibitor, C-75–induced LD inhibition led to inhibition of PGE₂ production. Accordingly, C-75, through mechanisms dependent on LD inhibition and independent of inhibition of the COX-2 enzyme, inhibited PGE₂ production in cancer cells (45) and mycobacterial-infected macrophages (11, 32). Interestingly, when other inflammatory mediators were analyzed, infected SCs secreted increasing levels of the anti-inflammatory cytokine IL-10 and reduced levels of IL-12 and NO compared with the basal levels observed in un. Also, inhibition of either PG production or LD formation resulted in downregulation of IL-10 production. Furthermore, we observed an increase in IL-12 and nitrite detection in culture supernatants, suggesting that LRs and/or LD-derived PGE₂ may negatively modulate the immune response of SCs toward intracellular infection. PGE₂ is a potent immune modulator that downregulates Th1 responses and bactericidal activity toward intracellular organisms (12–14, 49). Our data are consistent with these observations, because a cytokine profile characterized by predominant IL-10 secretion and low secretion of IL-12 was dependent on PGE₂ production by *M. leprae*-infected SCs. The signaling pathways that regulate this immune cascade in SCs and whether the high IL-10 production involves an autocrine circuit of PGE₂, as
PGE2, favors the inhibition of SCs’ bactericidal activities and the M. leprae BCG infections (11, 32). The role of LD inhibition in leading to T. cruzi has been associated with intracellular pathogen survival advantage. LDs that constitute active catalytic sites of PGE2 production and modulation of the cytokine milieu. In addition, LDs are being suggested as a rich source of nutrients for mycobacterial intracellular growth (12, 53–55). Accordingly, we demonstrated that LDs are promptly recruited to the bacterial-containing phagosomes during M. leprae internalization in SCs and that this event contributes to bacterial survival inside the cell (22).

In conclusion, a model can be proposed in which the lipid-storage phenomenon observed in M. leprae-infected SCs plays a key role in leprosy pathogenesis by facilitating bacterial persistence in the host through at least two mechanisms (Fig. 7). As indicated in our previous study, during M. leprae attachment and internalization, LDs are promptly recruited to bacterial-containing phagosomes, where they accumulate (22). LD recruitment probably constitutes an effective M. leprae intracellular strategy to acquire host SC lipids as a nutritional source and promote bacterial survival. Second, as shown in the current study, the accumulation of host-derived lipids in infected SCs favors the generation of an innate immune response that may contribute to a permissive environment for M. leprae proliferation within LL nerves. Sensing M. leprae by TLR6 leads to the formation of new LDs that constitute active catalytic sites of PGE2 synthesis. M. leprae-infected SCs also produce IL-10, which, together with PGE2, favors the inhibition of SCs’ bactericidal activities and the downregulation of Th1-type cytokines. Thus, suppression of normal inflammatory function of TLR6 by M. leprae in SCs results in the expression of an anti-inflammatory phenotype dependent on LD biogenesis.

The data presented in this article extend our knowledge of the underlying disease mechanism in M. leprae-infected nerves. Moreover, they support the idea of SCs as immunocompetent cells that form parts of the local immune circuitry within the peripheral nerve (42). Finally, our data confirm that the lipid modulation induced by M. leprae has pathophysiological consequences for the persistence of infection and open avenues for developing novel therapies for controlling mycobacterial infections, based either on inhibition of PG production or LD formation.

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Disclosures
The authors have no financial conflicts of interest.

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