Monocyte Chemoattractant Protein-1/CC Chemokine Ligand 2 Controls Microtubule-Driven Biogenesis and Leukotriene B $_4$ -Synthesizing Function of Macrophage Lipid Bodies Elicited by Innate Immune Response

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Monocyte Chemoattractant Protein-1/CC Chemokine Ligand 2 Controls Microtubule-Driven Biogenesis and Leukotriene B₄-Synthesizing Function of Macrophage Lipid Bodies Elicited by Innate Immune Response[1]

Patricia Pacheco,* Adriana Vieira-de-Abreu,* Rachel N. Gomes,* Giselle Barbosa-Lima,* Leticia B. Wermelinger,* Clarissa M. Maya-Monteiro,* Adriana R. Silva,* Marcelo T. Bozza,[†] Hugo C. Castro-Faria-Neto,* Christianne Bandeira-Melo,[2‡] and Patricia T. Bozza[2]*

Lipid bodies (also known as lipid droplets) are emerging as inflammatory organelles with roles in the innate immune response to infections and inflammatory processes. In this study, we identified MCP-1 as a key endogenous mediator of lipid body biogenesis in infection-driven inflammatory disorders and we described the cellular mechanisms and signaling pathways involved in the ability of MCP-1 to regulate the biogenesis and leukotriene B₄ (LTB₄) synthetic function of lipid bodies. In vivo assays in MCP-1$^{-/-}$ mice revealed that endogenous MCP-1 produced during polymicrobial infection or LPS-driven inflammatory responses has a critical role on the activation of lipid body-assembling machinery, as well as on empowering enzymatically these newly formed lipid bodies with LTB₄ synthetic function within macrophages. MCP-1 triggered directly the rapid biogenesis of distinctive LTB₄-synthesizing lipid bodies via CCR2-driven ERK- and PI3K-dependent intracellular signaling in in vitro-stimulated macrophages. Disturbance of microtubule organization by microtubule-active drugs demonstrated that MCP-1-induced lipid body biogenesis also signals through a pathway dependent on microtubular dynamics. Besides biogenic process, microtubules control LTB₄-synthesizing function of MCP-1-elicited lipid bodies, in part by regulating the compartmentalization of key proteins, as adipose differentiation-related protein and 5-lipoxygenase. Therefore, infection-elicited MCP-1, besides its known CCR2-driven chemotactic function, appears as a key activator of lipid body biogenic and functional machineries, signaling through a microtubule-dependent manner. *The Journal of Immunology,* **2007, 179: 8500–8508.**

L ipid bodies (also known as lipid droplets) are recognized as dynamic organelles with key roles in regulating storage and turnover of lipids in different cells and organisms. Beyond their function on lipid homeostasis, recent studies have placed lipid bodies as multifunctional organelles with roles in cell signaling, intracellular trafficking, and cell activation (1–3). Biogenesis of lipid bodies is a biological process that has been intensively studied over the past few years. The contemporary view of lipid body biogenesis establishes lipid bodies as endoplasmic reticulum (ER)[3]-derived organelles. Two main models have been proposed: 1) formation of a neutral lipid mass synthesized by ER enzymes that is deposited in a hydrophobic domain between the two leaflets of the ER membrane, followed by budding off of this lipid structure into the cytoplasm that ends up surrounded by a half-unit membrane of phospholipids directly derived from the cytoplasmic leaflet of the ER (4); 2) formation of lipid bodies by incorporation of multiple loops of ER membranous domains, with accumulation of neutral lipids among the membranes within lipid bodies (5). In addition, proteins of the PAT family, named after perilipin, adipose differentiation-related protein (adipophilin (ADRP)), and tail-interacting protein of 47 kDa (TIP47), are associated with lipid bodies and have previously been implicated in lipid body assembling and biogenesis (reviewed in Refs. 6 and 7).

Different from neutral lipids storing cells, leukocytes have virtually no lipid bodies under resting conditions. Yet, increased numbers of cytoplasmic lipid bodies are often associated with inflammatory and other pathological conditions.

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[3] Abbreviations used in this paper: ER, endoplasmic reticulum; ADRP, adipose differentiation related protein; LDL, low-density lipoprotein; oxLDL, oxidized LDL; 5-LO, 5-lipoxygenase; LTB₄, leukotriene B₄; CLP, cecum ligation and puncture; EDAC, 1-ethyl-3 (3-dimethylamino-propyl) carbodiimide.

Moreover, newly formed leukocyte lipid bodies have been implicated as key organelles in regulated arachidonate-derived inflammatory mediator synthesis with roles in immunity and inflammation. The detailed molecular mechanisms controlling the inducible lipid body biogenic process within leukocytes remains largely unclear (reviewed in Refs. 3 and 8). Specifically in macrophages, it has been shown that the genesis of new lipid bodies can be evoked by a variety of inflammation-related stimuli, including oxidized low-density lipoprotein (oxLDL) and oxLDL-components (but not native LDL) acting through membrane and nuclear receptors (9–12). Pathogens including Gram-positive (*Mycobacterium bovis* bacillus Calmette-Guérin) and Gram-negative bacteria (*Escherichia coli*) (13–16), *Chlamydia pneumoniae* (17) and *Trypanosoma cruzi* (18), as well as pathogen-related molecules (LPS and lipoarabinomannan) (13, 14) acting through their specific receptors (CD14, TLR4, and TLR2) (13, 14) were also shown to elicit signaling that culminate with activation of ER-assembling lipid body machinery. Downstream to the activation by pathogen-derived molecules, endogenous molecules—yet to be characterized—may also trigger lipid body biogenesis during infection-driven inflammation therefore comprising potential therapeutical targets for lipid body regulated pathologies.

MCP-1 is the prototype of CC chemokines β subfamily and exhibits the most potent chemotactic activity for monocytes. Collectively, both experimental and clinical studies have clearly established a key role of MCP-1 in the pathogenesis of macrophage-driven inflammatory disturbs, such as atherosclerosis and sepsis (reviewed in Ref. 19). Beyond its conventional role in leukocyte recruitment, MCP-1 and its receptor CCR2 contributions to cell activation and inflammatory mediator production are beginning to be unveiled. The current study shows that MCP-1 directly activates the biogenesis of lipid bodies equipped with active leukotriene B_4 (LTB_4)-synthesizing machinery within macrophages. MCP-1-induced lipid body formation and function signals through MCP-1 receptor CCR2 and the ERK and PI3K. Moreover, we demonstrated that MCP-1-driven lipid body biogenesis is a highly regulated phenomenon that culminates in microtubule-dependent lipid body assembly and protein compartmentalization leading to enhanced LTB_4-synthesizing lipid bodies during inflammatory response such as the observed during sepsis or endotoxemia.

Materials and Methods

Animals

The C3H/HeJ, LPS hyporesponsive mouse strain that has an inactivating point mutation within the signal transducing domain of the *Tlr4* gene (20), and the LPS-responsive mouse strain C3H/He of both sexes weighing 20–25 g were obtained from the Fundação Oswaldo Cruz Breeding Unit. MCP-1-deficient mice (MCP-1$^{-/-}$) of C57BL/6 genetic background and wild-type litter mates (21) were provided from Dr. C. Gerard (Harvard Medical School, Boston, MA) and CCR2-deficient mice (CCR2$^{-/-}$; in a homogeneous C57BL/6 background) (22) were provided by Dr. W. Kuziel (PDL BioPharma, Fremont, CA) and bred at the Departamento de Fisiologia e Farmacodinâmica (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil) experimental animal facility. 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 to the animal care guidelines of the National Institute of Health and were approved by the Oswaldo Cruz Animal Welfare Committee.

Mouse model of endotoxemia and sepsis: cecal ligation and puncture (CLP)

A murine model of endotoxemia and of polymicrobial sepsis was induced by CLP as previously described (16). Briefly, endotoxemia was induced in wild-type and MCP-1$^{-/-}$ mice with LPS (from *E. coli* 0111:B4; 300 μg/cavity i.p.) diluted in sterile saline. Control groups received equal volumes

of vehicle. All endotoxin-stimulated animals appeared acutely ill and displayed signs of lethargy and diarrhea.

Polymicrobial sepsis was induced in MCP-1$^{-/-}$ and wild-type mice anesthetized with a mixture of thiopental (40 mg/kg) and ketamine (80 mg/kg) diluted in sterile saline and administered i.p. (0.2 ml), as previously described (23). In brief, the cecum was punctured once with an 18-gauge needle and was then squeezed to empty its contents through the puncture. Immediately after the surgery, 0.5 ml of sterile saline was administered s.c. to the animals for volume resuscitation. Sham-treated mice were subjected to identical procedures except that ligation and puncture of the cecum were omitted. Animals subjected to CLP developed early signs of sepsis, including lethargy, piloerection, and diarrhea.

In vitro stimulation and treatment of macrophages

Peritoneal exudate cells removed from CCR2$^{-/-}$, MCP-1$^{-/-}$ or C57BL/6 wild-type mice were cultured in RPMI 1640 medium with L-glutamine, sodium bicarbonate, 1% of antibiotic (10,000 U/ml penicillin and 10,000 μg/ml streptomycin), 0.5% FCS at 2×10^6 cells/ml on 24-well plates. Cells were pretreated with anti-MCP-1/CCL2 (10 μg/ml; R&D Systems). To study the effect of inhibitors, cells were pretreated with LY294002, PD98059, U0126 (1 or 10 μM), taxol or colchicine (0.1 or 1 μM) at 37°C for 30 min before the stimulation with rMCP-1 (25, 50 or 100 nM; PeproTech) or LPS (500 ng/ml; from *E. coli* 0127:B8) for different time intervals at 37°C. The cell viability, determined by trypan blue dye exclusion at the end of each experiment, was >90% for each drug.

Lipid body analysis

Macrophages were stained with oil red O (Sigma-Aldrich) or osmium tetroxide (Electron Microscopy Science). In brief, macrophages were fixed in 3.7% formaldehyde in Ca^{2+}-Mg^{2+}-free HBSS (pH 7.4) for 30 min. For oil red O staining, cells were rinsed in 85% propylene glycol, stained in 0.5% oil red O for 10 min, rinsed in 85% propylene glycol (5 min) and counterstained with hematoxylin for 30 s. For osmium staining, cells were rinsed in 0.1 M cacodylate buffer, 1.5% OsO$_4$ (30 min), rinsed in dH$_2$O, immersed in 1.0% thiocarbohydrazide (5 min), rinsed in 0.1 M cacodylate buffer, restained in 1.5% OsO$_4$ (3 min), rinsed in H$_2$O, and then dried and mounted. The morphology of fixed cells was observed, and lipid bodies were enumerated by light microscopy with a ×100 objective lens in 50 consecutively scanned macrophages.

LTB$_4$ measurement

LTB$_4$ were measured directly in the cell-free supernatants from peritoneal lavage obtained 6 h after vehicle, LPS, or CLP, or from in vitro-stimulated macrophages. In indicated experiments, vehicle, LPS, or rMCP-1 in vitro-stimulated macrophages (6 h) were restimulated with A23187 (0.5 μM) for 15 min. Stimulatory reaction was stopped on ice, and the samples were centrifuged at 500 × g for 10 min at 4°C. The levels of LTB$_4$ in cell supernatants were assayed by an enzyme immunoassay kit according to the manufacturer's instructions (Cayman Chemical).

Intracellular immunodetection of newly synthesized LTB$_4$ within macrophages

To immunodetection of newly formed LTB$_4$ at its subcellular sites of synthesis within in vivo LPS-stimulated or in vitro MCP-1-stimulated macrophages, the cell preparations were mixed with 500 μl of water-soluble 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC in HBSS; 0.5% final concentration with cells; Sigma-Aldrich), used to cross-link eicosanoid carboxyl groups to amines in adjacent proteins. After 30–40 min incubation at 37°C with EDAC to promote both cell fixation and permeabilization, peritoneal macrophages were then washed with HBSS, cytospun onto glass slides, and blocked with HBSS containing 2% normal donkey serum for 10 min. The cells were then sequentially incubated with a rabbit anti-LTB$_4$ antiserum (Cayman Chemical) for 45 min. Then, a guinea pig anti-ADRP polyclonal Ab (Research Diagnostics) or Bodipy 493/503 (Molecular Probes) was added for 45 min to distinguish cytoplasmic lipid bodies within leukocytes. The cells were then washed with HBSS for 10 min (three times) and incubated with Cy2-labeled anti-rabbit IgG plus Cy3-labeled anti-guinea pig IgG secondary Abs for 1 h. The specificity of the LTB$_4$ immunolabeling was ascertained by three different approaches: 1) a nonimmune rabbit IgG used as an irrelevant control to anti-LTB$_4$ Ab; 2) incubation (30 min prior EDAC) with the inhibitor of 5-lipoxygenase (5-LO) zileuton (1 μM) to avoid the synthesis of LTB$_4$; and 3) the analysis of LTB$_4$ staining within nonstimulated leukocytes.

The images were obtained using an Olympus BX-FLA fluorescence microscope and CoolSNAP-Pro CF digital camera in conjunction with Image-Pro Plus version 4.5.1.3 software (MediaCybernetics). The images were analyzed using Adobe Photoshop 5.5 software (Adobe Systems).

Immunodetection of microtubular system of macrophages

Immunolabeling of microtubular network of MCP-1-stimulated resident peritoneal macrophages of wild-type animals was performed by immunostaining α-tubulin. In brief, macrophages stimulated for 6 h with rMCP-1 (100 nM) were fixed in 4% formaldehyde plus 4% sucrose (20 min) and permeabilized with ammonium chloride/PBS 50 nM (30 min) plus 0.05% saponin/PBS solution (30 min). After wash, macrophages were incubated for 1 h with mouse anti-α-tubulin mAbs diluted in 0.05% saponin plus 0.2% gelatin/PBS solution (overnight). After three washes with 0.05% saponin with 0.2% gelatin/PBS solution (10 min each), the preparations were incubated for 1 h with the Cy2-labeled anti-mouse IgG detection Ab (Jackson ImmunoResearch Laboratories) for 1 h. Slides were then washed three times, stained with 4',6'-diamidino-2-phenylindole (0.5 μg/ml, 5 s; Molecular Probes), and washed with PBS and distilled water (10 min). To distinguish cytoplasmic lipid bodies within α-tubulin-labeled macrophages, anti-ADRP was added, as described above. Alternatively, α-tubulin-labeled cells were costained with anti-5-LO. Briefly, after fixation and permeabilization, slides were incubated for 1 h with anti-5-LO polyclonal Abs (Cayman Chemical) diluted in 0.05% saponin/HBSS solution. Nonimmune rabbit serum was used as control. Immunodetection was achieved with a Cy3-labeled anti-guinea pig detection Ab (Jackson ImmunoResearch Laboratories). Cell images were obtained and analyzed as described above.

Statistical analysis

The data are represented as mean $\pm$ SEM and were statistically analyzed by means of ANOVA, followed by the Newman-Keuls-Student test, with a significance level set at $p < 0.05$.

Results

Lipid body biogenesis and LTB$_4$-synthesizing function elicited by infection-driven inflammation are mediated by endogenous MCP-1

A putative role of MCP-1 as an endogenous regulator of lipid body biogenesis and LTB$_4$-synthesizing function during infection-driven inflammation was investigated using two mouse models of peritonitis, polymicrobial infection sepsis induced by CLP (Fig. 1A) and LPS-induced endotoxemia (Fig. 1B), in MCP-1$^{-/-}$ vs wild-type mice. As previously reported, the numbers of cytoplasmic lipid bodies found within peritoneal macrophages of wild-type animals undergoing either a septic condition or endotoxemia were markedly increased (13, 16, 24). As shown in Fig. 1, A and B, basal levels of lipid bodies were not altered within macrophages of MCP-1$^{-/-}$ mice. In both experimental models, lipid body biogenesis was virtually absent in MCP-1$^{-/-}$ mice (Fig. 1, A and B), but very pronounced in the wild-type mice. Therefore, endogenous MCP-1 produced during sepsis- and/or endotoxin-driven inflammatory responses has a critical role on activation of lipid body assembling machinery within responsive macrophages. Of note, participation of endogenous MCP-1 in lipid body biogenesis depends on the stimulus. For instance, mycobacterial infection-induced lipid bodies in macrophages are MCP-1 independent (14).

In both experimental models of infection and concurring with sepsis- and endotoxin-induced in vivo lipid body biogenesis, the production of LTB$_4$ was also prominent in wild-type animals, but negligible in MCP$^{-/-}$ animals (Fig. 1, A and B). This parallelism between reduced numbers of lipid bodies and levels of secreted LTB$_4$ observed in MCP$^{-/-}$ animals turned up to be a functional correlation. Intracellular compartments of LTB$_4$ synthesis have never been determined. As illustrated in Fig. 1C, by using a new methodology that cross-linked and immunolabeled eicosanoids at its sites of synthesis (25), resident macrophages were identified as the cell population responsible for LTB$_4$ production during endotoxin-induced inflammatory reaction. The specificity of the LTB$_4$

immunolabeling was ascertained by the lack of LTB$_4$ immunolabeling within zileuton pretreated LPS-stimulated macrophages or within nonstimulated macrophages (Fig. 1C).

A detailed analysis revealed that the intracellular LTB$_4$-synthesizing compartment was in a punctate cytoplasmic pattern, proximate to, but separate from the nucleus, and fully consistent in size and form with macrophage lipid bodies. In fact, the compartmentalization of newly formed LTB$_4$ at macrophage lipid bodies was ascertained by the colocalization with ADRP (Fig. 1D). Virtually no LTB$_4$ immunolabeling was observed within LPS-stimulated macrophages of MCP$^{-/-}$ animals (Fig. 1D), thus showing that the newly formed lipid bodies of in vivo LPS-stimulated macrophages are inducible MCP-1-elicited organelles, which are enzymatically skilled for an effective LTB$_4$ synthesis.

MCP-1 triggers biogenesis of LTB$_4$-synthesizing lipid bodies within macrophages

To investigate whether MCP-1 was indeed capable of inducing formation of new lipid bodies, we have directly stimulated in vitro mouse macrophages with rMCP-1. MCP-1 induced a dose-dependent increase in the numbers of cytoplasmic lipid bodies within resident peritoneal macrophages isolated from naive mice (Fig. 2A). As shown in Fig. 2B, rMCP-1-induced lipid body biogenesis was a rapid phenomenon, which was significant within 2 h and maximum within 6 h at the concentration of 100 nM. In parallel to the increased number of newly assembled lipid bodies (within 6 h), rMCP-1 (100 nM) also caused the release of LTB$_4$ by macrophages (Fig. 2C). MCP-1-induced lipid body biogenesis and LTB$_4$-synthesizing function are not due to LPS contamination, because: 1) the neutralizing Ab against MCP-1 abolished MCP-1 effect (17.8 $\pm$ 0.9 lipid bodies per rMCP-1-stimulated cell vs 4.2 $\pm$ 0.1 lipid bodies found in anti-MCP-1-treated MCP-1-stimulated cell; $n = 6$); and 2) the resident peritoneal macrophages isolated from TLR4-deficient mice respond to rMCP-1 (100 nM), but not to LPS, with rapid (6 h) lipid body biogenesis and LTB$_4$ synthesis and similar magnitude to that observed with macrophages isolated from wild-type mice (Table I).

As shown in Fig. 2D, LTB$_4$ was synthesized within the cytoplasmic lipid bodies found in MCP-1-stimulated macrophages, because immunofluorescent LTB$_4$ was clearly compartmentalized within punctate bodipy labeled organelles. Therefore, MCP-1 induces the rapid formation of distinctive lipid bodies assembled with a specific protein composition that enable LTB$_4$ synthesis by macrophages. Of note, eicosanoid-synthesizing function may vary according with leukocyte type and stimulatory condition, inasmuch as protein composition of newly formed lipid bodies can be heterogeneous.

Cellular mechanisms of infection-related lipid body biogenesis and LTB$_4$-synthesizing function depend on an autocrine/paracrine activity of macrophage-derived MCP-1

To further investigate the mechanisms of MCP-1-driven lipid body biogenesis and LTB$_4$-synthesizing function, macrophages stimulated in vitro with LPS were treated with a neutralizing Ab anti-MCP-1. The neutralization of MCP-1 inhibited both lipid body formation and LTB$_4$ production (Table II), indicating that LPS-stimulated macrophages secrete MCP-1 acting autocrinally and/or paracrinally on macrophages triggers the assembling of LTB$_4$-synthesizing lipid bodies. Confirming the results with the neutralizing Ab to MCP-1, MCP-1$^{-/-}$ mouse-derived resident macrophages stimulated in vitro with LPS displayed negligible lipid body biogenesis (Table II). The addition of exogenous MCP-1 to MCP-1$^{-/-}$ macrophages was able to induce per se a significant increase in lipid body number (data not shown) as well as, to completely
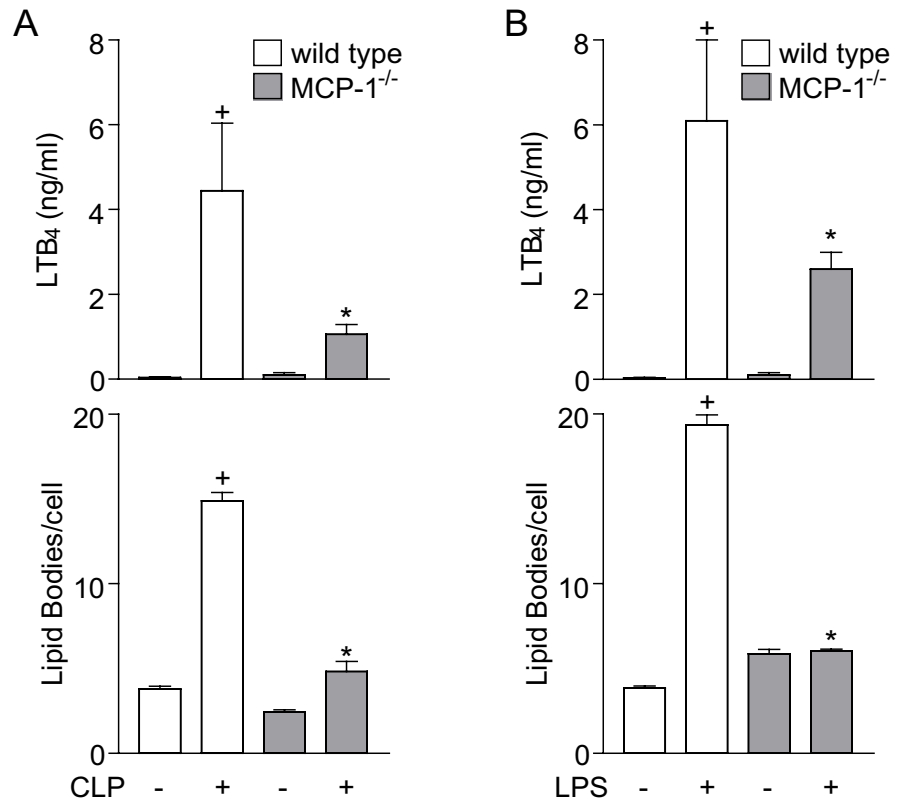
FIGURE 1. MCP-1 mediates lipid body formation and LTB$_4$ synthesis induced by infection-driven inflammatory response. In *A* and *B*, sepsis (CLP) and endotoxemia models of inflammation, respectively, were performed in wild-type vs MCP$^{-/-}$ mice. Analyses of lipid body formation and LTB$_4$ production were performed 6 h after stimulation. Results were expressed as means ± SEM from at least eight animals; +, $p \leq 0.05$ compared with wild-type control group; *, $p \leq 0.05$ compared with wild-type stimulated-mice. In *C*, leukocytes from in vivo vehicle- (*right panel*) or LPS-stimulated animals (*middle panel*) were immunolabeled for newly formed LTB$_4$ 6 h after stimulation. LPS-stimulated animals were treated with zileuton (1 μM), 30 min before incubation with EDAC (*left panel*). Images show that LTB$_4$ immunolabeling has a punctate cytoplasmic pattern that is absent in nonstimulated cells, inducible by LPS, and sensitive to zileuton. In *D*, anti-LTB$_4$ was merged with identical field of anti-ADRP fluorescent images of macrophages recovered from wild-type (*top panels*) vs MCP$^{-/-}$ (*bottom panels*) mice stimulated with LPS. Images show LTB$_4$ immunoreactive lipid bodies (as identified by anti-ADRP) of peritoneal macrophages. Scale bars, 5 μm.
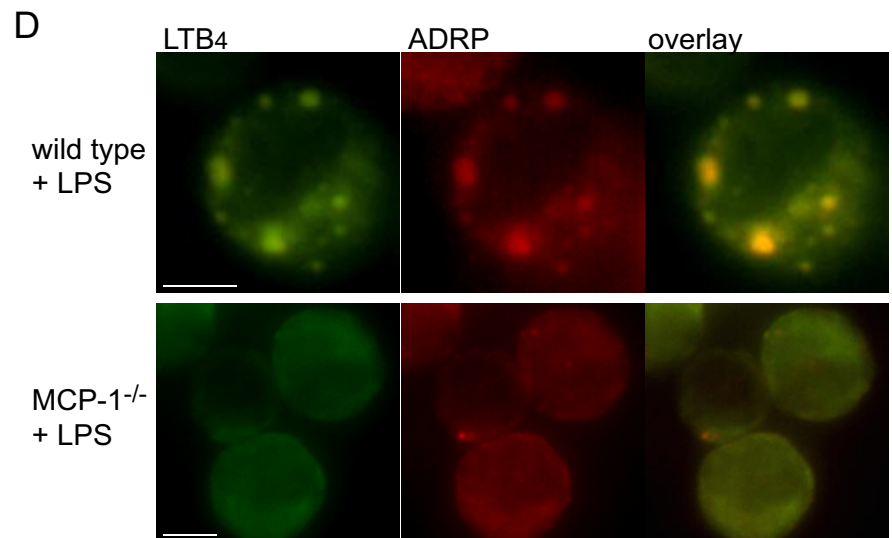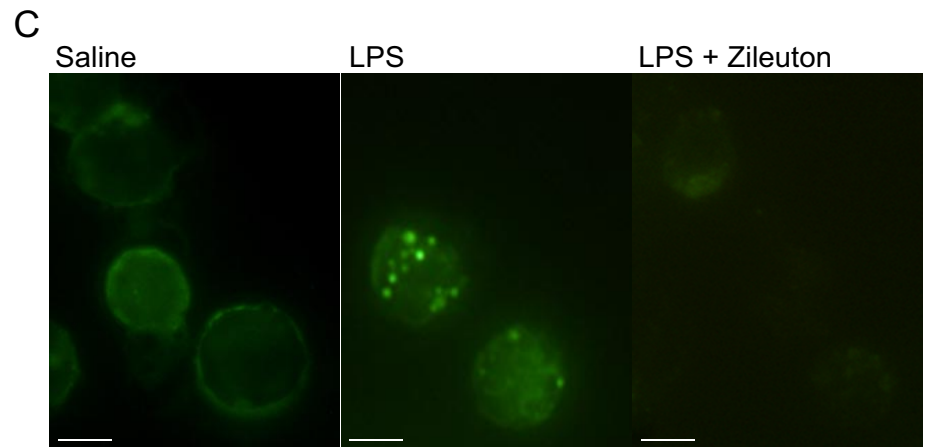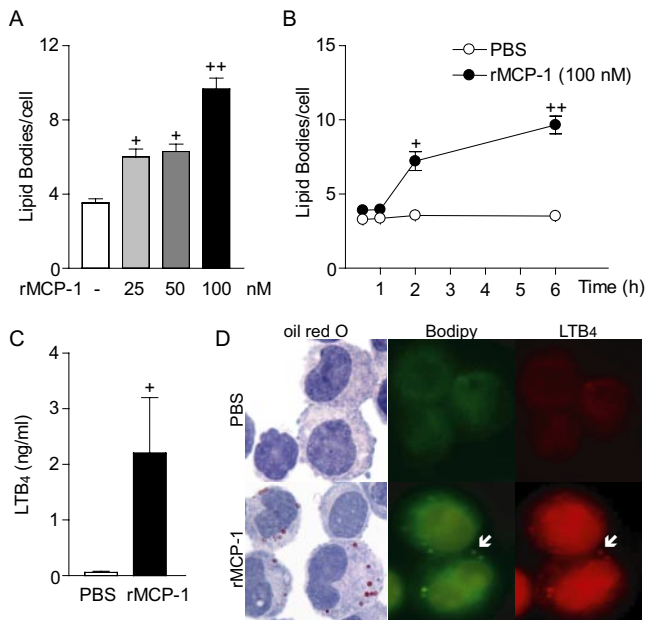
FIGURE 2. MCP-1 induces lipid body biogenesis and LTB$_4$ synthesis within newly formed lipid bodies of in vitro-stimulated macrophages. Dose-response (*A*) and kinetic (*B*) curves of lipid body biogenesis triggered by direct in vitro stimulation of macrophages with MCP-1. In *C*, analysis of LTB$_4$ production was performed 6 h after MCP-1 (100 nM) stimulation. Results were expressed as means $\pm$ SEM from at least three different animals; +, $p \leq 0.05$ and ++, $p \leq 0.01$ compared with PBS-stimulated cells. In *D*, macrophages stimulated in vitro with PBS (*top panels*) or MCP-1 (100 nM; *bottom panels*) were stained with oil red O (*left panels*), bodipy (*middle panels*), or anti-LTB$_4$. Images show that newly formed lipid bodies (as identified by oil red O and bodipy labeling) are sites of LTB$_4$ synthesis within MCP-1-stimulated macrophages. Scale bars, 5 μm.

Table II. *Autocrine/paracrine effect of endogenous MCP-1 secreted by LPS-stimulated macrophages in vitro*[a]

Condition	Animal Strain	Lipid Bodies/Cell	LTB$_4$ (ng/ml)
PBS	Wild type	2.3 ± 0.1	0.6 ± 0.0
LPS	Wild type	15.0 ± 0.8^b	2.7 ± 0.8^b
LPS plus anti-MCP-1	Wild type	3.2 ± 0.3^c	0.6 ± 0.1^c
PBS	MCP-1$^{-/-}$	3.0 ± 0.1	0.6 ± 0.4
LPS	MCP-1$^{-/-}$	4.9 ± 0.2^c	1.4 ± 0.1^c
LPS plus rMCP-1	MCP-1$^{-/-}$	10.7 ± 0.2^d	2.1 ± 0.6^d

[a] Macrophages isolated from wild-type and MCP-1$^{-/-}$ mice were stimulated in vitro with LPS alone or costimulated with rMCP-1 (100 nM) for 6 h. Pretreatment with neutralizing Ab anti-MCP-1 was performed 30 min before stimulation. Vehicle, LPS, or rMCP-1 in vitro-stimulated macrophages (6 h) were restimulated with A23187 (0.5 μM) for 15 min prior to LTB$_4$ analysis. Results were expressed as means $\pm$ SEM from at least three distinct animals.
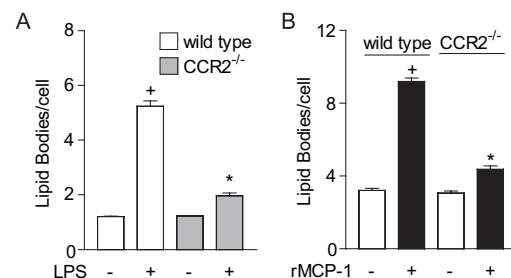[b] Value of $p \leq 0.05$ compared to PBS-stimulated cells.
[c] Value of $p \leq 0.05$ compared to LPS-stimulated cells isolated from wild-type mice.
[d] Value of $p \leq 0.05$ compared to LPS-stimulated cells isolated from MCP-1$^{-/-}$ mice.

Molecular mechanisms involved in MCP-1-driven assembling of LTB$_4$-synthesizing lipid bodies depend on CCR2, ERK, and PI3K activation

New lipid bodies assembled in ER membranes are not a simply sign of injury or excess lipid substrate. In fact, the biogenic process of lipid bodies is a complex cellular outcome triggered in a stimulus- and cell-dependent fashion by a variety of distinct signaling pathways. Here, our attempts to characterize the molecular signals committed to MCP-1-induced biogenesis of LTB$_4$-synthesizing lipid bodies revealed that CCR2 receptors and downstream signaling through ERK and PI3K are involved. As illustrated in Fig. 3, resident macrophages isolated from the MCP-1 receptor CCR2$^{-/-}$ mice were not able to assemble new lipid bodies in response to in vitro stimulation with LPS or rMCP-1. Moreover, two inhibitors of ERK 1/2 (PD98059 and U0126), even though did not alter the basal numbers of lipid bodies found in nonstimulated macrophages (data not shown), inhibited in a dose-dependent manner the MCP-1-induced lipid body biogenesis and the concurrent LTB$_4$ production (Fig. 4*A*). Similarly, an inhibitor of PI3K (LY294002) also blocked both formation and LTB$_4$-synthesizing function of lipid bodies induced by rMCP-1 (Fig. 4*B*). Collectively, our findings indicate that CCR2-elicited intracellular signaling cascade, characterized by ERK 1/2 and PI3K activation, switches on biogenesis of lipid bodies, particularly equipped with active LTB$_4$-synthesizing machinery. Of note, ERK and PI3K activation are known to be

restore the reduced LPS-induced biogenesis of LTB$_4$-synthesizing lipid bodies observed within MCP-1$^{-/-}$ macrophages (Table II). Therefore, MCP-1$^{-/-}$ mice express functional MCP-1 receptors on macrophages capable of eliciting lipid body biogenesis and compartmentalized LTB$_4$ synthesis. Altogether, these findings indicate that macrophages facing infection-related stimulatory conditions will respond initially secreting MCP-1, which in an autocrine/paracrine fashion may activate receptors expressed on the extracellular surface of macrophages.

Table I. *In vitro direct effects of rMCP-1 on lipid body biogenesis and LTB$_4$ synthesis are not due LPS contamination, because activation of TLR4 is not involved in these phenomena*[a]

Condition	Animal Strain	Lipid Bodies/Cell	LTB$_4$ (ng/ml)
PBS	C3H/He	2.45 ± 0.09	0.01 ± 0.00
rMCP-1	C3H/He	9.30 ± 0.17^b	0.73 ± 0.27^b
PBS	C3H/He	3.71 ± 0.09	0.01 ± 0.00
LPS	C3H/He	9.26 ± 0.09^b	0.42 ± 0.10^b
PBS	C3H/HeJ	3.87 ± 0.08	0.06 ± 0.00
rMCP-1	C3H/HeJ	9.64 ± 0.19^b	0.55 ± 0.17^b
PBS	C3H/HeJ	4.32 ± 0.09	0.01 ± 0.00
LPS	C3H/HeJ	4.56 ± 0.10	0.01 ± 0.00

[a] Macrophages isolated from TLR4-sensitive (C3H/He) and nonsensitive (C3H/HeJ) mice were stimulated in vitro with rMCP-1 (100 nM) or LPS (500 ng/ml) for 6 h. In vitro PBS-, rMCP-1- or LPS-stimulated macrophages were restimulated with A23187 (0.5 μM) for 15 min prior to LTB$_4$ analysis. Results were expressed as means $\pm$ SEM from at least three distinct animals.
[b] Value of $p \leq 0.05$ compared to proper PBS-stimulated cells.

FIGURE 3. MCP-1 induces lipid body formation and LTB$_4$ synthesis in macrophages through CCR2 activation. In vitro stimulation with LPS (*A*) or MCP-1 (*B*) trigger formation of new lipid bodies within macrophages isolated from wild-type, but not from CCR2$^{-/-}$, animals. Analysis of lipid body formation was performed 6 h after in vitro stimulation. Results were expressed as the means $\pm$ SEM from at least three different animals; +, $p \leq 0.05$ compared with wild-type control group; ∗, $p \leq 0.05$ compared with wild-type stimulated mice.
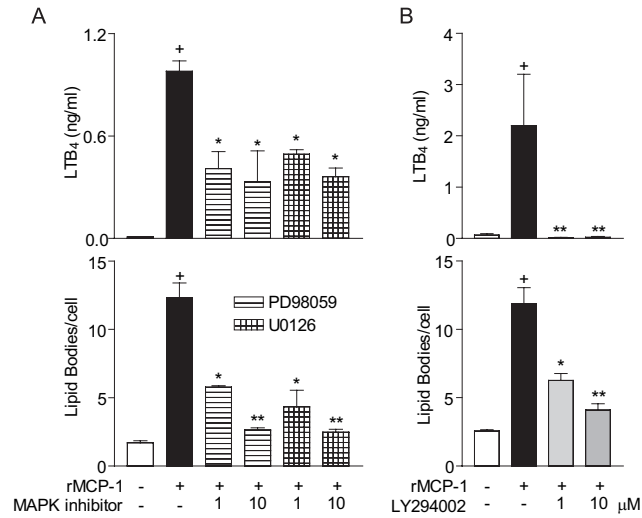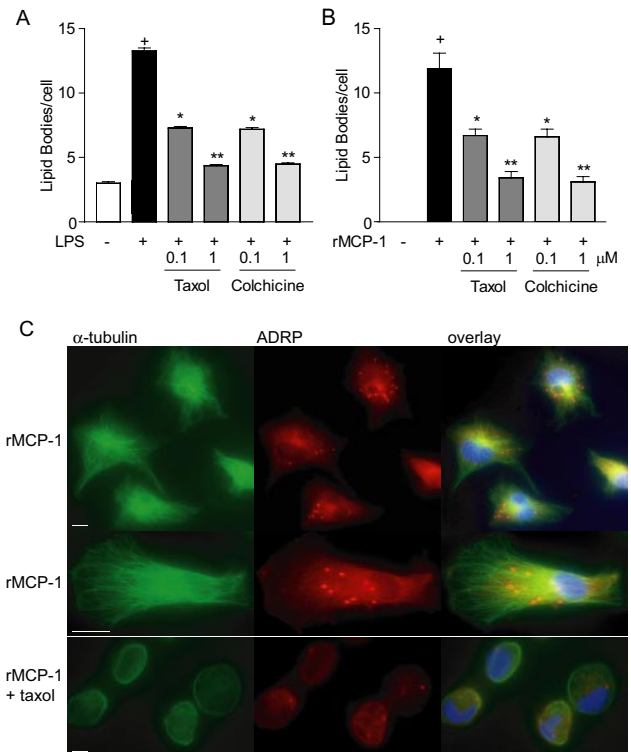
FIGURE 4. ERK 1/2 and PI3K kinases mediate MCP-1-induced lipid body formation and LTB$_4$ synthesis in macrophages. Macrophages stimulated in vitro with MCP-1 (100 nM) were pretreated with ERK 1/2 kinases inhibitors PD98059 and U0126 (*A*) or with PI3K inhibitor LY294002 (*B*) for 30 min before stimulation with indicated concentrations. Analysis of lipid body formation and LTB$_4$ production were performed 6 h after in vitro stimulation. Results were expressed as means ± SEM from at least three different animals; +, $p \leq 0.05$ compared with control groups; ∗, $p \leq 0.05$ and ∗∗, $p \leq 0.01$ compared with MCP-1-stimulated cells.

FIGURE 5. MCP-1-induced lipid body biogenesis depends on microtubular dynamics within macrophages. To assess microtubule-driven role on lipid body biogenesis, macrophages were pretreated with microtubule active drugs, including taxol and colchicines, 30 min before MCP-1 (100 nM) stimulation. Concentrations of drugs are indicated in *A* and *B*, and taxol concentration in *C* was 1 μM. In *A* and *B*, LPS- and MCP-1-elicited lipid body formation was analyzed 6 h after stimulation. Results were expressed as means ± SEM from at least different three animals; +, $p \leq 0.05$ compared with control groups; ∗, $p \leq 0.05$ and ∗∗, $p \leq 0.01$ compared with LPS- or MCP-1-stimulated cells. In *C*, macrophages stimulated in vitro with MCP-1 for 6 h were stained with anti-tubulin (*left panels*) and anti-ADRP (*middle panels*). *Right panels*, Merged images illustrating the distribution of newly formed lipid bodies enmeshed in microtubule network of MCP-1-stimulated macrophages. Scale bars, 5 μm.

important players of conventional MCP-1-elicited process of cell polarization/motility (26), a cytoskeleton-mediated cellular activity that seemed to require microtubular dynamics (27, 28).

Microtubule dynamics controls MCP-1-induced assembling of LTB$_4$-synthesizing lipid bodies

Microtubules play a well-demonstrated role in the cytoplasmic transport and spatial distribution of organelles; however, microtubular function on organelle biogenesis is not well-defined (28). Here, to study a putative role of microtubules on lipid body biogenesis and function, microtubule-active drugs were used. To understand the effect of microtubule depolymerization, peritoneal resident macrophages were pretreated with colchicine, a microtubule-destabilizing drug. Colchicine inhibited in a dose-dependent manner the assembling of new lipid bodies and synthesis of LTB$_4$ induced in vitro by LPS (Figs. 5*A* and 6*A*) or rMCP-1 (Figs. 5*B* and 6*B*). Therefore, disassembly of microtubules may disrupt ER biogenic machinery of LTB$_4$-synthesizing lipid bodies that may be in part dependent on the maintenance of a well-organized microtubule network. To further evaluate the effect of sustained microtubular polymerization, peritoneal resident macrophages were pretreated with taxol, a microtubule-stabilizing drug. As colchicine, taxol inhibited in a dose-dependent manner the assembling of new lipid bodies and synthesis of LTB$_4$ induced in vitro by LPS (Figs. 5*A* and 6*A*) or rMCP-1 (Figs. 5*B* and 6*B*). In as much as both microtubule disrupting vs stabilizing drugs (colchicine and taxol, respectively) have inhibitory effects on lipid body biogenesis and LTB$_4$-synthesizing function, we conclude that MCP-1-elicited highly regulated assembly of a lipid body depends on microtubule dynamics.

The inhibitory effect on biogenesis of LTB$_4$-synthesizing lipid bodies observed in the presence of taxol was accompanied by derangement of microtubule distributions, as visualized in MCP-1-stimulated peritoneal resident macrophages labeled with immunofluorescent α-tubulin. As illustrated in Figs. 5*C* and 6*C*, the

microtubular system of MCP-1-stimulated macrophages appears as a delicate network that radiates from the nucleus. Enmeshed in this microtubule network, immunolabeled ADRP-positive lipid bodies appeared as peripheral circumferential staining found either adjacent to or distant from the nucleus within macrophage cytoplasm (Fig. 5*C*), suggestive of MCP-1-induced motility of newly assembled lipid body on microtubule tracks. As ADRP is a key regulator of lipid body biogenesis, 5-lipoxygenase (5-LO) is a limiting step of the LTB$_4$-synthesizing pathway. As shown in Fig. 6*C*, differently from external ring-like ADRP immunostaining, immunofluorescent 5-LO was found as a filled punctate immunostaining, confirming previous ultrastructural data suggesting that 5-LO pervades the lipid body core (29). Both lipid body relevant biogenic and functional proteins were affected by taxol-induced microtubule stabilization. As shown in the bottom images of Figs. 5*C* and 6*C*, pretreatment with taxol for 30 min reorganizes the MCP-1-induced microtubule orientation in a typical peripheralization of microtubule bundles and impairs the formation of ADRP-circumscribed (Fig. 5*C*) 5-LO-packed (Fig. 6*C*) lipid bodies at the immunofluorescence levels. Therefore, active microtubule derangement seems to represent one of the molecular events involved in the activation of ADRP-regulated biogenic machinery and 5-LO-driven
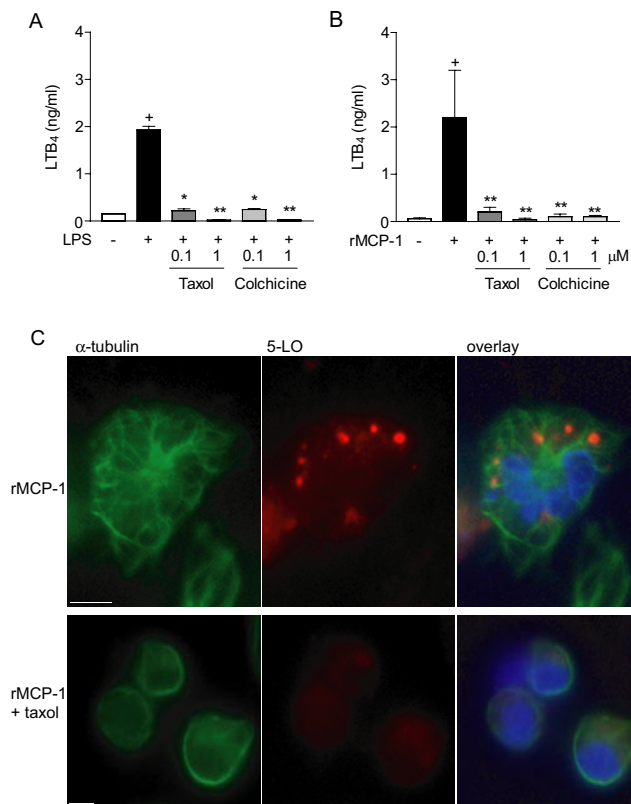
FIGURE 6. MCP-1-induced LTB$_4$-synthesizing function of newly formed lipid bodies depends on microtubular dynamics within macrophages. To assess microtubule role on lipid body function as active compartment of LTB$_4$ synthesis, macrophages were pretreated with microtubule active drugs, including taxol and colchicine, 30 min before MCP-1 (100 nM) stimulation. Concentrations of drugs were indicated in *A* and *B*, and taxol concentration in *C* was 1 μM. Respectively in *A* and *B*, LPS- and MCP-1-elicited LTB$_4$ production was analyzed 6 h after stimulation. Results were expressed as means ± SEM from at least three different animals; +, $p \leq 0.05$ compared with control groups; *, $p \leq 0.05$ and **, $p \leq 0.01$ compared with LPS- or MCP-1-stimulated cells. In *C*, macrophages stimulated in vitro with MCP-1 for 6 h were stained with anti-tubulin (*left panels*) and anti-5-LO (*middle panels*). *Right panels*, Merged images illustrating the distribution of 5-LO-containing lipid bodies enmeshed in microtubule network of MCP-1-stimulated macrophages. Scale bars, 5 μm.

LTB$_4$-synthesizing function of lipid bodies within MCP-1-stimulated macrophages.

Discussion

Biogenesis of lipid bodies is a central event to whole body energy homeostasis, as well as to several important human diseases. In inflammatory reactions, leukocytes constantly show increased numbers of cytoplasmic lipid bodies. Advances on the mechanisms behind the enhanced biogenesis of leukocyte lipid bodies are of paramount importance for understanding the pathogenesis of inflammatory diseases, such as allergic, atherosclerotic, and infection-driven disorders. Here, focusing on macrophage-driven pathologies, we have unveiled MCP-1 as an endogenous and potent biogenic stimulus of enzymatically active lipid bodies, and placed MCP-1-driven lipid bodies as key organelles involved on LTB$_4$ synthesis by macrophages. The MCP-1-elicited lipid body biogenic process was dependent on activation of a CCR2-elicited ERK and PI3K signaling, as well as, on an intact and dynamic microtubular system.

MCP-1 is a major mediator of leukocyte trafficking into the sites of the immune response. Here, the chemokine MCP-1 (CCL2) was

identified as a molecule capable of initiate lipid body biogenesis in macrophages. How does a professional chemoattractant arrange an effective signaling to activate the lipid body biogenic machinery within macrophages? The activating mechanisms involved in MCP-1-induced lipid body formation within macrophages were analyzed. Cells that respond to MCP-1 stimulation with a chemotactic activity do so through activation of CCR2. MCP-1-induced macrophage lipid body formation was drastically inhibited in CCR2-deficient mice, indicating a requisite role for CCR2 in lipid body assembly. It is noteworthy that besides inducing chemotactic functions, chemokine receptors can evoke leukocyte activation characterized by enhanced biogenesis of lipid bodies. For instance, activation of the eosinophilotactic receptor CCR3 by eotaxin (CCL11), eotaxin-2 (CCL24), eotaxin-3 (CCL26), or RANTES (CCL5) stimulates lipid body biogenesis in eosinophils (25, 30, 31). Our findings indicate that CCR2-driven lipid body biogenesis triggered by MCP-1 is dependent on downstream activation of PI3K and ERK1/2 kinases. Accordingly, activation of ERK 1/2 and PI3K in response to CC chemokines has been shown to participate in the regulated activation of lipid body biogenesis in other cell systems (25, 32), as well as in other MCP-1-induced leukocyte functions (26, 33). The specific isoform(s) involved in MCP-1-induced lipid body biogenesis are presently unknown. In addition to controlling lipid body biogenesis, activation of kinases within lipid bodies may have impacts to lipid body functions and interactions with organelles and cytoskeleton. In fact, Yu et al. (34, 35) demonstrated that PI3K regulatory and catalytic subunits, as well as ERK1, ERK2, and p38 MAPK, are compartmentalized and active within macrophage lipid bodies, suggesting that kinase-mediated signaling may take place within cytoplasmic lipid bodies in leukocytes.

Besides signaling molecules, structural molecules may also participate in downstream activation of lipid body biogenic machinery in macrophage ER. In fact, one can speculate that the role of MAPK and PI3K in lipid body biogenesis is presumably to provide activation of structural proteins actually involved in assembling lipid bodies. Recent studies using NIH 3T3 cells stimulated with insulin have established that dynein, a microtubule motor, has a central role in the growth of lipid bodies by fusing them (36). Dynein required phosphorylation, performed by the MAPK ERK2, to interact with its cargos, such as lipid bodies and may potentially control lipid body assembly by providing the structural conditions required to let the lipid body arise from the ER membrane (32). In agreement with this hypothesis, here we showed that lipid body assembly triggered by activation of CCR2 by MCP-1 depends on activities of both ERK 1/2 kinases and the microtubular system of macrophages.

It is well-established that microtubules have roles in cellular processes affecting lipid body size and location, such as lipid body growth (36) and motility (37). However, to best of our knowledge, no function of microtubules had been described on assembly of new lipid bodies. We hypothesized that a link between the source of the biogenic signal at cell membrane (e.g., CCR2 activation by MCP-1) and the ER-assembling machinery may involve cytoskeletal filaments, inasmuch as nascent lipid bodies are typically found enmeshed in them (38–40). To directly assess microtubular function, we have used a pharmacological approach by pretreating MCP-1-stimulated macrophages with microtubule-active drugs. Both microtubule disrupting vs stabilizing drugs (colchicine and taxol, respectively) blocked lipid body biogenesis, indicating that MCP-1-elicited ER lipid body assembly depends on activity of microtubule network. Whether the mechanism involved in microtubule-driven formation of lipid bodies depends on either a

function as scaffold to the ER-assembling machinery (42) or an intricate process affecting other microtubule-associated proteins such as PI3K and dynein, needs to be established. Alternatively, microtubule-regulated ADRP availability, localization, or activity may correspond to a more refined mechanism. Our analysis of ADRP compartmentalization within MCP-1-stimulated macrophages showed that the ADRP immunofluorescent halo is gone within a reoriented microtubular system, suggesting an effect on ADRP-regulated biogenic machinery.

Cross-talk between MCP-1/CCL2 and leukotrienes has been suggested in sepsis and atherosclerosis (11, 43, 44), however, mechanisms involved in MCP-1-induced increased leukotriene synthesis are unknown. In leukocytes, newly assembled lipid bodies are committed to specific inflammatory functions that may vary according to the cell type, specific biogenic stimulus and subsequent intracellular signaling that set off its biogenic machinery. Even though 5-LO—an enzyme with membrane insertion domains—needs to associate with membranes for its activation and catalysis, 5-LO have been found distributed throughout lipid bodies of activated leukocytes (13, 29). The presence of ER-derived membranous structures within lipid body cores of leukocytes may provide the means for membrane-associated proteins, like 5-LO, to be localized within lipid bodies (5). Despite large probability, synthesis of LTB_4 within 5-LO-containing lipid bodies was more hypothetical than based on firm evidence. Although it is well-established that 5-LO is compartmentalized within lipid bodies of activated leukocytes (45), the putative intracellular compartmentalization of the terminal enzyme of LTB_4 synthesis (LTA_4 hydrolase) within lipid bodies was never studied. In fact, there is no consensus in literature about the intracellular compartment of LTB_4 synthesis (46). Inferring intracellular eicosanoid-synthesizing compartments by immunolocalizing terminal enzymes represents an experimental strategy that has been recently overcome by an approach that directly targets the newly synthesized lipids. Here, by directly localizing the newly formed LTB_4 at its sites of synthesis, we have for the first time demonstrated that cytoplasmic lipid bodies could synthesize LTB_4 under proper stimulation. Both MCP-1- and LPS-stimulated macrophages assemble lipid bodies with enzymatic machinery proper to LTB_4 synthesis. Similar to the biogenic process, the LTB_4-synthesizing functional capability of MCP-1-elicited lipid bodies depended on CCR2-driven activation of PI3K and ERK1/2, as well as, microtubular dynamics within MCP-1-stimulated macrophages. Specifically regarding putative mechanisms involved in how disturbance of microtubules affects LTB_4 synthesis, the enzymatic composition of MCP-1-elicited lipid bodies appears to represent a target. We observed that the compartmentalization of 5-LO within MCP-1-elicited lipid bodies was shifted by the pretreatment with taxol, indicating that disruption of microtubule network affect the organization of 5-LO-limiting enzymatic complex of LTB_4 synthesis. The mechanism involved may be due an interference on the rapid translocation that 5-LO undergoes under stimulation from the cytosol of nonstimulated leukocytes to the cores of nascent lipid bodies of activated leukocytes (29).

In conclusion, our results indicate that MCP-1 plays a requisite role in macrophage lipid body biogenesis and functions during innate immune response to infection. Moreover, we demonstrated that MCP-1-driven lipid body biogenesis is a highly regulated phenomenon that culminates in microtubule-dependent lipid body assembly and protein compartmentalization leading to enhanced LTB_4-synthesizing lipid bodies during infection-related inflammatory response, such as the observed during sepsis or endotoxemia. Our data provide the basis to support the lipid body as dynamic organelles involved in amplification of the inflammatory response

and, as such, inhibition of lipid body biogenesis may provide targets for anti-inflammatory therapy.

Disclosures

The authors have no financial conflict of interest.

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