The Journal of Immunology

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J Immunol 2002; 169:6498-6506; ;
doi: 10.4049/jimmunol.169.11.6498
http://www.jimmunol.org/content/169/11/6498

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Lipopolysaccharide-Induced Leukocyte Lipid Body Formation In Vivo: Innate Immunity Elicited Intracellular Loci Involved in Eicosanoid Metabolism[1]

Patrícia Pacheco,* Fernando A. Bozza,*† Rachel N. Gomes,* Marcelo Bozza,‡ Peter F. Weller,§ Hugo C. Castro-Faria-Neto,* and Patrícia T. Bozza[2]*

Lipid bodies are rapidly inducible, specialized cytoplasmic domains for eicosanoid-forming enzyme localization, which we hypothesize to have specific roles in enhanced inflammatory mediator production during pathological conditions, including sepsis. However, little is known about the origins, composition, or functions of lipid bodies in vivo. We show that lipid body numbers were increased in leukocytes from septic patients in comparison with healthy subjects. Analogously, the intrathoracic administration of LPS into mice induced a dose- and time-dependent increase in lipid body numbers. Pretreatment with anti-CD14 or anti-CD11b/CD18 mAb drastically inhibited LPS-induced lipid body formation. Moreover, LPS failed to form lipid bodies in C3H/HeJ (*TLR4* mutated) mice, demonstrating a requisite role for LPS receptors in lipid body formation. LPS-induced lipid body formation was also inhibited by the platelet-activating factor-receptor antagonists, suggesting a role for endogenous platelet-activating factor. The eicosanoid-forming enzymes, 5-lipoxygenase and cyclooxygenase-2, were immunolocalized within experimentally induced (LPS in mice) or naturally occurring (septic patients) lipid bodies. The proinflammatory cytokine involved in the pathogenesis of sepsis, TNF-α, was also shown to colocalize within lipid bodies. Prior stimulation of leukocytes to form lipid bodies enhanced the capacity of leukocytes to produce leukotriene B_4 and PGE_2. In conclusion, our studies indicate that lipid bodies formed after LPS stimulation and sepsis are sites for eicosanoid-forming enzymes and cytokine localization and may develop and function as structurally distinct, intracellular sites for paracrine eicosanoid synthesis during inflammatory conditions. ***The Journal of Immunology*****, 2002, 169: 6498–6506.**

The innate immune system has a fundamental role in host defense against bacteria by means of effector mechanisms that are activated immediately after infection through pattern recognition receptors leading to inflammatory mediator production and cell activation (1). Sepsis, septic shock, and multiple organ failure are serious consequences of Gram-negative bacterial infections that often lead to death (2). The interaction of LPS from Gram-negative bacteria with the host initiates the production of a cascade of proinflammatory mediators from monocytes and macrophages that are responsible for its effects (reviewed in Refs. 1 and 3). In the case of septic shock, inflammatory mediators are produced in excess, resulting in hemodynamic instability, activation of the complement and clotting cascades, and multiorgan failure. Lipid mediators including platelet-activating factor (PAF),[3] PGs, and leukotrienes (LTs) have been implicated in the pathogenesis of many inflammatory diseases, including acute respiratory distress syndrome and sepsis (4, 5).

LTs and PGs are enzymatically formed oxidative derivatives of arachidonic acid (AA) that have a wide range of biological activities, including roles as paracrine mediators of inflammation as well as intracellular signals (6). Arachidonate is released from arachidonyl phospholipids, which may reside in various membranes within cells, by the actions of phospholipases (7). Although the enzymatic pathways for eicosanoid formation are well understood, the intracellular sites of action of these enzymes and the cellular sources of AA during inflammation remain less clear. Different studies have focused on the intracellular localization of eicosanoid-forming enzymes. Cyclooxygenases (COXs) are associated with cellular membranes, including the endoplasmic reticulum and nuclear membrane (8, 9). In contrast, 5- lipoxygenase (5-LO) localization is cell type specific and also varies according to the activation state of the cell. 5-LO was shown to localize within the nuclear environment of alveolar macrophages but was predominantly cytosolic in human neutrophils and resting peritoneal macrophages (10–12). Although translocation from cytosol to membrane may facilitate interactions of cytosol enzymes with membrane-bound arachidonate, there is increasing evidence that specific compartmentalization of eicosanoid formation within cells may relate to the different paracrine and intracrine functions of eicosanoids (9, 13). In addition to membranes, another lipid-bearing domain in cells contains lipid bodies.

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Received for publication May 31, 2002. Accepted for publication October 2, 2002.

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[1] This work was supported by Howard Hughes Medical Institute (to P.T.B.), Conselho Nacional de Pesquisa (Brazil), Fundação de Amparo à Pesquisa do Rio de Janeiro, and National Institute of Health Grant AI22571.

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[3] Abbreviations used in this paper: PAF, platelet-activating factor; AA, arachidonic acid; LT, leukotriene; PG, prostaglandin; COX, cyclooxygenase; LO, lipoxygenase; PL, phospholipase; i.t., intrathoracic.

0022-1767/02/$02.00

Lipid bodies are non-membrane-bound, lipid-rich cytoplasmic inclusions that are candidates to play a major role in the formation of eicosanoid mediators during inflammation. Lipid bodies have been recognized to be abundant in cells engaged in inflammatory and degenerative processes (14, 15). For instance, increased lipid body numbers have been noted in eosinophils from patients with hypereosinophilic syndrome (16, 17), in leukocytes from inflammatory arthritis (18, 19), and from bronchoalveolar lavage of patients with acute respiratory distress syndrome (20). In addition to being sites of storage of neutral lipid and phospholipids, it has been recently shown that lipid bodies are also sites of localization of several enzymes related to eicosanoid metabolism including COX, LO, phospholipase (PL) A_2, mitogen-activated protein kinase and phosphatidylinositide 3-kinase (17, 21–24). That lipid bodies in leukocytes contribute to 5-LO and COX pathway eicosanoid formation is supported by findings that increase in lipid body numbers correlated with increased LTB_4, LTC_4 and PGE_2 release by these cells when activated with submaximal concentrations of A23187 (19, 21, 25–27). Conversely, agents that inhibit lipid body formation in vitro inhibited the priming response for enhanced eicosanoid release (14, 19, 21, 28). Recently, direct proof of the involvement of lipid bodies as sites of leukotriene production was provided by the demonstration of intracellular immunofluorescent localization of newly formed LTC_4 within lipid bodies in chemokine-stimulated human eosinophils (26, 29).

Despite the recent advances on the understanding of the roles of leukocyte lipid bodies in arachidonate metabolism, little is known about the origins, composition, or functions of leukocyte lipid bodies in vivo. In this study, we have evaluated the occurrence and composition of lipid bodies from naturally occurring or experimentally induced sepsis and the functions that these structures play in innate immunity as inducible intracellular compartments involved in the heightened production of inflammatory mediators.

Materials and Methods

Materials

LPS from *Escherichia coli* (serotype 0127:b8) was obtained from Sigma (St. Louis, MO). Calcium ionophore A23187 was obtained from Calbiochem Novabiochem (La Jolla, CA). 1-acyl-2-(7-octyl BODIPY-1-pentanoyl)-*sn*-glycerol was obtained from Molecular Probes (Eugene, OR). Osmium tetroxide (OsO_4) was obtained from Electron Microscopy Science (Fort Washington, PA), WEB2086 was from Boehringer-Ingelheim (Ingelheim, Germany), and SR27417 and BN52021 was kindly provided by Dr. P. Braquet (Institut Henri Beaufour, Paris, France).

Patients with severe sepsis and septic shock

Leukocyte lipid bodies were analyzed from 10 septic patients (median age, 53 years old) hospitalized in the critical care unit, University Hospital, Federal University of Rio de Janeiro or in Spanish Hospital, Rio de Janeiro, Brazil. Eight patients had septic shock, and two had severe sepsis, as defined by the Consensus Conference of the American College of Chest Physician and Society of Critical Care Medicine (30). Among septic patients, there were four women and six men. The median acute physiology assessment and chronic health evaluation were 17 (range, 6–25) at the time of hospital admission. Blood was collected within 72 h of the sepsis diagnostic. Six healthy volunteers (median age, 43 years old) were used as controls. Patients and volunteers were recruited after ethical committee approval and informed consent was signed.

Animals

CBA/J, C3H/HeN, and C3H/HeJ mice of both sexes weighing 20–25 g were used. CBA/J and C3H/HeN mice were obtained from the Oswaldo Cruz Foundation breeding unit. C3H/HeJ mice were obtained from Fluminense Federal University (Niterói, Brazil). The C3H/HeJ and C3H/HeN mice used as controls were age and sex matched and were raised and maintained under similar housing conditions. The animals were maintained in a room with constant temperature (25°C) and alternating light/dark cycle of 12 h and had free access to pelleted diet and water. Animals were maintained and treated according to the animal care guidelines of the Council for International Organizations of Medical Sciences and the protocols were approved by the Oswaldo Cruz Animal Welfare Committee.

Pleurisy

Pleurisy was induced in anesthetized mice through the intrathoracic (i.t) injection of LPS (125–1000 ng/cavity) or vehicle (sterile saline) in a final volume of 0.1 ml. The animals were killed in a CO_2 chamber at different time points after LPS administration (1–96 h). The thoracic cavity was opened and rinsed with 1 ml of saline containing heparin (10 IU/ml). Leukocyte counts were performed in Neubauer chambers after diluting the samples in Turk solution (2% acetic acid). Differential leukocyte counts were performed in cytospin smears stained by the May-Grünwald-Giemsa method.

Polymorphonuclear neutrophil and mononuclear cell purification

The pleural fluid from LPS-stimulated or control animals were centrifuged for 10 min at 400 × *g*. The pellet was resuspended in 5 ml of Ca^{2+}/Mg^{2+}-free HBSS and placed on the top of a discontinuous Percoll gradient with densities of 57–72%. Cells were centrifuged for 30 min at 600 × *g*. Mononuclear cells were recovered from the interface, whereas polymorphonuclear neutrophils were recovered from the pellet (purity, >95%). The cells were washed twice in 10 ml of Ca^{2+}-Mg^{2+}-free HBSS.

Mouse peritoneal macrophages

Peritoneal macrophages (>90% macrophages) were collected by rinsing the peritoneal cavity from naive mice with 5 ml of PBS containing heparin (20 IU/ml).

Lipid body induction and treatments

LPS (125–1000 ng/cavity) was injected into the pleural cavity of mice, and its effect was analyzed at different time points (1–96 h). To study the effect of inhibitors, animals were pretreated with PAF receptor antagonists SR27417 (1 mg/kg), BN52021 (20 mg/kg), and WEB2086 (20 mg/kg i.p) 1 h before the injection of LPS (i.t., 500 ng/cavity). WEB 2086 and SR27417 were diluted in 0.1 N NaOH, neutralized with 0.1 N HCl, and volume completed with saline. BN52021 was diluted in saline. Vehicle alone were used as a control of experiment. Mouse peritoneal macrophages (10^6 cells/ml) were pretreated with anti-CD14 and anti-CD11b/CD18 mAb (BD PharMingen, San Diego, CA; 1 and 10 μg/ml), 5 min before the LPS administration (500 ng/ml) for 1 h at 37°C.

Lipid body staining and enumeration

While still moist, leukocytes on cytospin slides were fixed in 3.7% formaldehyde in Ca^{2+}-Mg^{2+}-free HBSS (pH 7.4), rinsed in 0.1 M cacodylate buffer, 1.5% OsO_4 (30 min), rinsed in dH_2O, immersed in 1.0% thiocarbohydrazide (5 min), rinsed in 0.1 M cacodylate buffer, restained in 1.5% OsO_4 (3 min), rinsed in dH_2O, 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 leukocytes.

LTB_4 and PGE_2 assays

Animals were stimulated i.t. with LPS (125–1000 ng/cavity) or vehicle for 6 h for lipid body formation. After that, pleural leukocytes were obtained for lipid body enumeration, and leukocytes were washed in Ca^{2+}-Mg^{2+}-free HBSS. Leukocytes (1×10^6 cells/ml) were resuspended in HBSS containing Ca^{2+}-Mg^{2+} and then stimulated with A23187 (0.5 μM) for 15 min. Reactions were stopped on ice, and the samples were centrifuged at 500 × *g* for 10 min at 4°C. LTB_4 and PGE_2 in the supernatants were assayed by ELISA according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

Immunocytochemistry

Mouse leukocytes obtained 6 h after i.t. injection of LPS (500 ng/cavity) or human leukocytes obtained from septic patients were incubated or not with 1 μM of the fluorescent fatty acid-containing diglyceride, 1-acyl-2-(7-octyl-BODIPY-1-pentanoyl)-*sn*-glycerol, to fluorescently label lipid bodies. After the incubation, cells were washed twice in Ca^{2+}-Mg^{2+}- free HBSS, cytospin onto slides, and fixed in 3% formaldehyde at room temperature for 10 min. Fixed cells were permeabilized with 0.05% saponin/HBSS solution (5 min) and then blocked with 10% normal serum from animals where secondary Ab was raised. After a washing, cytospin preparations were incubated for 1 h at room temperature with the following primary Abs

which were diluted in 0.05% saponin/HBSS solution: rabbit polyclonal serum anti-5-LO (1/150 dilution; Cayman Chemical, Ann Arbor, MI), polyclonal Ab anti-murine COX 2 (C terminus; Oxford Biomedical Research, Oxford, MI) (1:150 dilution) and purified goat IgG anti-murine TNF (1/50 dilution) and purified mouse IgG1 anti-hCOX-2 (1/50 dilution) (Transduction Laboratories, San Diego, CA). Nonimmune rabbit or goat IgG, at the same concentration as the primary Abs, were used as control. After three washes of 5 min in 0.05% saponin-HBSS, the preparations were incubated with biotin-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) or biotin-conjugated rabbit anti-goat IgG (Sigma, St. Louis, MO). The immunoreactive in cells were then identified by the ABC Vectastain glucose-oxidase kit following the manufacturer's instructions (Vector Laboratories). Glucose oxidase immunostaining was visualized under light microscopy, and fluorescent lipid bodies were identified under FITC filter.

Statistical analysis

Results were expressed as mean ± SEM and were analyzed statistically by ANOVA followed by the Newman-Keuls Student test with the level of significance set at $p < 0.05$. Correlation coefficients were determined by linear regression followed by analysis by Spearman correlation rank.

Results

LPS induces lipid body formation in mouse leukocytes

The i.t. injection of LPS (500 ng/cavity) induced an intense cell accumulation within 6 h; this augmentation was due to a marked increase in neutrophil numbers followed by significant increases in mononuclear cell and eosinophil numbers at later time points (data not shown) (31). The effect of LPS on lipid body formation in leukocytes was investigated. We observed that LPS was capable of inducing a dose-dependent increase on lipid body formation, with maximum lipid body induction observed at dose of 500 ng/cavity (Fig. 1*a*). This lipid body formation was apparent within 1 h, maximum within 6 h and decreased thereafter (Fig. 1*b*). Interestingly, blood leukocyte lipid body number were not modified after i.t. LPS stimulation, thus indicating that lipid body formation depends on leukocyte activation at the inflammatory site (not shown).

May-Grünwald-Giemsa staining cause dissolution of lipid bodies (Fig. 1*c*), which precludes their recognition in LPS-stimulated leukocytes. However, with appropriate lipid fixation and staining with osmium tetroxide, increased lipid body numbers have been observed in leukocytes after LPS administration (Fig. 1*d*), compared with the control (Fig. 1*e*).

To analyze the effect of LPS on lipid body formation in different pleural cell population, a discontinuous Percoll gradient was performed for cellular separation. As shown in Fig. 2, LPS-induced a significant increase in lipid body formation in neutrophils and mononuclear cells alike.

Immunolocalization of eicosanoid-forming enzymes and TNF-α in leukocyte lipid bodies

We evaluated whether key eicosanoid-forming enzymes and TNF-α were localized at leukocyte lipid bodies after LPS stimulation in vivo. The compartmentalization of 5-LO and COX-2, as well as the proinflammatory cytokine, TNF-α, to lipid bodies was analyzed by immunocytochemistry using conditions of cell fixation and permeabilization that prevent dissolution of lipid bodies. Lipid bodies were visualized by endogenous labeling with the fluorescent fatty acid-containing diglyceride, 1-acyl-2-(7-octyl BODIPY-1-pentanoyl)-*sn*-glycerol (1 μM) (21). Leukocytes stained with rabbit polyclonal serum anti-5-LO (Fig. 3*a*), anti-COX-2 (Fig. 3*c*) polyclonal Abs, in addition to present perinuclear membrane and cytosolic staining, show punctate cytoplasmic staining that matched with fluorescent fatty acid-labeled lipid bodies (Fig. 3, *b* and *d*, respectively). There was no immunoreactivity when control nonimmune rabbit were used as control for 5-LO and COX-2 Abs (Fig. 3*g*), although fluorescent fatty acid-labeled lipid bodies were strongly visualized (Fig. 3*h*). As shown in Fig. 3*e*, there was also a significant intracellular staining of TNF-α in LPS-stimulated leukocytes that coincidentally matched with fluorescent fatty acid-labeled lipid bodies (Fig. 3*f*). There was no immunoreactivity when control nonimmune goat serum was used (data not shown).

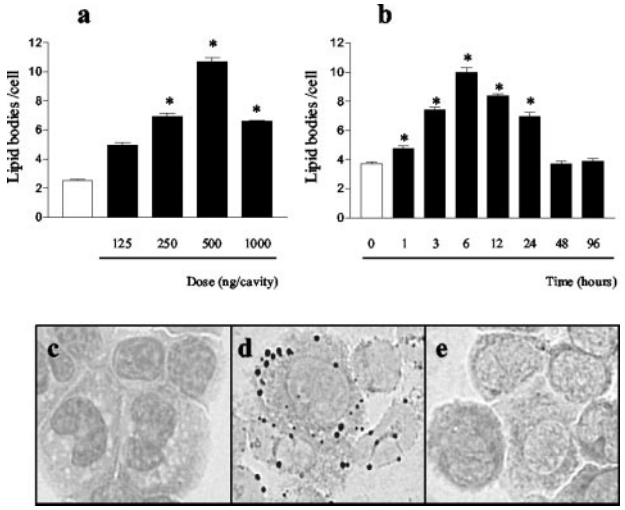

FIGURE 1. Effect of LPS on lipid body formation in vivo *a*, Dose-response curve of LPS-induced lipid body formation in leukocytes. Lipid body formation was evaluated in leukocytes 6 h after the i.t. injection of different doses of LPS (125–1000 ng/cavity). *b*, Kinetics of LPS-induced lipid body formation in vivo. Cells were recovered from a CBA/J mouse pleural cavity at different time points after stimulation with LPS (500 ng/cavity). ■, LPS; □, vehicle. Lipid bodies were enumerated using light microscopy after osmium staining. Each bar represents the mean ± SEM lipid bodies per cell in 50 consecutively counted cells from at least 6 animals. ∗, Statistically significant increases in lipid body numbers elicited by LPS compared with controls. Lipid bodies are sparse in normal cells (*e*) but very prominent in leukocytes after LPS administration in vivo (*d*), using appropriate lipid fixation and staining with osmium tetroxide. *c*, Common alcohol-based hematological stains cause dissolution of lipid body formation, which precludes their recognition. Cytocentrifuged leukocytes were fixed in 3.7% formaldehyde in HBSS and stained with 1.5% osmium tetroxide (*d* and *e*), or fixed in methanol and stained by May-Grünwald-Giemsa (*c*).

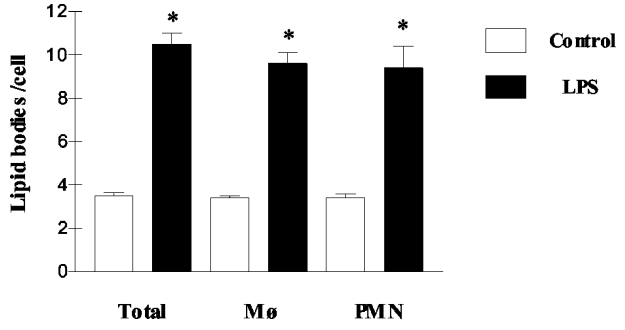

FIGURE 2. Analyses of lipid body formation in different populations of pleural leukocytes after LPS administration. CBA/J mice were stimulated i.t. with LPS (500 ng/cavity) for 6 h. The cells recovered from the pleural cavity were purified under a discontinuous Percoll gradient (56 and 72%) before lipid body enumeration. ■, LPS; □, vehicle. Lipid bodies were enumerated using light microscopy after osmium staining. Each bar represents the mean ± SEM lipid bodies/cell in 50 consecutively counted cells from at least 6 animals. ∗, Statistically significant increases in lipid body numbers elicited by LPS compared with controls. Mϕ, Macrophages.

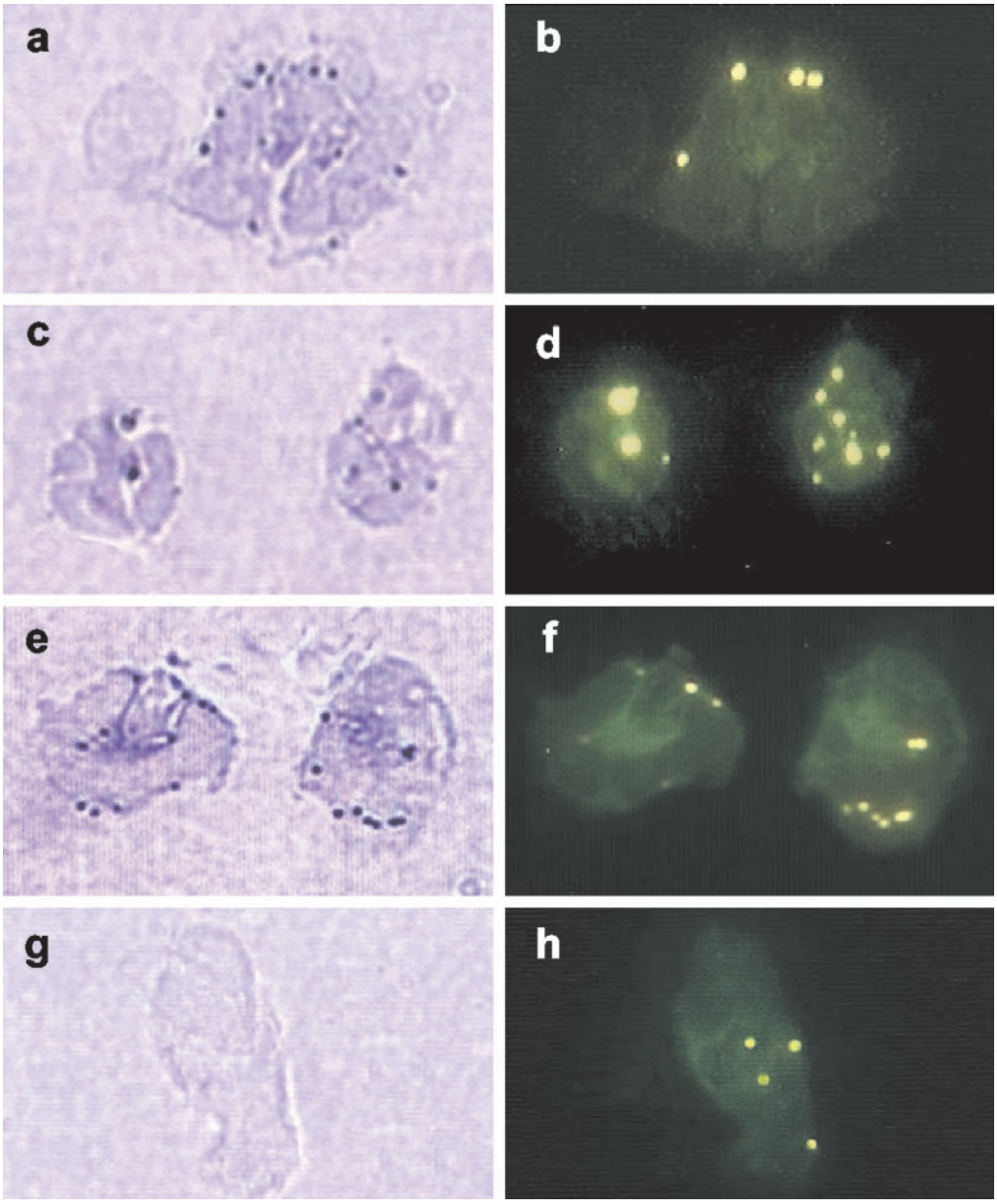

FIGURE 3. Intracellular localization of COX-2, 5-LO, and TNF-α after in vivo LPS stimulation in mice. Cells were stimulated with LPS (500 ng/cavity, for 6 h) to induce lipid body formation, and lipid bodies were visualized by endogenous labeling with the fluorescent fatty acid-containing diglyceride, 1-acyl-2-(7-octyl-BODIPY-1-pentanoyl)-*sn*-glycerol (1 μM, for 1 h). Fluorescent fatty acid-labeled lipid bodies were visualized as green punctate intracytoplasmic inclusions under FITC excitation (*b*, *d*, *f*, and *h*). Lipid bodies exhibit dark punctate staining in *a*, *c*, and *e*) which matched fluorescent lipids (some fluorescent lipid bodies are out of the plane of focus and not visible). 5-LO (*a*), COX-2 (*c*), and TNF-α (*e*) were localized on lipid bodies using anti-5-LO, anti-COX-2, and anti-TNF-α, respectively. Purified rabbit IgG (*g*) and goat IgG (data not shown) were used as a control.

CD14, CD11b/CD18, and Toll receptor are key receptors for LPS-induced lipid body formation

Most LPS effects occur via interaction with membrane receptors. The existence of membrane-bound receptors for LPS has been postulated and an increasing number of membrane-bound LPS-binding proteins have been identified (1, 3). As shown in Fig. 4*a*, pretreatment with anti-CD14 (1 and 10 μg/ml) or anti-CD11b/CD18 (1 and 10 μg/ml) mAb significantly inhibited lipid body formation in vitro. The specificity of the treatment was confirmed by the lack of effect of the irrelevant Abs in inhibiting LPS-induced lipid body formation (Fig. 4*a*). Furthermore, anti-CD14 and anti-CD11b/CD18 mAb were not able to inhibit PAF-induced lipid body formation (Fig. 4*b*).

Moreover, C3H/HeJ mice, animals resistant to endotoxin due to nonfunctional *Tlr4*, when stimulated with LPS (500 ng/cavity) were not able to form lipid bodies in vivo (Fig. 5*a*) or in vitro (not shown), demonstrating a requisite role for LPS receptor on lipid body induction. Lipid body formation induced by PAF was equally effective when cells from C3H/HeN and C3H/HeJ were compared after in vivo (Fig. 5*a*) or in vitro stimulation (not shown).

We evaluated whether key eicosanoid-forming enzymes were localized at C3H/HeN and C3H-HeJ mouse leukocyte lipid bodies after LPS stimulation in vivo. Similar to the observed in CBA/J leukocytes, 5-LO and COX-2 were colocalized to lipid bodies in C3H/HeN leukocytes recovered from LPS-stimulated animals. In contrast, there was no immunoreactivity in lipid bodies for these

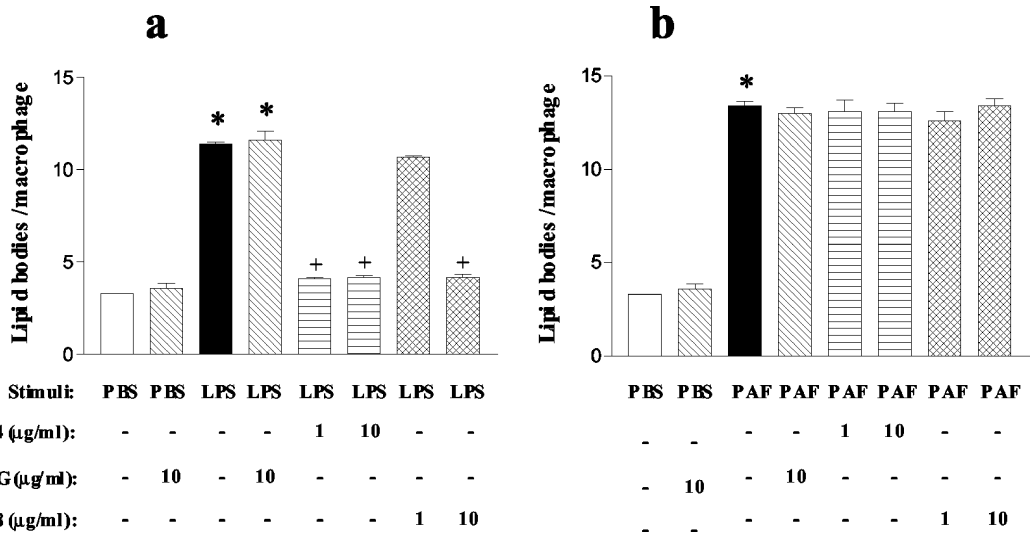

FIGURE 4. Effect of anti-CD14 and anti-CD11b/CD18 mAb on LPS (*a*) or PAF (*b*)-induced lipid body formation in vitro. Mouse peritoneal macrophages (10^6 cells/ml) were pretreated with mAb anti-CD14 (1 and 10 μg/ml), anti-CD11b/CD18 (1 and 10 μg/ml), or rat IgG (10 μg/ml) for 5 min before LPS (500 ng/ml) or PAF (10^{-6} M) stimulation for 1 h at 37°C. Lipid bodies were enumerated in 50 consecutive cells using light microscopy after osmium staining. Each bar represents the mean ± SEM from three to six experiments. +, Statistically significant increases in lipid body numbers elicited by LPS or PAF; ∗, Statistically significant differences in lipid body numbers between treated and untreated cells.

enzymes in leukocytes recovered from LPS-stimulated C3H/HeJ animals (Fig. 5*b*).

Endogenous PAF signals for LPS-induced lipid body formation

PAF and PAF-like lipids are potent inductors of lipid bodies in human and mouse leukocytes (21, 25, 32). Moreover, lipid mediators, especially PAF, have been implicated as mediators of LPS-induced inflammation (5). The role of PAF on LPS-induced lipid body formation was investigated through the use of three structurally unrelated PAF receptor antagonists. The animals were pretreated (i.p.) with PAF receptor antagonists BN52021 (20 mg/kg), SR27417A (1 mg/kg), and WEB2086 (20 mg/kg) 1 h before LPS stimulation. Confirming previous results (33), the pretreatment with PAF-receptor antagonists significantly inhibited the pleural neutrophil infiltration induced by i.t. injection of LPS within 6 h, suggesting the involvement of PAF in LPS-induced pleurisy (Ref. 33 and data not shown). As shown in Fig. 6, PAF antagonists inhibited LPS-induced lipid body formation, suggesting an important role for endogenous PAF in lipid body formation.

Lipid bodies enhance the generation of eicosanoids by LPS-stimulated leukocytes

Because leukocyte lipid bodies are sites of intracellular localization of eicosanoid-forming enzymes (Refs. 17, 21, and 24 and Fig. 3) and also stores of the eicosanoid precursor AA (16), we analyzed whether LPS-induced increases in lipid body numbers on leukocytes isolated from the mice pleural cavity would correlate with LTB_4 and PGE_2 production. After leukocytes were stimulated in vivo with increasing concentrations of LPS for 6 h, lipid bodies were enumerated, and replicated leukocytes were stimulated with A23187 (0.5 μM). As shown in Fig. 7, LPS dose-dependently induced significant concordant increases in lipid body numbers and priming for enhanced LTB_4 ($r = 0.917$, $p < 0.001$) and PGE_2 ($r = 0.986$, $p < 0.001$) generation. Similar results were obtained when peritoneal macrophages were stimulated in vitro with LPS (data not shown).

5-LO and COX-2 are localized at leukocyte lipid bodies from septic patients

As shown in Fig. 8*a*, lipid body numbers in leukocytes from septic patients were significantly increased compared with control healthy subjects. We also evaluated whether key eicosanoid-forming enzymes were localized at leukocyte lipid bodies from septic patients. The compartmentalization of 5-LO (Fig. 8*b*) and COX-2 (Fig. 8*c*) to lipid bodies was demonstrable in leukocytes from septic patients.

Discussion

The innate immune inflammatory reaction triggered by LPS is characterized by early neutrophil influx followed by late accumulation of mononuclear cells and eosinophils (34–36). Leukocyte influx and activation are regulated by chemotactic factors including soluble lipid mediators, cytokines, and chemokines that are induced after LPS engagement of membrane receptors in host targeted cells (1, 3, 37). LPS is a potent trigger of priming for enhanced inflammatory mediator release by leukocytes. Mechanisms involved in LPS-induced priming for eicosanoid production are not fully defined and may involve different processes. Indeed, it has been demonstrated that LPS increases AA availability and induces eicosanoid-forming enzyme expression (38–41). Another mechanism that might contribute to the enhanced eicosanoid production is the compartmentalization of eicosanoid production at discrete sites, as in lipid bodies. The compartmentalization of arachidonate substrate, cytosolic PLA_2, and eicosanoid-forming enzymes provides in one locale an efficient means to regulate arachidonate release and directly couple it with the enzymes to form eicosanoids. Lipid bodies, although few, are normal constituents of leukocytes and are characteristically increased in both size and number in vivo in cells associated with inflammation (14, 15). In this study, we showed that stimulation of leukocytes in vivo with LPS induced formation of lipid bodies that are sites of immunolocalization for eicosanoid-forming enzymes and correlates with enhanced generation of both prostaglandins and leukotrienes.

Mechanisms involved in LPS-induced lipid body formation were analyzed. First, we investigated the need of LPS to interact

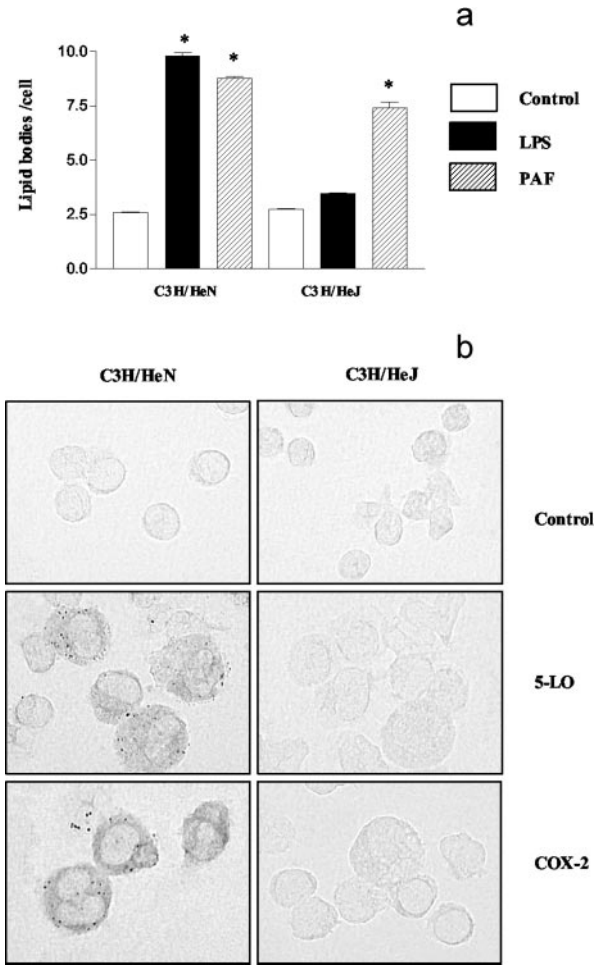

FIGURE 5. Effect of LPS on lipid body formation and composition in leukocytes from C3H/HeN or C3H/HeJ mice. *a*, Cells were recovered from the mouse pleural cavity 6 h after the i.t. injection of LPS (500 ng/cavity; ■), PAF (1 μg/cavity; ▨), or vehicle (□). Each bar represents the mean ± SEM lipid bodies per cell in 50 consecutively counted cells from at least 6 animals. ∗, Statistically significant increases in lipid body numbers elicited by LPS or PAF compared with control. *b*, Immunolocalization of eicosanoid-forming enzymes of C3H/HeN and C3H/HeJ mouse leukocytes after LPS stimulation. 5-LO and COX-2 were localized on lipid bodies using rabbit polyclonal Ab anti-5-LO and anti-COX-2, respectively. Rabbit IgG was used as a control. The glucose oxidase immunostaining was visualized under light microscopy.

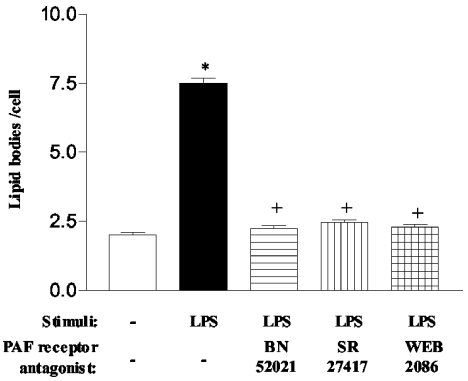

FIGURE 6. Effect of three structurally unrelated PAF receptor antagonists on LPS-induced lipid body formation in leukocytes. CBA/J mice were pretreated (i.p.) with PAF receptor antagonists BN52021 (20 mg/kg), SR27417A (1 mg/kg), and WEB2086 (20 mg/kg) 1 h before the injection of LPS (500 ng/cavity) or vehicle. Lipid bodies were enumerated using light microscopy after osmium staining. Each point represents the mean ± SEM in 50 consecutively counted cells from at least 6 animals per group. Each bar represents the mean ± SEM lipid bodies per cell in 50 consecutively counted cells from at least 6 animals. ∗, Statistically significant increases in lipid body numbers elicited by LPS. +, Statistically significant differences in lipid body numbers between treated and untreated cells.

with surface receptors to induce lipid bodies in leukocytes. The current understanding of the LPS receptor system consists of multiple LPS receptors on the surface of phagocytes which, after interacting with LPS, mediate remarkably diverse events. CD14 is likely the LPS receptor primarily responsible for initiating LPS-induced cellular activation. This is supported in part by the observation that pretreatment with anti-CD14 drastically inhibit LPS-induced inflammatory mediator production, cell activation, and death and by the findings that CD14-deficient mice are highly resistant to LPS and Gram-negative bacteria (42–44). The β_2 integrin CD11/CD18 is important for phagocytosis of invading Gram-negative bacteria and has been shown to bind LPS (45). Moreover, CD11/CD18 is involved in LPS-induced activation of gene expression, including induction of COX-2 and IL-12 (46–48). LPS-induced macrophage lipid body formation occurs in a dose-dependent manner, consistent with a receptor-dependent phenomenon. In agreement, LPS-induced lipid body formation was inhibited by anti-CD14 and anti-CD11b/CD18 mAb (Fig. 4), suggesting a role for both CD14 and CD11b/CD18 receptors in lipid body formation.

Recent data have demonstrated that mammalian TLR proteins participate in intracellular signaling initiated by Gram-negative bacterial LPS. The Toll protein is a transmembrane receptor that displays resemblance in its intracellular portion to the signaling domains of members of the IL-1R family that mediate activation of

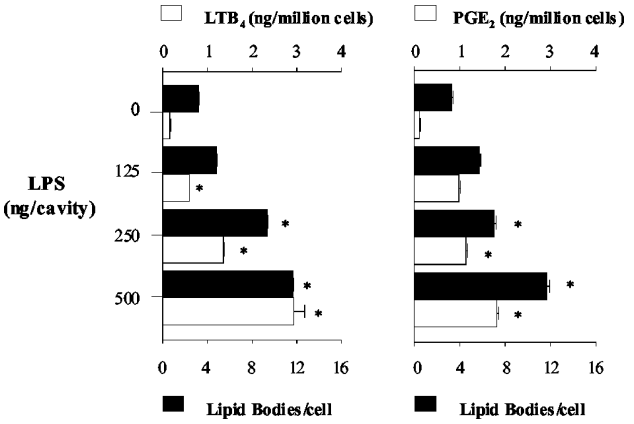

FIGURE 7. LPS induced both lipid body formation (■) and priming for enhanced LTB_4 and PGE_2 generation (□). Animals were stimulated i.t. with LPS or vehicle for 6 h for lipid body formation. After that, pleural leukocytes were obtained for lipid body enumeration, and leukocytes were washed in Ca^{2+}-Mg^{2+}-free HBSS. Leukocytes (1×10^6 cells/ml) were resuspended in HBSS containing Ca^{2+}-Mg^{2+} and then stimulated with A23187 (0.5 μM) for 15 min. The baseline level production within a 15-min period (mean ± SD picograms per milliliter) in the absence of A23187 stimulation of LTB_4 was 0.01 ± 0.01, 0.03 ± 0.01, 0.01 ± 0.01, and 0.01 ± 0.01 and that of PGE_2 was 0.62 ± 0.24, 2.37 ± 1.24, 0.76 ± 0.67, and 0.65 ± 0.56 for PBS and 125, 250, and 500 ng/cavity LPS, respectively. Lipid bodies were enumerated microscopically after osmium staining. LTB_4 and PGE_2 in supernatants were measured by EIA. Data are means ± SEM from at least six animals. LPS-induced increasing numbers of lipid bodies correlated with increased production of each eicosanoid ($r > 0.9$, $P<0.001$, Spearman correlation rank). ∗, Significantly greater than value without LPS stimulation ($p < 0.05$, Student's t test).

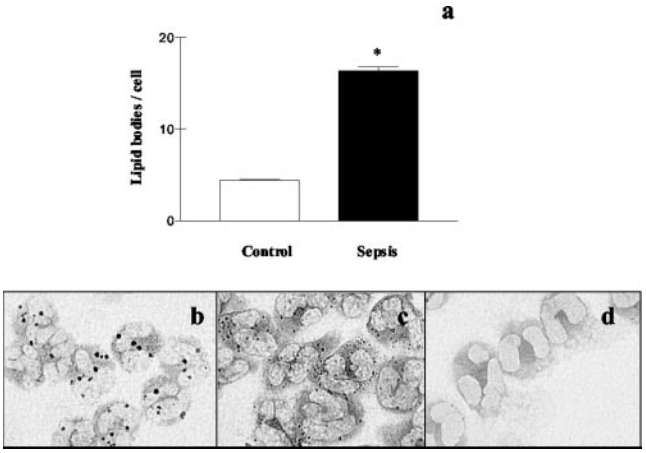

FIGURE 8. Analysis of blood leukocyte lipid bodies from septic patients. *a*, Leukocyte lipid bodies from septic patients or control subjects were enumerated using light microscopy after osmium staining. ■, Septic patients; □, control patients. Each bar represents the mean ± SEM from 10 septic patients and 6 healthy volunteers. ∗, Statistically significant increases in lipid body numbers from septic patients compared with healthy volunteers. Immunolocalization of eicosanoid-forming enzymes on blood leukocytes from septic patients. 5-LO (*b*) and COX-2 (*c*) were localized on lipid bodies using rabbit pAb anti-5-LO or mouse mAb anti-COX-2, respectively. Rabbit or mouse IgG were used as a control (*d*). The glucose oxidase immunostaining was visualized under light microscopy.

NF-κB (1, 3). In mice, *Tlr4* appears to be the main LPS response pathway involved in cell activation (49–52). The involvement of *Tlr4* in LPS-induced lipid body formation was investigated by using C3H/HeJ mice.

It has been recognized for several years that mice of the C3H/HeJ strain have a defective response to bacterial endotoxin (53, 54). Recently, the inability of C3H/HeJ mice to respond to LPS was characterized to be linked to a truncated and inactive *Tlr4* (49–52). Indeed, we observed that LPS administration into C3H/HeJ failed to induce pleural inflammation as measured by neutrophil influx (data not shown). Moreover, leukocytes from these animals did not present lipid body formation or punctate eicosanoid-forming enzyme localization after LPS administration indicating a requisite role for *Tlr4* in LPS-induced lipid body formation. Our findings suggest that for lipid body induction CD14, CD11b/CD18, and *Tlr4* must be coordinately engaged to deliver optimal signaling to leukocytes.

Several LPS-induced effects occur indirectly through the generation of cytokines and lipid mediators. One such mediator is the glycerophospholipid PAF. Indeed, pretreatment with PAF receptor antagonists are capable of significantly inhibit the inflammatory process and cardiovascular collapse induced by endotoxin in different experimental models (5). LPS have also been shown to induce PAF production both in vivo and in vitro (55, 56). Moreover, many groups have shown that patients undergoing septic shock and systemic inflammatory response syndrome have elevated levels of PAF or PAF-like substances in their plasma (57). We demonstrated that the pretreatment with three structurally unrelated PAF receptor antagonists inhibited the lipid body formation induced by LPS in vivo, suggesting that endogenous PAF plays an important role in this phenomenon. In agreement, we and others have previously demonstrated the ability of PAF and PAF-like lipids to induce lipid body formation in a receptor-mediated manner in human and murine leukocytes (21, 25, 27, 32). Although PAF may induce cell activation at intracellular binding sites (58), our results suggest that PAF is acting in a paracrine/autocrine way to induce lipid body formation, because the PAF receptor antagonist used in this study would act preferentially at membrane receptors.

The two major enzymes, 5-LO and COX-2, involved in the enzymatic conversion of AA into eicosanoids during inflammatory conditions were shown to localize within in vivo formed lipid bodies from septic patients or experimentally elicited by LPS administration in mice. In agreement, by using immunocytochemistry at the light level and ultrastructural postembedding immunogold, COX has been reported to localize at eosinophil lipid bodies, both in naturally formed lipid bodies in eosinophils from hypereosinophilic syndrome patients and in PAF-induced lipid bodies (17, 21, 24). Previous reports have used a nonselective anti-COX Ab, and our results are the first description of COX-2 localization in lipid bodies. Previous studies on the intracellular localization of 5-LO have shown that 5-LO localization is cell type specific and also varies according to the activation state of the cell. 5-LO was shown to localize within the nuclear environment of alveolar macrophages, whereas 5-LO was predominantly cytosolic in human neutrophils and resting peritoneal macrophages (10–12). In addition to the nuclear environment, we have demonstrated the compartmentalization of the key enzyme for LT production, 5-LO, within human eosinophil lipid bodies (17, 21, 26). Accordingly, in this study we observed a significant correlation between lipid body formation induced by LPS and priming for PGE_2 and LTB_4 production in leukocytes stimulated by submaximal concentrations of the ionophore A23187, suggesting that lipid bodies are early response structures involved in the production of lipid mediators of inflammation. Recently, direct evidence for LT synthesis derived from eosinophil lipid bodies has been provided by using intracellular immunofluorescent localization of LTC_4 (26, 29). Together, our findings support a role for lipid bodies to function as specific sites for eicosanoid formation. Of special interest for inflammatory cells where increased eicosanoid synthesis is observed, lipid body arachidonyl phospholipids might provide a source of substrate AA without requiring the perturbation of the integrity of membranes and could be replenished from the arachidonyl triglycerides abundant in lipid bodies (59, 60).

Interestingly, the proinflammatory cytokine involved in the pathogenesis of sepsis, TNF-α, were also shown to colocalize within leukocyte lipid bodies formed after LPS stimulation in vivo (Fig. 3) and from septic patients (not shown). Accordingly, the lipid body localization of TNF-α in eosinophils from Crohn's disease has been shown previously (61). Accumulating evidence indicate that TNF-α are involved with AA release and synthesis of its metabolites; conversely, AA and eicosanoids have been implicated in TNF-α synthesis and function(s) (62–67). The colocalization of eicosanoid-forming enzymes and TNF-α within lipid bodies might be of importance for intracellular signaling in sepsis.

In conclusion, our studies indicate that leukocyte lipid bodies formed in response to LPS stimulation or sepsis are sites for eicosanoid-forming enzymes and cytokine localization and may develop and function as structurally distinct, intracellular sites for paracrine eicosanoid synthesis during inflammatory conditions.

Acknowledgments

We thank Dr. João Viola for helpful comments on the work and the manuscript and Dr. Sonia Altenburg for C3H/HeJ mice.

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